

QUALITY CONTROL MANUAL FOR CATTLE FEED PLANTS



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FOREWORD

Apart from locally available feed resources, compounded cattle feeds and strategic feed supplements are important source of nutrients for growing and lactating animals. To ensure that these feeds and feed supplements are manufactured in a cost effective manner and in accordance with standard specifications, various aspects related to least cost feed formulation, raw material quality testing, storage, production etc. need to be taken into consideration. It is essential that, feed milling plants have a qualified and trained animal nutritionist.

I am happy to note that NDDB's Animal Nutrition group has put in a great deal of effort to prepare a Quality Control Manual for Cattle Feed Plants based on research from authoritative sources, to serve as a practical guide on the subject, which would be useful not only for officers currently involved in quality control but also for those interested in knowing about systems for ensuring quality in feeds.

The manual has been carefully organized in numbered parts, chapters and sections for quality control in production, storage and common analytical procedures of feeds. The part on cattle feed production and storage includes recommended practices on a number of aspects such as sampling, spot tests for feed ingredients, rodent/pest control, production operations, feed formulation methods, pelleting, process for production of bypass protein and mineral mixture production.

The part on commonly used analytical procedures has useful information on laboratory practices, proximate analysis, analysis of fibre fractions, molasses, minerals, vitamins, bypass protein and fat supplements, aflatoxins, pesticide residues as well as procedures for measurement of methane emissions. The description of analytical procedures generally include relevant information on principle, apparatus, reagents/materials, procedure, calculation and reference source, along with photographs and/or illustrations where ever applicable. The appendices contain useful information on BIS standards for feed and feed supplements, glossary of terms, conversion factors etc. which would aid the readers.

I am sure that this manual will prove to be a valuable reference tool for animal nutrition officers involved in quality control in cattle feed plants as well as others who are interested in the subject.

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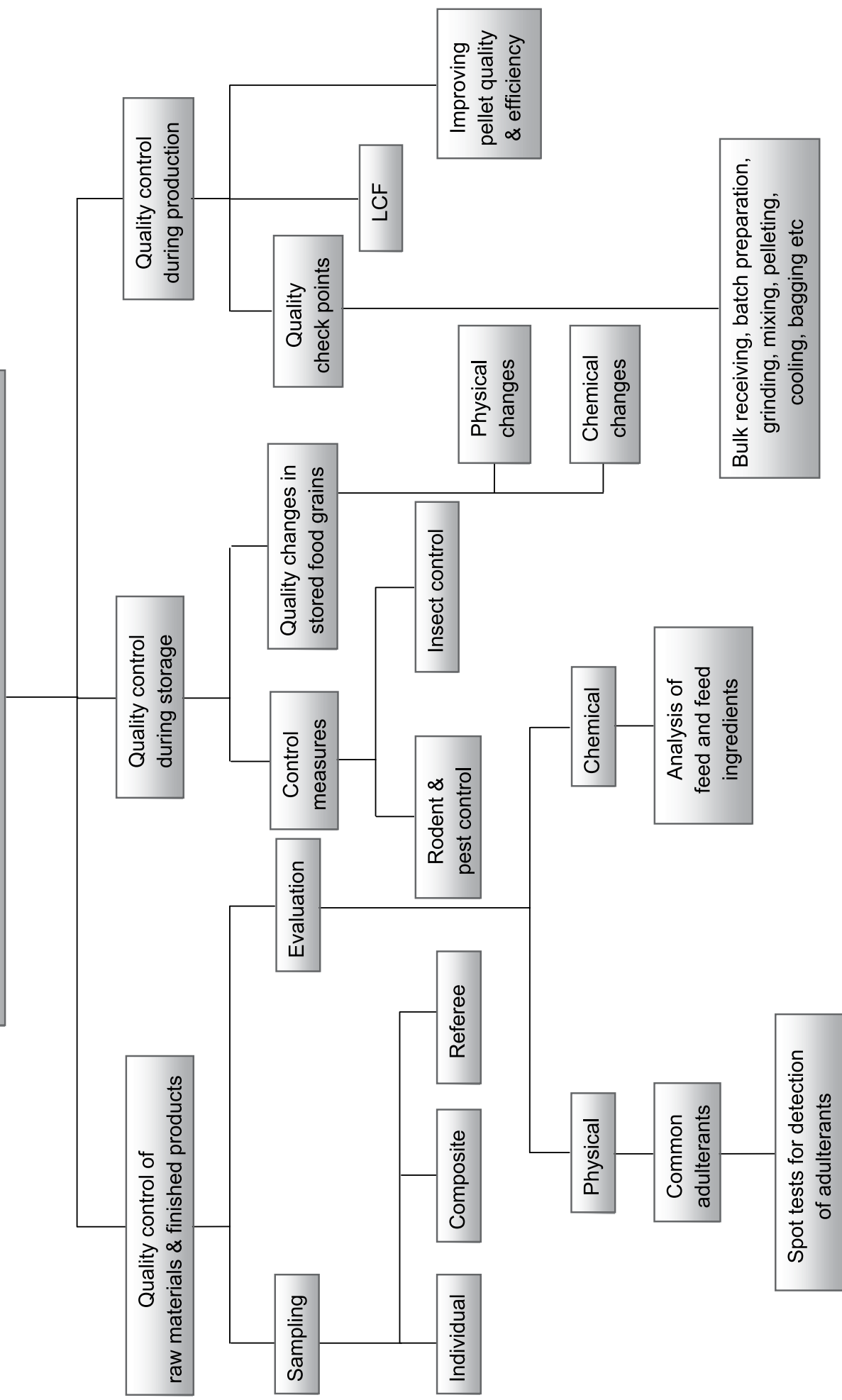
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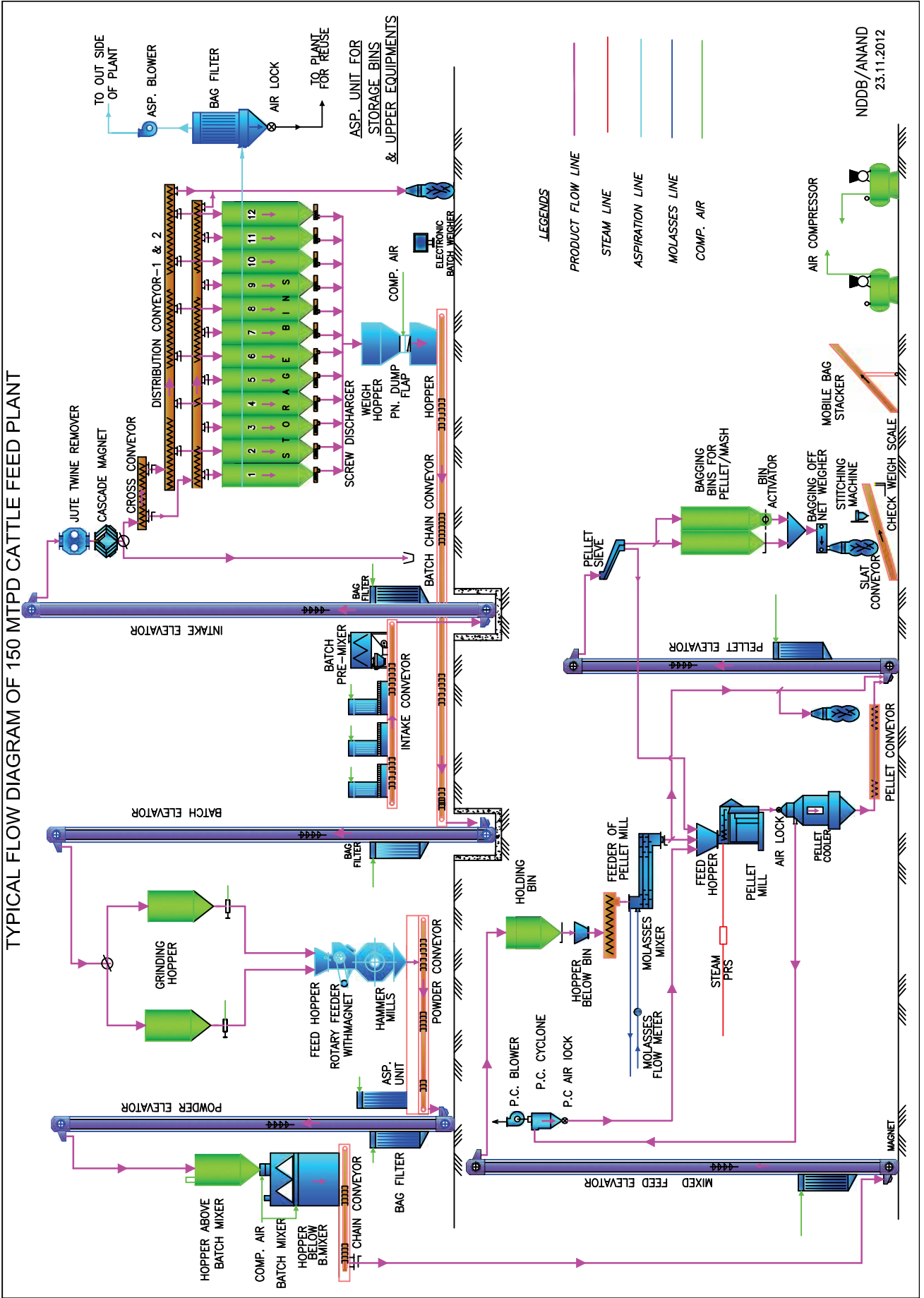
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Flowchart for quality control in a cattle feed plant



TYPICAL FLOW DIAGRAM OF 150 MTPD CATTLE FEED PLANT



PART - I

QUALITY CONTROL IN A CATTLE FEED PLANT

Quality Control Manual for Cattle Feed Plants

Introduction

Compound cattle feed is an important constituent of ration, considering the fact that dairy animals in India have limited access to cultivated green fodder and grasses. Most of the macro and micro nutrients to meet animals' requirement are provided by compound feed, especially on crop residue based diets. It is possible to formulate balanced rations for growing and lactating animals only if the feed used conforms to the laid down specifications, for energy, protein, minerals, vitamins etc. Quality control in cattle feed production is of utmost importance for overall success and profitability. The degree of quality is the consistency in which feed is formulated, processed, mixed and delivered as compared to what is expected. Animals respond better if the feed is low in nutrient variation; and is similar in moisture content, texture and rate of nutrients availability.

The relationship between feed quality and animal performance is important and thus, the challenge for animal nutritionists and others involved in cattle feed production is to consistently monitor all aspects of the feed production system being used and measure those variables that are good indicators of quality control. For the feed industry, quality control is the responsibility of the management and involves qualified personnel being trained to ensure a high grade of organization, documentation and the policing of various procedures and processes necessary to guarantee the basic quality of feed ingredients and feeds.

In order to provide animals with necessary nutrients to meet their requirements for maintenance, growth, pregnancy and production of milk, to reduce the risks of animal health and to minimize excretions and emissions into the environment, the chemical composition of cattle feed used in the diet has to be precisely known. A robust quality control system provides the mechanism to ensure that all quality criteria are met and provides a system to constantly monitor the cattle feed plant operations along with laboratory analysis results and identify opportunities for further improvement. It is extremely important that the chemical composition of the feeds and feed ingredients is analyzed with the same or similar standard methods. Therefore, in part-II of this manual, commonly used analytical procedures are described. Quality of raw materials during storage and proper functioning of different sections of feed plant are equally important for production of feed of consistent quality, which have been adequately covered in the manuscript.

In addition, details of mineral mixture and bypass protein supplement production are also covered, which are very important ingredients of compound cattle/bypass protein feed. For the better understanding of quality control officials, photographs of various components of feed milling plant and the testing equipment are also given. All the relevant information for production of compound cattle feed and feed supplements and the test methods compiled from various sources, including our own experience of decades in guiding the quality control officers of cattle feed plants in the dairy cooperative sector, is given in this manual.

QUALITY CONTROL IN A CATTLE FEED PLANT

Quality control in cattle feed plant is of utmost importance for overall success and profitability of animal enterprises. No other factor, directly or indirectly related to proper nutrition and high performance of animals is more critical than feed quality control and ration consistency. The degree of quality is the consistency in which feed is formulated, processed, produced and delivered as compared to what is expected. Quality has been defined as *“Degree to which a set of inherent characteristics fulfils requirements”*. This clearly indicates that achieving quality means fulfilling requirements. The requirements may come from customers and in some cases from regulatory authorities. Usually quality is verified by comparison with a known standard. However, relative value of quality over time is extremely valuable and useful in many situations.

The relationship between feed quality and animal performance is important and encompasses not only the quantitative amounts of all feed components but also the digestibility and metabolism of those components. Thus, the challenge for animal nutritionists and others involved in feed production is to consistently monitor all aspects of feed production system being used and measure those variables that are good indicators of quality control.

A well organized quality control section is essential for a cattle feed plant (Fig. 1.0). The aim of this section is to maintain high standards of the compounded feed products. Animal nutrition officer has to ensure not only the nutritional standards but also the least-cost product for the livestock consumption which the farmers can afford easily.

Objectives

The objective of quality control of feedstuff is to ensure that a consumer should obtain feeds that are unadulterated, true to their nature and produce desired results. Quality control is therefore, defined as the maintenance of quality at levels and tolerances acceptable to the buyer while minimizing the cost of processing. Main objectives of the quality control are detailed below:

1. Along with least cost of production, the quality of cattle feed should conform to the laid down specifications (for specific functions) so as to maintain the animals in optimum health and production.
2. By maintaining the quality of feed uniform and of desired standard, it will also help in keeping the goodwill of the farmers' organizations and maintain the faith of the producers in their organisation which will help in boosting the sale of feed and consequently plant capacity utilization.
3. The quality control helps the milk producer to ensure that the product offered is properly labelled and supplied wholesome as feed for his livestock. For the manufacturer, it is an attempt to protect him from unscrupulous competitors who might misrepresent their products to the consumers. Thus, it is meant to safeguard the interests of both the producer and the consumer.

Quality control is everyone's responsibility; it must be built in at every stage of the plant process, from identifying customer's needs, through planning and implementation right from the point of receiving raw materials to delivery of finished products. In some cases, quality needs to be checked even beyond delivery to the customer since customer satisfaction can have an enormous impact on quality as perceived by them. In view of this, monitoring of quality control at different points has been classified as under:

1. **Quality control of raw materials and finished products**
2. **Quality control during storage**
3. **Quality control during production**



Fig. 1.0 Cattle feed plant

1.0 QUALITY CONTROL OF RAW MATERIALS AND FINISHED PRODUCTS

Quality control of incoming ingredients is crucial to predicting the quality of a finished feed. A large number of raw materials are considered for the production of cattle feed, based on their chemical composition and current price structure. The processing techniques, such as oil extraction, polishing etc., are the factors, which affect the composition of raw materials. Adulteration of raw materials is also quite common. It is, therefore, desirable to lay down specifications for purchase of standard raw materials and negotiate prices effectively. Sampling of material for analysis should be such that it represents the consignment. Otherwise, the entire purpose of quality control will be defeated. It may be noted that the quality of a cattle feed will be no better than the qualities of the individual ingredients used in the formula. Hence, it is essential to observe strict quality control measures for purchase of raw materials.

1.1 Sampling

Since the samples forms the base for analysis, utmost attention should be taken in drawing out samples. It is generally found that the raw materials supplied are not of uniform quality. It is also common to note that some suppliers send the consignments with good quality materials in the periphery of gunny bag and inferior material in the centre. Hence, the sample should be drawn from the centre as well as from the periphery of the gunny bags. It is better to draw samples from all the bags (100 per cent sampling) as far as possible. 100 per cent sampling proves to be an efficient system for detecting adulteration during the receipt of material. Substandard material can be outright sorted and rejected. However, sometimes it may not be practical with a very large consignment. In such a case, a standard sampling procedure based on sound statistical techniques should be followed, where the quantity of each sample, number of samples and percentage of bags to be sampled is proportional to the size of consignment. In this way, the result would reflect the real picture of the entire consignment within the accepted range of accuracy. Sample collecting staff

should be adequately trained and sample should be collected strictly under the supervision of the animal nutrition officer. Every consignment should be given a separate code number so that it can be recorded for analysis, reporting and issuing.

In India, Bureau of Indian Standards (BIS) has laid down the following procedures and precautions for collecting the samples for analysis:

1.1.1 General requirements of sampling

- In drawing, preparing, storing and handling samples, care should be taken that the properties of feeds are not affected.
- Take sample at a protected place not exposed to damp air, dust or soot.
- The sampling instrument shall be clean, dry and sterile when used.
- Protect the samples, the sampling instrument and containers for samples from adventitious contamination.
- Preserve samples in clean, dry and sterile containers. The sample containers shall be of such a size that they are almost completely filled by the sample.
- Each container shall be sealed air-tight with a stopper or a suitable closer after filling in such a way that it is not possible to open and reseal it without detection. Mark full details of sampling i.e. the date of sampling, batch or code number, name of the manufacturer and other important particulars of the consignment.
- Sample shall be stored in sample storage room (Fig. 1.1) in such a manner that there is no deterioration of the material.
- Sampling shall be done by a person agreed to between the purchaser and the vendor and if desired by any of them, in the presence of the purchaser (or his representative) and the vendor (or his representative).

1.1.2 Scale of sampling

- The quantity of cattle feed of a particular type, produced under relatively similar conditions in a day shall constitute a lot.
- Samples shall be tested for each lot for ascertaining conformity of the material to the requirements of the standard.
- The number of bags to be selected from the lot shall depend on the size of the lot and shall be in accordance with table 1.1.



Fig. 1.1 Sample storage room

1.1.3 Preparation of individual samples

Draw with an appropriate sampling instrument, equal quantities of the material from the top, bottom and sides of each bag selected. The total quantity of material drawn from each bag shall be not less than 1.5 kg. Mix all the portions of the material drawn from the same bag thoroughly. Take about 0.75 kg of the material and divide it into

Lot size	No. of bags to be selected
Up to 50	1
51 – 100	3
101 – 300	4
301 – 500	5
501 and above	7

three equal parts. Each portion, thus obtained, shall constitute the test sample representing that particular bag and shall be transferred immediately to clean and dry sample containers, sealed airtight and labelled. The individual samples thus obtained shall be formed into three sets in such a way that each set has a test sample representing each bag selected. One of the sets shall be for the purchaser, another for the vendor and the third for the referee.

1.1.4 Preparation of composite samples

From the mixed material from each selected bag remaining after the individual samples have been taken, equal quantities of the material from each bag shall be taken and mixed up together so as to form a composite sample weighing not less than 0.75 kg. This composite sample shall be divided into three equal parts and transferred to clean and dry containers, labelled and sealed airtight. One of these samples shall be for the purchaser, another for the vendor and the third for the referee.

1.1.5 Referee samples

Referee samples shall consist of a set of test samples and a composite sample and shall bear the seal of the purchaser and the vendor and shall be kept at a place agreed to between the two.

1.1.6 Testing of samples

Samples shall be tested for each lot for ascertaining the conformity of the material to the requirements of the standards.

1.1.7 Criteria for conformity

A lot shall be considered as conformity to the specification when the test results on the individual samples satisfy the requirement.

1.2 Evaluation of feeds and feed ingredients for quality

The feeds are usually subject to the following types of tests:

1. Physical evaluation
2. Chemical evaluation

1.2.1 Physical evaluation

Physical evaluation is easy but rough in nature. One must be highly trained to identify the changes in the nature of the raw materials/ feeds.

Colour

The appearance of the ingredient will reveal its quality. Any change in the colour of the feed ingredients give an indication of the maturity of the grain, storage conditions, presence of toxins, contamination due to sand, possible use of insecticides/ fungicides which gives dull and dusty appearance. Orange to red colour of sorghum indicates high tannin content. Browning or blackening due to heat on improper storage reduces nutritive value.

Size

Size of the grains governs its energy value due to the proportional decrease/increase in seed and its coat. Smaller the grain, lower will be the metabolizable energy (ME) value due to more proportion of coater hulls. To evaluate the cereals, weight of a fixed number of grains usually 100 grains or fixed volume is taken. Higher weight indicates a higher ME value. This technique is called Test Weight.

Homogeneity

The presence of contaminants like other grains, husk, broken grains, weed seeds, infected seeds is viewed. In the oil seed cakes closer observation will reveal the presence of fibrous material, especially in de-oiled groundnut cake. Rice polish is contaminated with husk. Clumps in mineral ingredients are not suitable for premixing.

Smell

Smell is the next best indicator. Just standing near the stock itself will immediately indicate any difference in the normal smell. The plant manager should familiarize himself with the normal smell of the ingredients; any change in the normal smell of the ingredients should be viewed with suspicion. Musty odour indicates the beginning of fungal contamination or boring insects. To detect rancidity in oil rich feed ingredients this is the best method. Odour of petroleum products is suggestive of excessive pesticide or fungicides.

Taste

Each ingredient has a different taste, any change in the taste like bitterness in grains, soya, sunflower oil meal and groundnut cake indicates the presence of mycotoxins. The level of salt can be detected by tasting the ingredient and the feed. Bitter taste of rice polish indicates rancidity of fatty acids.

Touch

Feeling the raw material will indicate dryness. Chilliness indicates high moisture content. Clumps can be detected by inserting a hand inside the bag. Clumps may be formed due to high moisture content, improper storage, packing of fresh warm solvent extracted meal, which crumbles on application of light pressure. Clumps formed due to excess of moisture will be very hard. To evaluate rice polish, place about 25g of rice polish on the palm and close the fingers tightly and then open the fingers, the rice polish will become like a solid mass if the crude fibre level is below 12 per cent. If the fibre level is high, the mass will disintegrate once the fingers are opened. Further pressure will be felt when the hand is closed in high fibre rice polish.

Sound

Dry grains on pouring down or biting will produce sound of spilling coins.

1.2.1.1 Common adulterants in feeds

Adulteration is defined as the admixture of a pure substance with some cheaper and low quality substance. It is done intentionally usually to make money. In costly feed ingredients like oil seed cakes, adulteration is done by spraying urea in order to raise their protein content. However, sometimes brans are also added. Besides urea, oilseed cakes are adulterated with husk, non-edible oilseed cakes etc.

The common contaminant or adulterant is husk or sand (Table 1.2). Winnowing is the best method to detect husk in the feedstuffs. Sieving can be done to differentiate contaminants based on particle size.

Table 1.2 Common adulterants of different feed ingredients	
Feed ingredients	Common adulterants
Groundnut cake	Groundnut husk, urea, non-edible oil cakes
Mustard cake	<i>Argemone mexicana</i> seeds, fibrous feed ingredients, urea
Soybean meal	Urea, raw soybean, hulls
De-oiled rice bran, wheat bran	Ground rice husk, saw dust
Mineral mixture	Common salt, marble powder, sand, lime stone
Molasses	Water
Maize	Cobs, cob dust, sand
Rice kani	Marble, grit

1.2.1.2 Spot tests for detection of various types of adulteration

Mahua cake

To water extract of the test feed, add concentrated H_2SO_4 (sulphuric acid). Violet or pink colour indicates the presence of mahua cake.

Argemone seeds

To water extract of test feed, add conc. HNO_3 (nitric acid). Appearance of brown-reddish colour indicates the presence of argemone seeds.

Detection of castor cake in feedstuffs

When feed is treated with potassium chlorate, the castor cake is destroyed and settles down at the bottom.

Detection of neem seed cake in feedstuffs and edible oil cakes

The coarsely powdered feed stuffs are percolated three times at room temperature with 95 per cent alcohol. The total percolate is concentrated under reduced pressure till a thick syrupy amber coloured residue is obtained, which is treated with different solvents to extract a crystalline product (Nimbine). It is cautiously dissolved in concentrate sulphuric acid, the resultant brown solution changes to cherry red on addition of small quantity of concentrated nitric acid. The crystalline product gives a yellow colour with tetranitromethane. Alcoholic solution of the crystalline product shows a sky blue fluorescence under ultraviolet light.

Detection of linseed meal in animal feeds

A small quantity of feed is treated with 1 or 2 drops of dilute sulphuric acid in a test tube. It is some time necessary to add some granulated zinc and more acid. The mouth of the test tube is covered with a disk of filter paper moisten with a drop of reagent. Depending upon the amount of hydrogen cyanide produced a more or less intense blue colour appears on the reagent paper. Gentle warming in water bath is advisable when small quantity of cyanide is suspected.

1.2.1.3 Urea spot test

Procedure

- A) Weigh 2.5 g urease powder with small amount of distilled water, stir into paste and dilute to 50 ml with distilled water.
- B) Rub 0.15 g bromothymol blue indicator powder in mortar with 2.4 ml 0.1 N NaOH (Sodium Hydroxide) solution. After indicator dissolves, wash mortar and pestle with distilled water and dilute to 50 ml with distilled water. Solution should be green; pH approx. 7.0.
- C) Mix solution (A) and (B) in equal proportion.
 - Take 90 ml mixture solution of (C) and mix with 10 ml glycerol.
 - Pour mixture into watch glass. Dip pieces of heavy filter paper in solution.
 - Allow to dry papers in a place free from NH_3 fumes, strong air currents or heat.
 - Paper should be orange when dried.
 - Make strips of these papers and store in amber coloured glass bottle in a cool place.

Testing

- Dissolve small quantity of feed/ feed ingredients with distilled water in a beaker.
- Deep the strip in a beaker and allow to dry it.
- Appearance of blue colour indicates the presence of urea.

1.2.1.4 Identification of plant and animal protein in feed

1. Mix 1-2 g test sample with 100 ml boiling water or boil the mixture for 2-3 min. Place a few ml of the cooled mixture in test tube and add 5-6 drops of iodine solution. If starch is present, the mixture turns blue.
2. Spread 1-2 g test sample into a petri dish. Add 5-6 drops of iodine solution and let stands for 10 min. A purple brown colour indicated the presence of plant fibre, whereas yellow indicated animal fibre (protein) using a microscopic examination.

1.2.1.5 Spot tests for minerals in finished feed

Preparation of sample

Usually the minerals in mixed feed are powder or fine particles. Sieve the sample through fine sieve and put the fine portion in chloroform or carbon tetrachloride in a 100 ml beaker. Shake thoroughly and keep it for 5 minutes. Pour off the floating feed material. Sprinkle the residue mineral portion over the paper with a small spatula for spot test.

Test for cobalt (Co), copper (Cu) and iron (Fe)

Reagents

Solution A: Sodium potassium tartrate (Rochelle salt, $\text{KNaC}_4\text{H}_4\text{O}_6\cdot\text{H}_2\text{O}$). Dissolve 100 g of Rochelle salt and make volume 500 ml with distilled water.

Solution B: Dissolve 1 g of 1-nitroso-2-hydroxynaphthalene-3, 6 g disulfonic acid and make volume 500 ml with distilled water.

Procedure

Moisten the filter paper with 3 to 4 drops of solution A and B then sprinkle the feed sample on filter paper. Allow the filter paper to dry and check closely under low power stereoscopic microscope.

Positive results

Presence of cobalt will produce a pink colour, copper will produce light brown coloured ring, and iron gives a deep green colour.

Test for manganese (Mn)**Reagents**

Solution A: 2 N Sodium hydroxide

Solution B: Dissolve 0.07 g of benzidine dihydro chloride in 10 ml of glacial acetic acid and dilute to 100 ml with distilled water.

Procedure

Moisten filter paper with solution A. Sprinkle the feed sample immediately, let it stand for 1 minute. Add 2-3 drops of solution B. If no reaction occurs immediately, add solution B again, without flooding.

Positive results

Presence of manganese will produce a dark blue colour with a black centre.

Test for iodine (Potassium iodide)**Reagents**

- Starch paper.
- Bromine solution: Dissolve 1 ml of saturated bromine in water and add distilled water to make the volume 20 ml.

Procedure

Moisten starch paper with bromine solution. Sprinkle the feed sample on the starch paper.

Positive results

Iodine will produce a blue purple colour.

Test for magnesium (Mg)**Reagents**

Solution A: 1 N potassium hydroxide

Solution B: Dissolve 12.7 g of iodine and 40 g of potassium iodide in 100 ml distilled water.

Procedure

Mix solution A with excess amount of solution B to produce a very dark brown colour mixture and add 2 to 3 drops of solution A until it turns a pale yellow. Moisten the filter paper with pale yellow solution and sprinkle with feed sample.

Positive results

Magnesium gives yellow brown spots.

Note: The mixture of solution A and B should be freshly mixed for each test.

Test for zinc (Zn)**Reagents**

Solution A : 2N sodium hydroxide

Solution B: Dissolve 0.1 g dithiozone in 100 ml of carbon tetrachloride.

Procedure

Moisten filter paper with solution A, sprinkle the feed sample. Add 2 to 3 drops of solution B.

Positive results

Presence of zinc gives a strawberry red colour.

Test for phosphorus (P)**Reagents**

Solution A: Dissolve 5 g of ammonium molybdate in 100 ml of cold distilled water and add 36 ml concentrated nitric acid.

Solution B: Dissolve 0.05 g of benzidine (using base or its chloride) in 10 ml of conc. acetic acid and dilute with distilled water to 100 ml.

Solution C: Saturated sodium acetate solution.

Procedure

Moisten filter paper with solution A and dry it in an oven. Sprinkle the feed sample and add a drop of solution B and a drop of solution C.

Positive results

Presence of phosphorus will produce a blue ring.

1.2.1.6 Spot tests for toxic constituents**Nitrates****Reagents**

- Diphenylamine
- Concentrated sulphuric acid
- Distilled water

Procedure

Place the material to be tested in a white spot plate. Add 2 to 3 crystals of diphenylamine and a drop of water. Add a drop of concentrated sulphuric acid.

Positive results

Presence of nitrate will produce a deep blue colour.

Cyanogenic glycosides (HCN) in feeds**Procedure**

Prepare sodium picrate paper by dipping strips of filter paper into 1% picric acid solution and dry. Again dip it into 10% Na_2CO_3 solution and dry. Store these papers in stoppered bottle.

Take a small amount of ground feed sample in a test tube. Insert a piece of moistened sodium picrate paper in tube, taking care that it does not come in contact with sample. Add few drops of CHCl_3 (chloroform) and stopper tube tightly.

Results

If cyanogenic glycosides present in the feed, sodium picrate paper gradually turns orange, then brick red.

Note: Test is delicate and rapidity of change in colour depends upon amount of free HCN present. This test works well with fresh plant materials but relatively less sensitive to dry substances, particularly ground.

Aflatoxin

Reagents

- Methanol
- N-hexane
- Benzene
- Anhydrous sodium sulphate
- Green basic cupric carbonate

Procedure

Take 100 g of dry ground sample in a mixer and add 300 ml of solvent methanol: water (7:3). Mix the content at higher speed for 5 minutes. Allow to settle and then filter through double layer of muslin cloth using vacuum. Take 100 to 150 ml liquid filtrate in a separating funnel. Add 30 ml benzene and shake for one minute and add 200 ml distilled water. Allow to settle and discard lower layer. Place the upper layer into a beaker and evaporate to complete dryness. Re-suspend in 0.5 ml benzene. Spot 50 μ l on Whatman filter paper No. 4. Allow the spot to dry and place it under a long wave UV light.

Results

Development of a blue fluorescence colour on it clearly indicates that the sample contains aflatoxins. This method can detect aflatoxins at 10 to 15 ppb.

Notice: If the sample has a high fat soluble pigments, add 50 ml hexane to the separating funnel, shake for one minute and add 50-100 ml water. Take lower layer and discard the upper layer. Then proceed further with addition of 30 ml benzene.

Compounds other than aflatoxin present in benzene fraction and may produce fluorescence of their own or partially mask the fluorescence of the aflatoxin. To prevent this problem, collect benzene layer into a 50 ml beaker containing 10 g anhydrous sodium sulphate and 5 g of green basic cupric carbonate. Shake gently and filter through porcelain filter into a 50 ml flask and evaporate to dryness. Re-suspend in 0.5 ml benzene and follow the above procedure.

1.2.1.7 Other tests

Salt (NaCl)

Reagents

- Silver nitrate solution (5per cent)
- Nitric acid solution (1:2)
- Ammonium hydroxide solution (1:1)
- Standard sodium chloride solution (0, 0.1, 0.2 and 0.3 per cent)

Procedure

Weigh 1 g of feed sample and add 100 ml of distilled water. Stir and filter through Whatman filter paper no. 4. Take 1 ml of above feed solution and 8 ml of nitric acid solution in a test tube. Stir and add 1 ml of silver nitrate solution. Similarly add 1 ml standard solution (0, 0.1, 0.2 and 0.3 per cent) in different test tube and add 1 ml of silver nitrate solution. Compare the test sample with the standard sample. The test should be read within 5 minutes.

Positive results

Presence of salt will produce a white turbidity.

Rock phosphate or DCP**Reagents**

Hydrochloric acid (1:1)

Procedure

Take 5ml of hydrochloric acid (1:1) in a test tube. Add small amount of sample of rock phosphate in the tube.

Positive results

If it dissolves immediately means rock phosphate is present.

Carbonate**Reagents**

Hydrochloric acid (1:1)

Procedure

Put a small portion of the carbonate sample on watch glass. Add 4 to 5 drops of hydrochloric acid (1:1) and heat slightly. Observe it with hand lens.

Positive results

If carbonate is present, it will produce effervescence.

Meat cum bone meal and leather meal**Reagents**

Ammonium molybdate solution: Dissolve 5 g of ammonium molybdate in 100 ml of distilled water and add into 35 ml concentrated nitric acid.

Procedure

Place few feed particles in petri dish. Add 3 to 5 drops of ammonium molybdate and let it stand for 5 to 10 minutes.

Positive results

Presence of meat cum bone meal will produce a greenish yellow colour. Leather meal gives no colour change.

Blood**Reagents**

- Solution A: Dissolve 1 g of N, N-dimethylaniline in 100 ml of acetic acid and 150 ml of distilled water.
- Acetic acid
- Hydrogen peroxide (3 per cent)

Procedure

Place a few particles of feed sample on a slide. Mix 4 parts of solution A with 1 part of 3per cent hydrogen peroxide. Add 1 to 2 drops of this solution on feed sample.

Positive results

If blood is present, a dark green colour will develop around the feed particles. Low magnification stereo microscope can be used to observe the colour.

1.2.2 Chemical evaluation

An analytical laboratory for precise estimation of nutrient contents and contaminant is of utmost important. Analyze the feeds for proximate principles. This indicates possible constraints on usage due to the presence of excessive content of crude fibre, fat or total ash. Low crude protein (CP) and high crude fibre (CF) of oil seed meals indicates adulteration with fibrous materials. The high CP alone is indicative of adulteration with urea and or some inferior quality oil seed meals like mahua, castor or karanj cake. The amount of acid insoluble ash is a good guide to the amount of sand or other dirt which may be present.

It is also desirable to determine free fatty acid content of oily materials as this will affect palatability due to rancidity of oils. The specifications of various feeds and feed ingredients are laid down by the Bureau of Indian Standards (BIS) which acts as guidelines for the supplier, buyers and the users.

The commonly used analytical procedures for analysis of feeds and feed ingredients are given in part II of this manual.

Ingredient specifications

Ingredient specifications are essential in feed quality assurance programme. Specifications serve as the basis from which purchasing agreements are written, feeds are formulated and ingredient inspections are performed. Ingredient description and general nutritional specifications may be found in BIS specifications for feeds and feed ingredients in India.

Specifications of the feeds must be as comprehensive as possible, realistic and transmitted to the seller. These are the "measuring sticks" to which the delivered material must conform. Specifications are the foundation of a quality assurance programme because they serve as an understanding between nutritionist, purchase and production departments. A list of BIS specifications for feed and feed ingredients is presented as appendix in part III of this manual.

1.2.2.1 Analysis of feed and feed ingredients

Quality control laboratory should be well equipped for analysis of feed and feed ingredients. Since purchase and feed formulations are based on the analysis of raw materials, the highest degree of accuracy in analysis is desirable. All the samples should be analyzed in duplicate to minimize experimental error. The variation limit between duplication samples should be kept to minimum. In case of considerable variation, the samples should be re-analyzed. Accuracy of estimation can only be relied upon when pure reagents and precision instruments are used. The staff conducting analysis work should be qualified, experienced and efficient so that the analysis is completed as quickly as possible to facilitate prompt payment to the suppliers, usage of consignment and marketing of finished feed.

It is needless to emphasize that the authority of the animal nutrition officer should be final in the matter of accepting or rejecting raw materials and finished product. Keeping in view this important responsibility, it is felt that the animal nutrition officer should send reports directly to the administrative head of the organization.



Fig. 1.2 Generation of analysis report

Analysis report and reporting system

After the analysis of raw material and finished product, the results should be checked thoroughly and entered in the analysis register by the animal nutrition officer and not by any other staff of the quality control laboratory (Fig. 1.2). Analysis register and reports should be treated as confidential and kept under the safe custody of the animal nutrition officer.

1.2.3 Quality control of finished product

All measures which are concerned with the quality control of finished product should be carefully reviewed to maintain high standards. The finished product should be examined for colour, texture, pellet size, strength, aroma, palatability and chemical composition before delivery (Fig. 1.3). Periodical evaluation is also necessary to examine the health of the animals and effect of feed on their productivity through field survey. There may be seasonal variations in the standards or the basis of availability of basal diet and its quality and quantity during different seasons. The product manufactured should be analyzed and compared with set standards. The finished product lot should be cleared for marketing only after the approval of the animal nutrition officer. Besides quality, the net weight, proper packaging and safe transport to the retail sale outlets may also be ensured. It is of utmost importance for the production unit to take adequate care in compounding of the feed as per formula prescribed by the animal nutrition officer.

The wide variation in the chemical composition of the ingredients is obtained. This is the main constraint with which the nutritionists have to formulate the ration to maintain the quality of the feed at affordable costs. Choosing the best quality raw materials continuously throughout the year is nearly impossible. Further, nutritionists are not in a position to reject materials if there is variation in the specification since the availability is constant or lower and the demand is increasing. Therefore, fixing the cost of ingredients on the basis of nutrient content and using them in the formulation with certain additives is the most practical option possible.

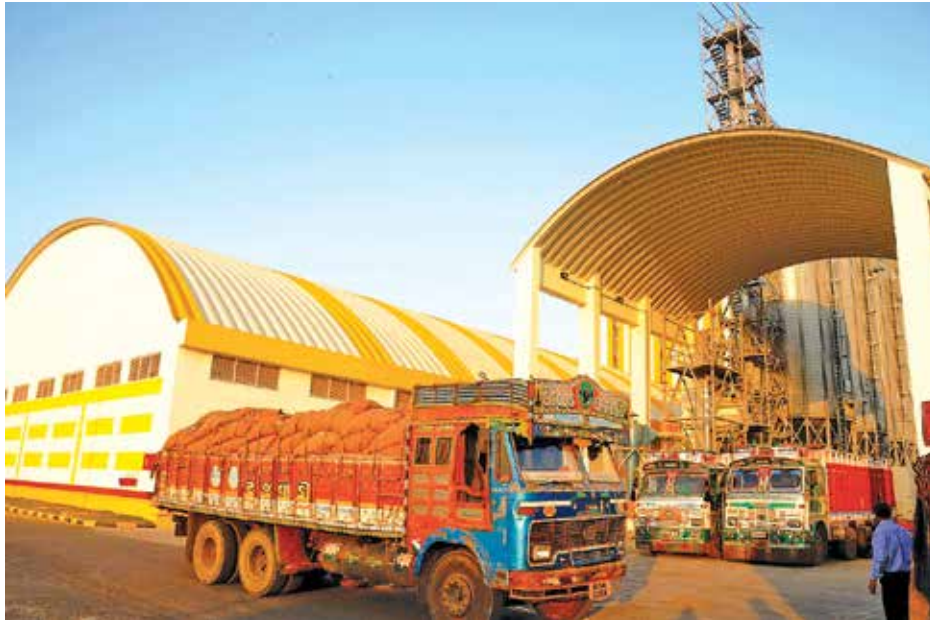


Fig. 1.3 Finished products should be tested before delivery

2.0 QUALITY CONTROL DURING STORAGE

2.1 Control measures

Every care must be taken in the storage of raw materials and finished products (Fig. 2.1), as one or more of the following problems may arise:

1. Infestation
2. Deterioration in quality
3. Rats and birds problem
4. Weight losses
5. Fire hazards

The storage godowns for raw materials and finished products should be separate. Raw materials are received from various sources and utilization of food grains unsuitable for human consumption is common. In such situations, infestations may occur and spread from one material to another. Cleaning, insecticide spraying and fumigations may help in checking infestation. Stacking of different materials should be done in such a way that there are minimum chances of cross infestation.

Deterioration of raw materials as well as finished products may occur due to high moisture content, rancidity, mould and fungi growth etc. Immediate removal of damaged materials from the godowns may help in checking further deterioration of the remaining stock. Further the deterioration of raw materials may occur due to long storage of raw materials, especially rice polish fine, rapeseed meal, grains. Normally such materials should not be stored in godown / silos not more than a month. First In First Out (FIFO) system should be adopted.



Fig. 2.1 Finished product

The raw materials with moisture content more than 10 per cent may result in drayage loss during storage. The longer the duration of storage, more will be the moisture loss. To compensate moisture loss in filled finished product bags, extra feed should be filled in each bag depending upon season and duration of storage in the godowns.

There are a number of raw materials which are inflammable. Fire due to auto-oxidation can also take place with materials having high moisture content. A certain limit should be fixed for accepting materials containing high moisture. To prevent fire hazards, 'no smoking' plates etc., should be affixed and strictly adhered to. The material and godown should be insured for fire. Fire extinguishers should be kept at all strategic points and the staff should be imparted fire fighting training.

Fire hazards in raw material godown/ silos /new gunny bag go-down may also take place due to following:

- Abnormally high stacking of filled raw material (RM) bags. Height of stack should not be more than 18 bags.
- Continuous length of stacking of RM bags. Size of stacking of bags should not be more than 5 M in length and 3 M in width. There should be a minimum gap of 600 mm between two consecutive bags to ensure proper air circulation.
- Storage of raw materials in silos without aeration and re-circulation system. To avoid rise in temperature in silos, aeration system (in case of grain silo) should be operated in night instead of in day time for better cooling efficiency. In case of DORB storage silos, re-circulation shall compulsorily be done at a regular interval at least once a day.
- Improper house-keeping in sorting area of new gunny bags godown. While sorting and printing new gunny bags, waste jute particles which are fire hazardous, are deposited on the floor. The area must be protected and floor should be cleaned regularly.

In certain situations, raw materials with relatively higher moisture content are accepted with rebates. Animal nutrition officer should advise the stores officer to exhaust such materials on priority. If the sample contains very high moisture or is of inferior quality/ wet/ damaged/ infested, it should be rejected partly and inform the stores and purchaser accordingly.

Sorting of inferior/ damaged consignments

Receipt of wet/ damaged consignment of raw material is also not uncommon. Inferior material in the interior of the bags is also observed. During sampling these things should be checked.

In case, the consignment has been transported by road, the vehicle driver should be shown wet or damaged consignment and his signature obtained on the goods receipt slip and register. If part of the consignment is either inferior or deteriorated or damaged, sorting of these materials should be done in supervision of the animal nutrition officer. Inferior materials should be rejected and supplier should be informed accordingly. If the supplier fails to lift the rejected material within a stipulated period, it should be disposed off at the supplier's cost. Sorting charges should be realized from the supplier. Lumps in de-oiled rice bran and rice polishing are quite common. Adulteration of rice polish fine with rice polish thick is also very frequent. The animal nutrition officer can immediately detect such inferior materials at the time of unloading and sampling. Depending upon the extent of damage, total or partial rejection of consignment should be decided.

Inspection of stores pertaining to quality control

The animal nutrition officer should regularly visit the stores. A careful and close examination for the presence of weevils, worms, moulds, fungal growth, rancid odour etc., is required. Evidence of damage to bags by rats and birds should be traced minutely. If there is any evidence of worms, weevils etc., immediate steps should be taken to undo the damage. The damaged material should be sorted and used or disposed immediately.

2.1.1 Rodent and pest control

Raw materials and finished product go-downs are the most desired places by rodents and pests, where they not only feed themselves but also multiply very fast. If proper control is not exercised there may be a heavy loss. Pests not only consume feed but also make feed unhygienic due to their excreta, dead body, skin casts, foul etc. A proper rodent and pest control programme should be planned and calendar of operation decided.

Rodent control

Rodents not only consume but also spoil a considerable portion of raw material, if they are allowed to inhabit in godowns. Squirrels, rats and mice are common rodents.

Following measures are suggested to control rodents:

1. **Trapping:** Various types of rat traps can be used for killing the rats
2. **Poison baiting:** This can be practiced as an effective measure to control rodents. Commonly used rodenticides are barium carbonate, zinc phosphate, red squill, phosphorus, tomorin, sodium fluoro-acetate etc.

The following techniques should be adopted:

- a) Spraying with malathion in high viscosity oil for repellent action.
- b) Placing poison baiting material at selected points.

Rodent proofing

Rodent proofing of go-down can be enforced by closing, protecting and preventing access to rats by using structural materials. Suitable guards are provided to check climbing, preventing entry through foundations, walls, floor, roofs, ventilators, doors, windows, etc.

2.1.2 Insect infestation and its control

This can be tackled by complete wiping out of the existing insects and safe guarding re-infestation.

These insects can multiply very fast under favourable conditions. For example, a small population of the flour beetle can multiply to 76 million within a period of six months at 28°C temperature and 65 to 80 per cent relative humidity. If infestation is heavy, the crawling insects can be detected on the bags, in between stacks, on floors and walls of the godown. To assess the extent of infestation about 3 kg samples should be collected from different stacks, sieved and examined. Table 2.1 may serve as a general guideline for insect infestation.

Table 2.1 General guidelines for insect infestation	
Clean	No insects in stacks or in the sieved sample or the sample incubated for 3 weeks
Light	2 to 3 adult insects in the sample with 1 per cent damaged grains
Medium	5 to 10 adult insects with 3 per cent damaged grains
Heavy	Crawling insects on the stacks, 10 to 20 adult insects in one kg sample, 5 per cent damaged grains
Very heavy	10 to 20 adults in 100 g sample. A rustling sound of insects can be heard near bags. Crawling insects on floor and walls

Common insects found in cattle feed raw materials



Fig. 2.2 Lesser grain borer



Fig. 2.3 Rice weevil

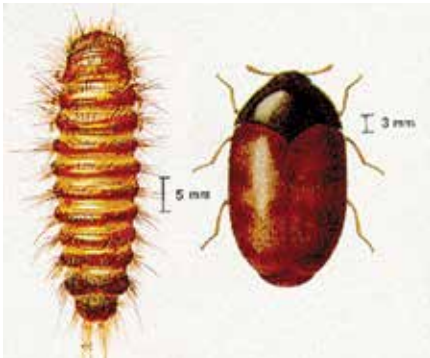


Fig. 2.4 Warehouse beetle



Fig. 2.5 Flat grain beetles



Fig. 2.6 Grain moth



Fig. 2.7 Confused flour beetle

Table 2.2 Common insects found in cattle feed raw materials	
Popular name	Product in which generally found
Rice weevil (<i>Sitophilus spp.</i>)	Maize, sorghum, wheat bran, rice polish, cowpea
Lesser grain borer (<i>Rhyzopertha spp.</i>)	Rice polish, pulses, tapioca
Warehouse beetle (<i>Trogoderma spp.</i>)	Groundnut
Flour beetles (<i>Tribolium spp.</i>) (bran bugs)	Maize gluten, cakes, meals, brans and polishes
Flat grain beetles (<i>Cryptolestes spp.</i>)	Maize, rice and wheat
Grain moth (<i>Coreysa spp.</i>)	Maize, wheat, milo and rice

Control of insect infestation

- i) **Spraying:** Spraying can be done to control light infestation. DDT/ lindane formulation, malathion, DDVP (Nuvon) are the usual insecticides used for spraying. DDVP can be used as 300 ppm solution to spray over the stacks and malathion 5per cent can be sprayed on the floor and walls up to 9 ft. height.
- ii) **Fumigation:** In case of heavy and very heavy infestation fumigation is advised. For this the stacks is covered with aluminum coated rubber sheets to make the stacks airtight and fumigation is done by supplying fumigants (e.g. aluminum phosphide) to the stacks.

Some of the fumigants used in cattle feed plants are listed below:

- ED:CT mixture 30 to 35 tables / 1,000 sq. ft. for 48 hours every 3 months.
- EDB and MB mixture 1 to 3 tables/1,000 cu ft.
- Celphos tables – 2 tabs/ton
- 16 per cent lindane in a smoke generator.

Precautions to be taken during the operation:

- No smoking
- Hand gloves, aprons, spectacles, gum-boots should be used
- Doors and windows should be kept opened.

Residual effect of pesticides and animal toxicity

Many pesticides have a residual effect. These residues at certain level may prove toxic to the animals. Thus these should be carefully used. Insecticides like ethylene dibromide, methyl bromide, aluminum phosphide, etc., generally have residual effect. The treated material should be exposed to air for some time before issuing for production.

Safeguard the infestation

It is better to adopt certain measures to prevent the store from infestation than to check it afterwards. Any raw material with moisture more than 10 per cent should not be stacked in the godown for longer period. The finished product should be free from feed dust or broken particles. Godown should be so constructed and stacking done in such a manner that cross ventilation is allowed. Cleaning of stacks and godowns should be done regularly.

Insecticides and pesticides residues

To control infestation of insects, pests and rodents in food grains, several insecticides, pesticides and rodenticides are used. The residues of these chemicals must be within the prescribed limits as per the Prevention of Food Adulteration (PFA) Act.

2.2 Quality changes in stored food grains

In India, the food grains produced are usually stored in bulk. Food grains during storage undergo certain physical, chemical and biological changes due to the presence of enzymes and bio-chemicals itself and the enzymes produced by the insects, pests and microbes or due to some other factors. These changes may deteriorate the quality of the grains. Usually the physical, chemical and biological changes occur in the food grains during harvesting, handling, transportation and storage.

Insects in stored grain can result in the reduction of both grain quantity and quality

2.2.1 Physical changes

The sound and the healthy grains are shining with good luster and show hardness. The various physical changes in the grains undergo during storage are dull colour, bores in grains, sprouting of seeds, damaged kernels due to bad weather conditions.

2.2.2 Chemical changes

Cereals are characterized by relatively low protein and high carbohydrate contents contained in kernel. The germ is rich in protein, fat, sugar and minerals whereas, the endosperm is low in protein, fat and ash contents.

The various chemical changes that occur during storage are due to increased activity of endogenous and exogenous enzyme responsible for quantitative and qualitative changes in carbohydrates, proteins and fats of the cereals in addition to colour, flavour and texture.

Carbohydrate

In India, the temperature and relative humidity varies greatly which causes physical and biochemical changes in grains during storage, such as bursting and gelatinization of starch and depending upon the moisture content. Amylases hydrolyse the starch into dextrose and maltose and significantly increase the content of reducing sugars during storage. Storage of wheat grains above 12 per cent moisture increased sucrose, glucose, fructose and raffinose contents. Storage of cereals with high moisture content also produces sour odour due to production of alcohols and acetic acid.



Fig. 2.8 Grain storage silo

Protein

The high temperature and use of chemicals in grains during storage (Fig. 2.8) denature the proteins and make them less dispersible in water, deteriorates the gluten quality and increase the free amino acid contents. The formation of certain sulphur containing amino acids impart bad odour. The free amino acids may also undergo Maillard reaction combining with the reducing sugars giving browning of the grains. The type of deterioration is possible at temperature above 20°C and relative humidity between 60-70 per cent.

Lipid

Oxidation of lipids especially the unsaturated fatty acids results in typical rancid flavour, odour and taste. Hydrolysis of lipids also increases free fatty acid contents, which is considered as a sensitive index for grain deterioration.

Contaminants

Food grains are usually contaminated with foreign materials viz stones, chaffs, poisonous weeds, excreta of insects, pests, rodents etc. which gives poor look to the grains. Limits of weed presence, uric acid and insect excreta should be as described by the Government of India (FCI) for the stored food grains. Before any recommendation is made in this regard, complete evaluation of these materials is required with respect to their chemical composition, toxins, residues of insecticides, pesticides and finally the *in vivo* feeding value of different categories of livestock.

3.0 QUALITY CONTROL DURING PRODUCTION

Quality control in feed production is of utmost importance in the overall success and profitability of dairy enterprise. There is no other factor, directly or indirectly related to the proper nutrition and high performance of animals that is more critical than feed quality control and ration consistency. The degree of quality is the consistency, in which feed is formulated, processed (Fig. 3.1), mixed and delivered as compared to what is expected. Animals respond better if the feed is low in nutrient variation as offered to them; and is similar in moisture content, texture and rate of energy availability.



Fig. 3.1 Controlling a plant operation

The relationship between feed quality and animal performance is important and encompasses not only the quantitative amounts of all feed components, but also the digestibility and metabolism of those components. Thus, the challenge for nutritionists and others involved in animal feed production is to consistently monitor all aspects of the feed production system being used and measure those variables that are good indicators of quality control. For the feed industry, a quality control system is the responsibility of management and involves personnel being properly trained to ensure a high level of organization, documentation, and the policing of various procedures and processes necessary to guarantee the basic quality of feedstuffs and feeds.

3.1 Quality check points during production

The activities of cattle feed production include transporting ingredients to the production block from raw material godown, cleaning and separating ingredients, mixing all ingredients in required proportions, crushing them into powder form wherever necessary, and to make the finished feed in mash or pellet form, weighing and packing in suitable bags. All these functions are performed under hygienic conditions by modern machinery.

The area of operation and vigilance on quality control by the animal nutrition officer is not restricted to a laboratory alone. He should make rounds of the plant and stores frequently to check the strategic points. Even minor negligence at these points may adversely affect the quality and cost of the product. However, making of rounds and checking all important points should be with the spirit of quality control only. The animal nutrition officer may counter check at the following points:

1. Bulk receiving operation
2. Intake of raw materials at the dumping hopper depending on aging time (FIFO) system)
3. Batch preparation operation
4. Grinding operation
5. Mixing operation-Dry
6. Molasses mixing operation
7. Pellet mill operation

8. Pellet Cooling operation
9. Moisture control operation
10. Bagging section
11. Bin sampling
12. Premix operation
13. Aspiration section

Today's high demands for cost effective, quality and high performance feeds, make it obvious to require a high level of specialized processing technology

3.1.1 Bulk receiving operation

The animal nutrition officer should check the incoming feed ingredients for their colour, texture, odour, foreign materials, wet spots, mould growth etc., to have a firsthand idea on the quality of raw material. Attention should be paid to ensure that the samples are collected systematically by the sampler and under his direct supervision. Identification of raw materials with barcode systems fit perfectly in a properly organized quality control system. Quality control officer should also instruct the supervisor for proper stacking of bags in raw material godown.

3.1.2 Intake of raw materials at the dumping hopper

Effective and dust free operated intake dumping points with a large conveying capacity are sufficient for reliable intake of materials. Raw materials are dumped as per a set schedule into various dumping hoppers provided in raw material godown (Fig. 3.2). The density of the material determines to a large extent the amount and speed of the conveyor. Raw materials issued for production are to be checked for their soundness prior to issue for production. Materials damaged during storage (due to high moisture, mould, pest, rodents or rancidity) have to be sorted and discarded. The dust extraction system provided at each dumping point must be monitored regularly and maintained periodically to ensure it is in proper working condition and is performing efficiently.



Fig. 3.2 Dumping of maize grain

An improperly functioning of dumping hopper aspiration unit not only leads to material (powder) loss but also an unhealthy environment for the dumping personnel. Magnetic grills if provided at the dumping hoppers must be cleaned periodically so that ferrous impurities do not carry forward. Raw materials dumped into the intake line conveyors are carried by bucket elevators to pre-weighing bins through cascade magnets (magnetic separators) and Jute twine removers (or drum sieves) to remove the ferrous and non ferrous impurities such as jute strings.

Quality control officer must periodically inspect the dumping stations and pre-weighing bins to determine whether impurities are being screened effectively both at the dumping station and at the entry point into the pre-weighing bins. Jute strings trapped at the dumping hopper grills or jute twine removers must be periodically removed and destroyed by burning or else they are bound to find their way back into the plant.

3.1.3 Batch preparation operation

Batching is considered to be one of the most critical and essential operations in feed manufacturing. Lack of proper batching can lead to reduced diet uniformity, affecting not only animal performance but regulatory compliance as well.

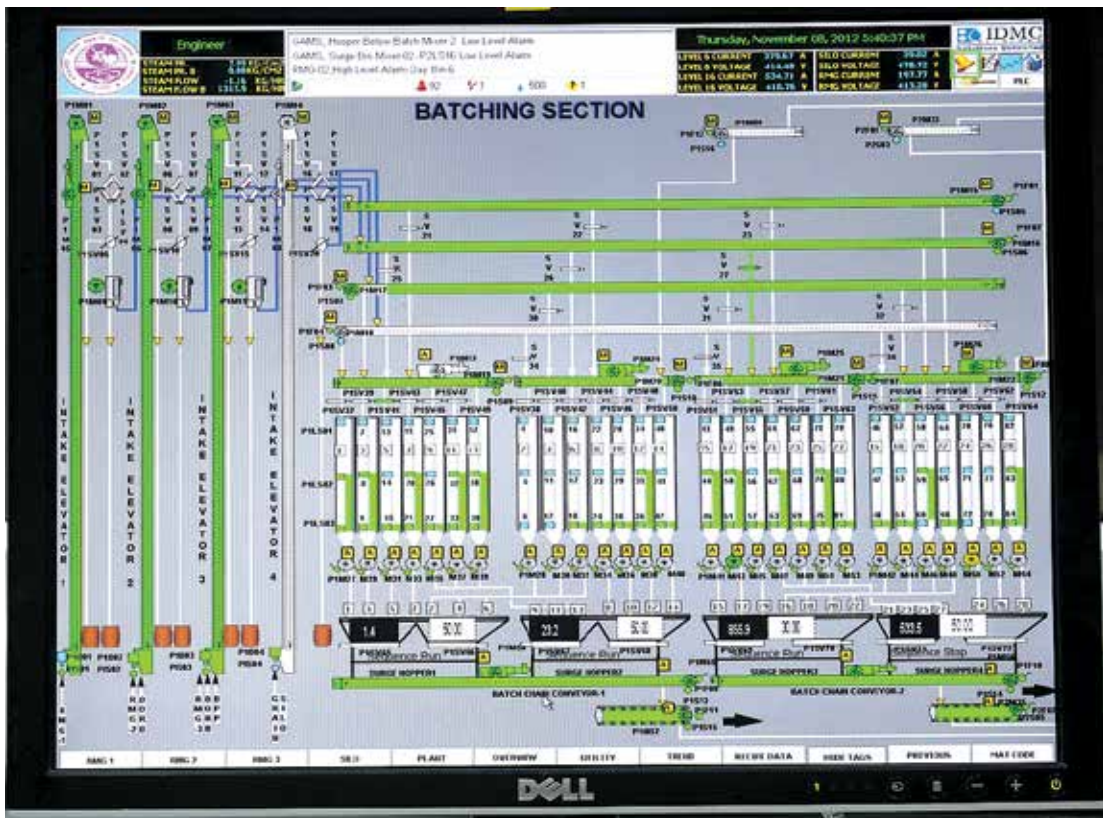


Fig. 3.3 Computerized batching

The batch section of the plant consists of mainly pre-weighing bins, bin dischargers, weigher, hopper below weigher, chain conveyor and elevator. Formulae containing various available ingredients stored in the RMG/ Silos based on least cost to produce a specific type balance concentrate feed are made available by the Nutrition Officer for production. All such ingredients are stored in various pre weighing bins. Plant is provided with automatic microprocessor controlled batching system for accurate batching (Fig. 3.3). As per feed formula, desired quantity of each ingredient is discharged from the pre-weighing bins (Fig. 3.4) into the batch weigher (through VFD operated bin discharger for more accurate batching) to make a complete batch of 1.0/ 1.5/ 2.0/ 2.5 MT (as per plant capacity) including molasses. Batching operation is done from the control room. Once the batch is ready, it is transferred from the batch weigher to the hopper below batch weigher through a pneumatic flap and the batch weigher starts getting the next batch. The complete batch is then mechanically conveyed to hopper above hammer mill in grinding section through batch chain conveyor and elevator.

The quality control officer must periodically check the accuracy reports of the computerized batch



Fig. 3.4 Pre-weighing bins

preparation system to ensure that weighing of individual raw material is in accordance with the feed formula and that large deviation of set and achieved weights are not taking place. Periodically, he should also check the accuracy of the weigh scale and calibrate the same if necessary.

3.1.4 Grinding operation

Grinding is the most effective processing technique to improve nutrient utilization and animal performance. For uniform particle size to ensure better quality pellets, the complete batch passes through the hammer mill (i.e. 100 per cent grinding after batching) prior to mixing. Thus grinding increases surface area of batch ingredients for improved rate of digestion, decreased segregation and mixing problems. Hence a grinding section has been provided with the plant to grind the complete batch materials to powder form. The grinding system consists of hopper, a rotary feeder with magnet, the grinding mill (Hammer mill; Fig. 3.5), collection hopper below mill, screw conveyor, elevator and aspiration system. The grindable raw materials are fed to the grinding mill by the rotary feeder through a magnet, where these materials are ground to powder. After grinding, the total powder batch is conveyed through conveyor and elevator and stored in hopper above mixer. Batch from this hopper is fed automatically to the batch mixer through a pneumatic flap. Quality control officer should ensure the particle size of grinded materials for proper mixing of raw materials. He should also ensure that the hammer mill sieve is of correct mm hole size and the same is not damaged so as to limit the ground particle micron sizes within the fixed norms.



Fig. 3.5 Hammer mill

3.1.5 Mixing operation-Dry

The mixing process determines, to a high degree, the capacity of the whole manufacturing process and the quality of the final product. Lack of proper mixing can lead to reduced diet uniformity, affecting not only animal performance but regulatory compliance as well. Mixing of batch in batch mixer (Fig. 3.6) is done generally for 3 to 4 minutes for proper mixing. The mixing accuracy/ homogeneity of a mixer indicates how efficiently a single ingredient is distributed among the totality of other ingredients. The homogeneity (Fig. 3.7) is expressed by the coefficient of variation CV (per cent). A good mixer should have a $CV \leq 5$ per cent in less mixing time. Interlocks are provided to ensure that only one batch at a time is mixed in the mixer. Quality control officer should also check for proper mixing of batch ingredients in mixed feed.



Fig. 3.6 Batch mixer

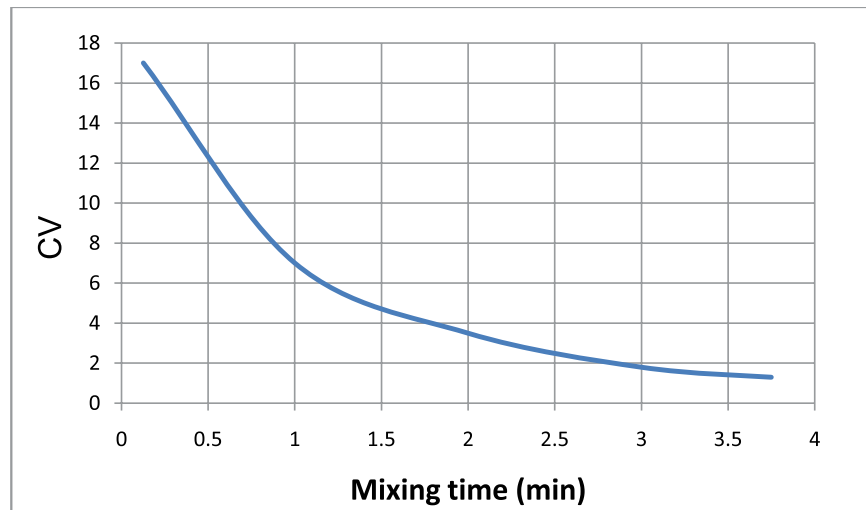


Fig. 3.7 Homogeneity is function of mixing time

The influencing factors on the mixing accuracy are:

- Properties of components: Best mixing results are achieved, if the particles in a mixture have only small differences in regard to granulation and specific weight

Rule of thumb:

- Granulation: Differences between components max. 3 mm
- Bulk density: Differences between the components smaller 0.5 t/m³
- Accuracy of proportioning: Proportioning has to fulfil the requirements
- Type of mixer (conical, U-shaped)
- Mixing time: Specific for each mixer type. The mixing time of conical shaped mixer: 5-20 min, circular horizontal single ribbon / peddle type mixer – 3 to 4 minutes and horizontal twin shaft paddles -1 to 2 minutes.
- Liquid addition: Liquids have to be added after the mixing process.

While going around the plant, the animal nutrition officer should ensure that the operators are cautious about the effects of using less or more raw materials. He should ensure that weighing of raw material is in accordance with the feed formula. Periodically, he should also check accuracy of the scale.

3.1.6 Molasses mixing operation

Molasses is an important cheap source of energy and hence is generally included in the feed formulation. Molasses is stored in MS storage tanks (Fig. 3.8). Quality of molasses should be checked for its density prior to transfer to storage tank.



Fig. 3.8 Molasses storage tank



Fig. 3.9 Molasses mixing

Batch after getting mixed in batch mixer is fed mechanically to the mixed feed bin above molasses mixer through elevator. The mixed feed is fed to the molasses mixer through loss in weight feeder for feed, where molasses at around 45-50°C from molasses heater is sprayed and mixed in controlled quantities (Fig. 3.9). The molassed feed is either conveyed to finished feed bins for bagging if the feed is required in mash form or is allowed to ripen in a small bin for few minutes. The molassed feed is finally fed to pellet mill through its feeder and conditioner, if feed is to be produced in pellet form.

Samples at random may be drawn from the feed coming out of the molasses mixer to test the quality of molasses mixed in feed. The material may be rubbed by hand and a typical texture and molasses aroma would approximately indicate the extent of molasses content in the material.

3.1.7 Pellet mill operation

The main purpose of this operation is to convert mash feed into pellet form by mixing with steam. Pelleting improves digestibility of feed and hence better the pellet, better the performance (Fig. 3.10 & 3.11). Pellet mill section consists of the feeder, conditioner, pellet mill, pellet cooler, elevator, pellet sieve, pellet cooler cyclone & its blower and air lock etc. The molassed feed is fed from the storage bin to the feeder and thereafter to the conditioner, where dry saturated steam is mixed with the feed and is fed to the pellet mill at a controlled rate.



Fig. 3.10 Better the pellets, better the performance



Fig. 3.11 Pelleting improves digestibility of feed

The feeder is driven by Variable Frequency Drive & conditioner is driven by mechanically controlled PIV gear. Both feeder and conditioner shall be of stainless steel AISI 304 to avoid rusting. Conditioner shall have machined steel rotor with adjustable and removable paddles and arrangement for electric heat shield of body for sanitary feed production along with all necessary insulation, earthing and thermostat. Apart from having arrangement of injecting and mixing dry saturated steam normally for 30 seconds, the conditioner shall have provision for injecting and mixing two different liquids uniformly.

The rate of feeding into pellet mill (Fig. 3.12) depends upon the condition of the pelleting die (if the die is new, it will not start immediate pelleting). Pelleting will start when all the holes of the die get polished and filled with the feed, condition of the feed (i.e. the feed should be neither too moist nor too dry). Pelleting takes place due to the process of extrusion. The press rolls provided inside the die, force the material to pass through the holes of the die and when the feed comes out of the die, it is in pellet form. The length of pellets can be adjusted by the knives provided in the machine.



Fig. 3.12 Pellet mill

To review the operation of the pellet mill, the animal nutrition officer should check the moisture per cent in feed before conditioning. He should collect sample after the ingredients are thoroughly mixed in the steam injection chamber and note the moisture content and temperature at which the material is being fed to the pellet mill matrix. This may also serve as an index of molasses content in the material. After pelleting, he should collect some hot pellets to have an idea about the quality of pellets (the broken portions, fines, size, pellets etc.). For efficient conditioning, the steam should be dry, temperature of feed in the conditioner should be in between 80-85°C and moisture before and after conditioning should be 12-13 per cent and 14 per cent, respectively.

3.1.8 Pellet cooling operation

Due to mixing of steam the pellets are hot, thus these must be cooled to avoid formation of lumps and deterioration of quality of the feed. Cooling of the pellets is done in the pellet cooler by induced cool air draft. While cooling the pellets, some of the powder and crumbled pellets are carried along with the exhaust hot air. These are separated by cyclone (Fig. 3.13) & air lock assembly and this material is again fed to the pellet mill for re-pelletizing. Cooled pellets are carried to the pellet sieve by an elevator. In the pellet sieve, crumbled pellets and powder are separated from the good pellets and sent to the pellet mill for re-pelletizing while good pellets are stored into the bagging bin.



Fig. 3.13 Cyclone

Quality control officer has to ascertain that the pellets coming out of the pellet cooler are properly cooled and do not contain excess moisture. Improperly cooled pellets will result in lump formation in the storage bags and bins.

3.1.9 Moisture control operation

Moisture control in the production of animal feed is desirable at several stages during the manufacturing process. With the ever varying moisture content in raw materials, on line measurement has become a key tool in ensuring optimum efficiency and quality control. The ability to measure moisture and make any adjustments during the feed manufacture process becomes critical at several stages during the production cycle.

Benefits

- Reduction in energy costs as the correct amount of energy required for drying can be accurately calculated
- Reduction in amount of wasted materials
- Consistent, repeatable quality for end product

Rising costs of raw materials increases the demand for product optimization and yields

Monitoring the moisture in the raw materials, controlling the water addition during the mixing/blending process and finally checking the moisture of the feed in pellet form, not only ensures an end product with consistent quality, but will enable substantial savings to be achieved in the production process. The producer should know the levels of moisture in the raw materials and should be able to accurately calculate the amount of moisture that needs to be added to the mix to achieve the correct consistency of the end product. These known levels will also enable the producer to accurately calculate the drying time needed and therefore potentially reduce energy costs.

3.1.10 Bagging section

This section deals with filling the required quantity of the finished product into bags, stitch them and transfer these to the finished product godown. The filling of bags is done by bagging machines (Fig. 3.14) and the filled bags are shifted to the finished product godown by a slat conveyor after being stitched by a stitching machine on their way. The bagging operation is semi-automatic. The bags are fixed manually and the filling is done automatically by the main feed and the fine feed mechanisms. Initially the main feed motor starts and when about 75 per cent of feed is bagged the supply switches over to fine feed to regulate the material at a slow rate for accurate filling.



Fig. 3.14 Filling of cattle feed in a bag

Quality control in charge should see that the pellets are properly cooled and their physical appearance is within the normal range before they are bagged and sealed. He should also ensure that the sample for analysis is collected from each bag and a composite sample of 100 bags is prepared. The packing material should be of standard specifications and free from contaminants.

3.1.11 Bin sampling

The animal nutrition officer should counter check the grinding and mixing operations. He should obtain bin samples from the mixing hopper and examine them for proper grinding and mixing up of ingredients (mixing of one ingredient into other). He should check the premix bin sample for proper grinding and mixing of the ingredients. If there is any discrepancy detected, it may be brought to the notice of the higher management for immediate rectification.

3.1.12 Premix section

In certain plants where the premix section is separate and complete with its own hopper, pre-mixer and storage bin for premix, the animal nutrition officer shall periodically check the accuracy of the weighing system provided for the individual weighment of the premix ingredients.

3.1.13 Aspiration section

To clear the dust that may have accumulated inside the equipment due to dumping of various ingredients in the hoppers, bins and other equipment, localized aspiration system (Fig. 3.15) operated with compressed air and having bag filters and blowers are provided in all dust generating areas like elevators, conveyors etc. in the plant. The dust powder collected by aspiration system is put back to the process line for minimizing the process loss. Air breathers are provided on top of hoppers/ bins for releasing air and



Fig. 3.15 Aspiration system

arrest dust. In some equipment, air balancing ducts are provided to neutralize the pressurized dust air. In case of pellet cooler aspiration system, instead of bag filter, cyclone with air lock, and blower with ducting are provided. The outlet emission of pellet cooler cyclone should be within 150 mg / cu m. All bolted joints in equipment, conveyors are provided with silicon sealant / good quality felt packing to arrest leakage of dust through joints.

QC officer should check periodically the proper functioning of the compressed air based jet filler system of these aspiration units and also ensure that these filler bags are not torn due to continuous use.

3.2 Determining excess or short usage of raw materials

Batching operation in most of the plants is now-a-days automatic and controlled through a computerized batch weighing system. Accuracy level of 5 to 10 kg is possible. If the error is more than 10 kg, it should be pointed out to the production management for rectification. Adopting the latest technology available in feed industry, now-a - days, all the new feed plants are equipped with auto batching operation, automatic bagging machine for bag filling, energy efficient equipment, new design empty gunny bag cleaning machine, and hence the handling and process losses are reduced drastically.

The verification is made from the daily raw materials usage statement and actually calculated quantity as per the formula. The difference between two statements gives daily excess/ short usage of raw materials. This should either be assessed by the production superintendent or

the animal nutrition officer. If this verification is not done, it will reflect on the stocking of ingredients and the chemical composition of finished products may vary beyond permissible limits. Verification also helps in controlling the usage of urea, minerals and vitamins, and production cost of feed.

3.3 Quality commitment and points to evaluate

To organize an in-plant quality control programme, an overview of the total operation is the primary consideration and the development of a quality control manual is logical first step as a useful guide to action. As an employee training tool and as a reference for all cattle feed plant's (CFP) personnel, typical quality control manuals will usually have the following:

- An index or outline of content.
- A statement of the CFP's quality control philosophy.
- In-plant quality control supervisory and operator duties and responsibilities.
- Sampling practices and procedures for ingredients and finished products.
- A suggested ingredient assay schedule.
- Laboratory report including interpretation as to their use.
- Regulation and compliance (Good Manufacturing Practices).
- Production record keeping and procedures.
- Package weight control, labelling and coding.
- Complaint procedures.
- Product recall procedures.
- Rework material guidelines.
- Housekeeping (sanitation) requirements.
- Ingredient purchasing specifications.
- Warehousing and pest control practices.
- Shelf-life and finished product turnover standards.
- Guidelines for medicated feed manufacturing and handling.
- Plant formula guidelines/standard operating practices for handling of new and old formula.
- Employee training in quality control.
- In-process sampling, testing method and test equipment for particle size reduction, batching and mixing, pellet quality etc.
- Maintenance practices and responsibilities.
- Assignment of one person for total coordination of the programme. The person should be given clear authority to articulate conditions and problems to management and should not be restricted in that by purchasing, production, sales or any other person or function.
- All plant personnel, including delivery personnel should be involved in the programme and trained to perform individual quality control duties.
- All quality control stations-receiving; the various processing locations such as grinding, mixing, pelleting and others should be provided with the necessary test equipment, forms for recording test results, sample bags and other supplies.
- Periodic, routine compliance inspections should be conducted by appropriate management personnel using checklist to ascertain that the CFP's quality commitment standard are being met and the results of those inspections should be shared with all levels of management as well as with plant's employees.

3.4 Computerized least cost feed formulation (LCF)

Genetic improvements are placing constant pressure on feed manufacturers to formulate and produce quality feeds that match the increased requirements of improved breeds without creating additional physiological or health stress. Increased understanding of animal nutrition and the environmental impact on animal performance caused feed manufacturers to alter business. In a cattle feed plant, one of the most significant aspect of production is to derive an appropriate formula to produce nutritionally balanced feed at the lowest possible cost. This formulation can be worked out by using a computer. Computer assistance should be sought to arrive at a nutritionally adequate LCF formulation. Today it is experienced that the linear programming model is a helping hand to the animal nutrition officer in his venture of quality control operation in cattle feed plant (Fig. 3.16).

LCF formulation is a mathematical solution based on linear programming technique. It combines many feed ingredients in a certain proportion to provide the target animal with a balanced feed at the least possible cost. Linear programming is a mathematical technique to investigate all possible formulae, which can meet the specific requirements and work out the cost. Within minutes the formulae and their costs are reviewed by the computer to provide the LCF formula. The National Dairy Development Board (NDDB) of India has established expertise in the field of LCF formulation, dynamic production and procurement system and is extending such facilities to interested organizations in dairy cooperative sector.



Fig. 3.16 Computerized control using LCF

In computing the least cost formula, the marginal value of each of the nutritional element in the total requirement of raw materials is found by linear programming technique. By adopting this technique, the values arrived at would mean increase or decrease of 0.1 per cent of the nutritional element in the cattle feed and would affect the cost of the cattle feed by the amount equivalent to the marginal value of that element. It is observed that if the raw materials which are mixed to get the cattle feed, involve least cost technique and provide for more than specified requirements of nutritional elements in the cattle feed, the addition or removal of 0.1 per cent of the nutritional elements will not significantly affect the nutritional value of the cattle feed. Therefore, the marginal value of this 0.1 per cent can be considered as zero. This aspect gains significance in the analysis, as the marginal value or the shadow price of each element in the cattle feed would tend to vary with the specific conditions under which optimal formula is prepared. The knowledge of this becomes more important in deciding whether a raw material whose market price is more or less equal to its intrinsic value (this can be arrived at by pricing the nutritional element at the shadow price) should be purchased or not.

3.4.1 Dynamic production and procurement system

Although adoption of least cost formulation system helps in the reduction of formula cost, the overall reduction in the cost of production can be brought about through a dynamic production and procurement system. It aims at minimizing the total cost of the ingredients procured over the whole year. It works on a series of "price forecasts" for all the ingredients. "Price forecasts" are based on various factors amongst which past experience plays an important role. Using these forecasts, it prepares purchase schedules and taking into

account the stocks available and likely purchases, it works out consumption patterns which help in deriving a monthly or seasonal formula. This also takes care of capital involvements in the purchase schedule. The prices of ingredients fluctuate sometimes very rapidly and often unpredictably. It is possible, therefore that in a given month, on the basis of the price forecast for an ingredient and the possible substitutes, the programme recommends purchase of an ingredient but in the next month prices of the substitute ingredients may fall unexpectedly. Then the question arises: should we take advantage of the low price and buy this ingredient: If so, in what quantity? This question is answerable by the dynamic production and procurement system.

Advantages of dynamic production and procurement system

1. It works on the estimation of prices over the succeeding 12 months and therefore, the purchases are scheduled when it is most advantageous. For example, suppose the presence of an ingredient in the formula can reduce the cost of the formula for the current month, but the stock has run out, then the better of the following two alternatives will be recommended:
 - Buy now at higher price if it can reduce the overall cost of formula.
 - Buy later when the price is likely to reduce, and bear a slightly higher cost of formula for the current month.
2. It recommends procurement schedule by thoroughly analyzing the stocks available.
3. It retains the use of a limited stock of any ingredients. The static method requires an arbitrary limit for each month, while the dynamic method needs a limit which is 'just right' the whole year.
4. It can also suggest selling ingredients back to the market whenever found to be the best course of action.
5. Dynamic procurement-production model can compete favourably with other methods of procurement and production planning, as it uses forecasts to plan the subsequent actions more systematically. Moreover, it is based on a rolling year and can view 12 months ahead to make monthly schedules.

3.4.2 Required information for computerized feed formulation

The following information is required for finalizing computerized LCF and procurement system:

1. Forecast of raw materials prices
2. Availability of raw materials
3. Composition of raw materials
4. Nutritional specifications for a particular quality of feed
5. Group constraints
6. Individual constraints
7. Numbers of bins in the plant
8. Production capacity of the plant
9. Storage capacity for raw materials and feed plant
10. Demand forecasts for the feed

A part of the information stated above will remain unchanged in a cattle feed factory. While the entire information would be needed to start with but later on only the changes would be required. The collection of above information can be coordinated by one officer and maintained at one central place and updated periodically. A discussion on the guidelines in furnishing this information, therefore, will be necessary.

Price forecast

Least cost formulation, dynamic production and procurement system require price forecast of raw materials. Since the prices fluctuate unpredictably, an analysis of past purchases can give an idea of future price variations. The price forecast so derived, may be adjudged by the purchase officer since he is always in touch with the market. If the past data are not available, the purchase officer should forecast the prices by consulting a sister organization or through regularly contacting the market, trade journals, brokers and by other means of communications.

The purchase officer should keep in mind a rough price forecast while planning procurement. Using the rough price forecast as a starting point, the dynamic production and procurement system can complete favourably with the help of other methods of planning and procurement. Finally, it may be possible for the purchase officer by continuously remaining in contact with suppliers, brokers and other trade media, to forecast reasonably accurate prices.

Availability of raw materials

The raw materials available in the stores and expected arrivals can be combined together to furnish data of raw materials for LCF and procurement system.

- Composition of raw materials: Average composition of each raw material in the godown or average of a large number of samples analyzed in past may form the basis to furnish information.
- Nutrient specifications for a particular quality of feed.

The animal nutrition officer must keep in mind the type of animal and the type of physiological function for which finished feed is desired while making specifications for minimum-maximum levels of nutrients and other constraints. There should be different specifications for different species such as cattle and buffalo, and various physiological functions like growth, milk production etc. Specifications formed on the basis of seasonal variation in the energy and protein contents. These can be lower for rainy/winter season and higher for summer season depending upon the quality and quantity of basal feed generally available to cattle and buffaloes during the season.

Constraints

Constraints are generally resorted to restrict the use of individual or a group of ingredients to a maximum or minimum limit. These constraints are based on the nutritional evaluation of the individual/ group of raw materials, and their stock position. For deriving a LCF in the real sense, it is also necessary that minimum possible constraints may be imposed in the data fed to computer. It may be noted that the computer works out all permutations and combinations from the data fed, to arrive at a LCF. Group constraints are allowed for a free choice by the computer among a listed similar type of ingredients e.g. grains, brans, oil cakes etc., subject to limitations of individual restrictions within the group.

Constraints or restriction should be provided by the animal nutrition officer. Individual restrictions should be generally based on toxic principles including anti-metabolites, palatability etc. For example, use of salseed oil meal cannot be left free with the computer as higher level in cattle feed will be hazardous for the animal. The price constraint is being taken care of in the least cost method.

Similarly, when there are many items, which are not very palatable and small quantity of each, added together may have an additive effect and render the feed unpalatable unless a group constraint on unpalatable items is applied. In yet another case, which is very common in these days, is the frequent use of unconventional items because of their lesser cost. As sufficient information is not available on many of these ingredients, to be on the safe side, it is desirable to put a maximum limit on the use of these unconventional ingredients together. A symbol code must be put against all concerned ingredients to indicate the group to which they belong.

3.4.3 LCF within a given number of bins

When the LCF is computed using linear programming, computer selects the ingredients out of a list of ingredients in such proportions that the required nutritional and other conditions are satisfied and the formula has the lowest cost among all formulae which can be obtained from these ingredients. However, there is no way of constraining the number of ingredients which appear in the LCF obtained through the technique of linear programming. This may make the formula impractical, if the number of ingredients appearing in the formula exceeds the number of bins in the plant. Another practical difficulty is that, often some ingredients may appear in quantities so low that the equipment in the plant may not be able to measure such small volumes with the necessary accuracy. In such cases, either the ingredients are dropped or they are adjusted to a measurable fraction of the scale number in the final composition of the formula.

A technique known as 'Mixed Integer Programming' can solve both these difficulties. It is thus possible to specify the maximum number of ingredients which may appear in the formula and the smallest quantity which can be accurately measured by the plant equipment. The cost of the formula obtained thus may be higher than cost of formula obtained by the use of linear programming technique. This is because of the additional constraints such as the one placed on the number of bins. However, the cost of the formula obtained by using Mixed Integer Programming is less than the cost of the formula obtained by manual adjustments to the linear programming solution in order to eliminate some ingredients from the formula. Mixed Integer Programming, therefore, results in further saving of cost.

3.4.4 LCF for various classes and physiological functions

Most of the cattle feed plants under dairy cooperative sector formulate and provide feed for cattle only with the objective to enhance milk production. Cattle feed plants may manufacture feed for buffalo also. In addition, milk replacers, calf starters, grower feed etc. may be manufactured by CFPs for various physiological functions of cow/ buffalo.

3.4.5 Addition or deletion of ingredients in LCF

Based on animal nutrition research and the possible utilization of various agro industrial by-products, new feed ingredients may be added in the list of concentrate feed ingredients. However, in many cases sufficient experimental evidences are not available to decide the inclusion limit. Inclusion of such materials in the formula on limited information may prove quite risky. The animal nutrition officer should remain in constant touch with the latest research and development activities as to suitably modify his approach. Any new feed ingredient should preferably be evaluated from the palatability, toxicity etc., angle and their limits for inclusion decided accordingly.

3.5 Losses in feed weight

Shortages/ losses can be estimated under following sub heads:

Handling loss

These are the losses due to loading, unloading and transportation from godown to intake hopper or railway siding to godown.

Storage loss

This is due to moisture evaporation, insect infestation, rodent and birds, putrefaction and formation due to bad storage conditions or rancidity.

Production loss

This dust loss is due to various processes, such as conveying, cleaning, mixing and grinding. Leakage of dust through various conveyers and machines can be kept to minimum by

using effective packing at all bolted joints and by maintaining effective aspiration system. In grinding, loss occurs due to removal of moisture. Powdered material going out as losses can be minimized by properly maintaining the suction fans and bag filters of the dust extraction systems. However, de-oiled cakes (light material such as rice bran) suffer maximum loss due to grinding.

3.6 Improving pellet quality and efficiency

The first step for improving pellet quality is to design a quality testing programme that is meaningful and reflects the actual conditions that product is manufactured under. To accomplish this goal, plant management staffs need to understand the methods of describing pellet quality and then apply these methods to the products they manufacture. Once these tests are in place, a well defined monitoring programme, using data analysis techniques that can improve the process, needs to be established. Once these steps are undertaken, plant management and staff can isolate process problems and correct them, while continually improving pellet quality.

3.6.1 Pellet quality issues

Since its introduction, pelleting has become an important process to the feed industry. Pelleted diet can affect animal performance in a variety of ways. In addition, pelleting allows the use of a wider variety of ingredients without obvious changes in the physical properties of the diet. Pellet quality issues can be partitioned into several individual components and their contribution of each component.

3.6.2 Factors affecting pellet quality

Issue number one in pelleting of course is pellet quality. There are several factors in the pelleting process that influence pellet quality.

3.6.2.1 Formulation

Keith Behnke of Kansas State University concludes that formulation has the largest impact on pellet quality, followed by grinding and conditioning (Fig. 3.17). Formulation is based on meeting the nutrient requirements of the animal at the least possible cost.

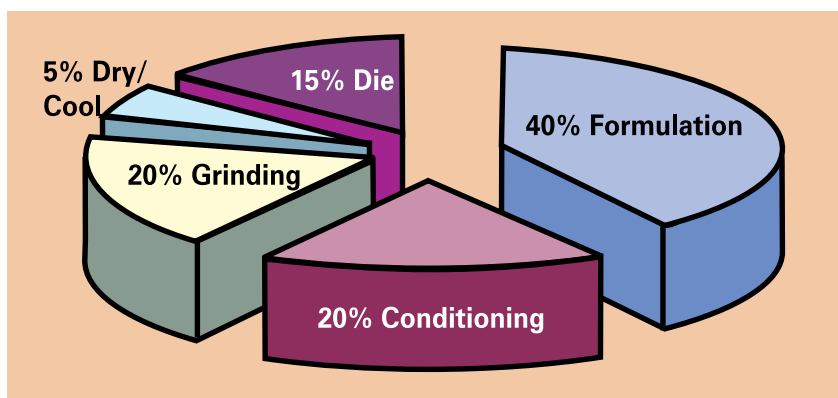


Fig. 3.17 Factors affecting pellet quality

3.6.2.2 Conditioning

Conditioning can be defined as “any modification or addition made to the mash after it leaves the mixer and before it reaches the pellet die chamber”. One has to consider that pellet quality is primarily established in the conditioner, not in the pellet die.

Conditioning is the most critical step to manufacture quality pellets. Regardless of conditioning technique used, the fundamentals are the same: heat and moisture are added to the dry meal. Conditioned meal is then readied for plasticization during the pelleting process. The moisture is critical for the ease of passage

In order to obtain a quality pellet, the steps taken before pressing the meal through the die are more important than the actual pelleting itself

of the meal through the die, and for proper agglomeration. Heat energy is critical for uniform transportation of moisture to the centre of the meal particles. Conditioning can improve pellet quality and reduce the amount of energy required to pellet. Proper conditioning of the meal prior to physical agglomeration also facilitates the pelleting process.

Atmospheric conditioners are fairly simple in the design, essentially being mixing vessels with steam and liquid ports. However, several criteria that are essential to the proper operation and maintenance of conditioner are:

- Effective steam hardness (quality & heat content)
- Proper steam pressure
- Correct pick configuration
- Retention time

The most critical aspect of conditioning involves regulation of steam. Typically this is accomplished through the use of steam hardness. It is essential that good steam has the right quantity of heat to optimize the migration of moisture into the interior of the meal particles. Unfortunately, good quality pellets cannot be achieved by maximizing the conditioning temperature alone, but also by understanding the critical factors that influence steam quality and conditioner performance. These factors include good, clean steam, a firm grasp on mixed meal, moisture & temperature and, proper steam pressure adjustment capability.

3.6.2.3 Grinding

Grinding has a considerable impact on pellet quality (20 Per cent). As a rule finer the grind, better the pellet quality. Generally, compound feed will have an average particle size of approx. 0.5 to 0.7 mm, with no particles > 1.0-1.5 mm. Otherwise; such a particle will act as a kind of predetermined breaking point in the pellet.

3.6.2.4 Die/roller

The die is still responsible for 15 per cent in the quality of the pellet. Once the pellet is forced through the die little can go wrong when the pellets are properly cooled. The rollers in the conjunction with the die are the most significant elements of a pellet mill. It is vital to ensure the most uniform spreading of the mash across the entire width of the die and the rollers in a pellet mill in order to achieve the most consistent pelleting conditions possible (Fig. 3.18).

3.6.2.5 Cooling

The pellets are additionally dried during the cooling phase. The temperature of the air flowing through the counter current cooler is increased by hot pellets. This enables air to absorb more humidity, and the pellets are dried. This shows that high air volume is not necessary to achieve a good drying result. Large air volume means high air



Fig. 3.18 Uniform pelleting should be desirable

flow velocity and therefore a shorter period of contact with the product. Air is heated to lesser degree and can therefore absorb less humidity, which in turn degrades the drying result. For this reason, the air volume must be optimised relative to the required values.

Other factors

Storage

During pellet storage, temperature is crucial. Pellets must be sufficiently cooled after the pelleting process. Pellet temperature should be in a range lower than 10°C above the ambient temperature. This will prevent condensation inside the storage bins. The storage bins should be equipped with an autonomous exhaust system to absorb major fluctuations in the climate which might affect the pellet quality.

Handling

During transportation and handling of pellets, attention must focus on minimising production of fines. As a result, agitation of the pellets must be minimised. At the same time, care must be taken to ensure movements that are easy on the pellets: low conveying velocities, low friction and low falling heights. The term pellet quality in this case means hardness and stability.

Two basic different measurement methods exist to assess and describe the durability and stability of pellets. One is "hardness" testing which measures the force required to destroy the pellet. The other measurement method is the so-called "durability" test. It uses test devices to investigate the actual loads acting during transportation and handling on the pellets, simulating them and measuring the weight (percentage) of fines produced or the weight of the pellets left over.

3.6.3 Advantages of pelleted feeds

1. Compound cattle feeds (Fig. 3.19) are composed of a variety of ingredients. Pelleting helps in disallowing the ingredients to segregate. It does not allow the animals to sort out certain ingredients and reject others.
2. It reduces wastage of feeds.
3. Pellets are less subject to infestations by insects and moulds.
4. Pelleting preserves vitamin A potency and ensures less storage losses and prevents disintegration of nutrients after the feed is mixed.
5. It reduces the possibility of adulteration of feeds with undesirable substances.
6. It is easy to handle.
7. Pelleting kills bacteria like Salmonella and E. coli, if any happen to be present in the feed due to exposure to high temperature.
8. Increases palatability and digestibility.
9. Reduces to certain extent, the microbial degradation of protein.



Fig. 3.19 Compound cattle feed

3.6.4 Pellet durability index (PDI)

Texture of feed pellets is considered an important criterion for the acceptance of compound cattle feed by milk producers. Cattle feed plants in routine test the feed pellets for chemical composition, but not for texture. Sometimes, the feed pellets are either too soft or too hard, much to the dissatisfaction of customers. Texture of feed pellets largely depends upon the composition and mode of processing in the feed plant. Fineness of feed particles, fibre, fat and silica content in the formulation play important role in the quality and texture of feed pellets. Pellet durability tester is a useful device for testing the texture of feed pellets (Fig. 3.20).



Fig. 3.20 Pellet durability tester

Take test pellets of diameters 9 to 20 mm, length up to 30 mm, totalling approximately 100 grams. After testing, express the final weight as percentage of original weight to give the durability value. For example, original weight of pellets is 100 gm and the weight after testing is 97.5 gm. Thus, the sample has a "Pellet Durability Index" of 97.5 per cent.

Guidelines for using pellet durability tester

1. Sample should be properly sealed, firm and dust free.
2. Minimum sample size is 1 kg.
3. Two runs of 30 sec. are sufficient in duplicate sample.
4. Select only premium pellet.
5. Clean the tester at regular interval.

3.7 Contamination/ cross-contamination

- Contamination is the presence of substances in a feed which does not belong to the recipe.
- If these substances belong to a previous processed batch, it is treated as a cross-contamination.
- Impurities from macro – ingredients are not critical.
- Cross-contamination through highly concentrated additives can be critical for animals.
- Measures for minimizing the cross-contamination risk:
 - Separate mixing line for each species.
 - Smaller and more flexible mixing lines
 - Hygienic design of the machines + conveying elements minimizing the remaining residues.
 - Bins and hoppers must have mass flow characteristics.
 - Avoid long conveying distances
 - Central aspiration system after the addition of the additives is to be avoided. Localized aspiration system having bag filters with compressed air, blowers is to be provided.

3.8 Plant design

Co-operatives are facing direct competition with private players and hence it is necessary to produce quality product at a competitive price.

Modern feed plants require high capacity throughput and a compact design

In cattle feed industry, the feed cost is gradually increasing due to increase in cost of infrastructure facility, cost of raw materials as well as increase in processing cost due to increasing cost of labour, energy & consumables. As such it is difficult for co-operatives to compete with the price of inferior quality of feed produced by private manufacturers. In order to produce better quality of feed at competitive price, all the new cattle feed plants being executed by the NDDDB are designed by incorporating the following:

- Adopting latest technology available in feed industry
- Incorporating Energy Efficient & Hygienic Designed Equipment
- Changing the process flow
- Providing mechanical conveying system for material handling in place of suction pneumatic system
- Providing localized aspiration system in all dust generation areas for dust free operation
- Increasing the level of automation to reduce labour cost and dependence on labour.
- Facilitate for easy handling of raw materials in RMGs and finished feed.

3.9 Future trends

Feed cost represents the major item in the cost of animal production. Without doubt, efforts will continue to refine feed manufacturing techniques to reduce cost of feed and to increase its value to the target animal. Some of the following future trends will play a role for improving the quality of feeds.

Odour control

Given current trend in dust emission control, the next step may be odour control for CFPs. Technology is presently available to reduce odour emission to near zero. However, it is costly and adds no value to the product.

In-line pellet moisture monitor

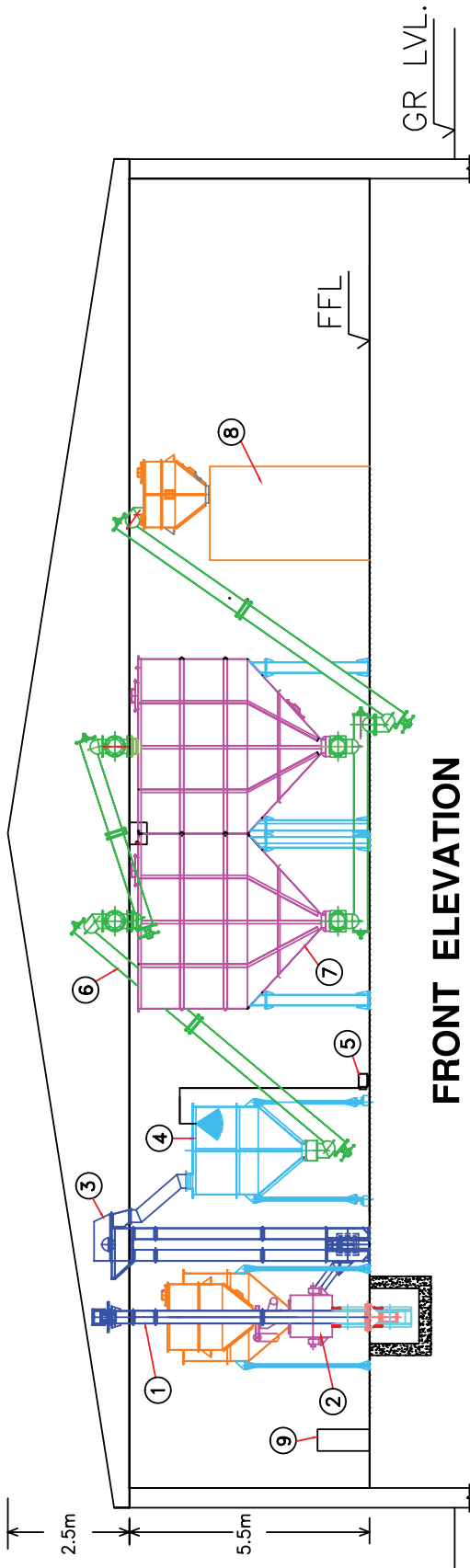
Rising costs of raw materials increase the demand for product optimization and yields. Now, the means of accurately controlling the moisture content within a batch flow process has been finally breached within the industry (Fig. 3.21).

Several prototype and production models of in-line pellet moisture monitors are available. For successful adoption, these monitors must be given feedback control capabilities to be able to control residence time and air flow in coolers. Both temperature and moisture content are important to shelf-life and must be controlled. In addition to controlling coolers and dryers, in-line moisture monitoring and control is being adopted in the pre-pellet area. The concept is to refine management of feed moisture to improve pellet quality and production rate while controlling moisture shrink in the final product.

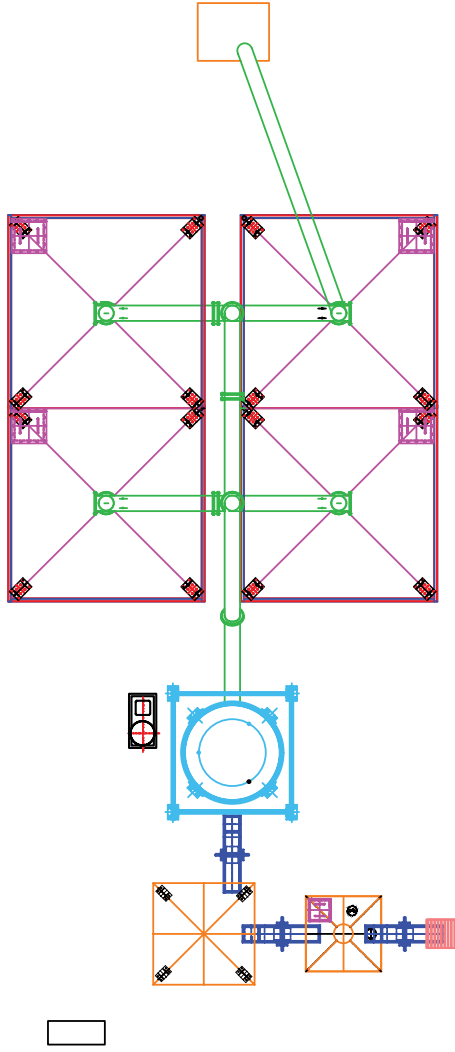


Fig. 3.21 Moisture meter installed in a conveyor

BYPASS PROTEIN PLANT LAY OUT (CAPACITY 50 MT / DAY)



FRONT ELEVATION



PLAN

LEGEND: --

- 1. INTAKE ELEVATOR
- 2. FULL CIRCLE HAMMER MILL
- 3. BUCKET ELEVATOR (POWDER)
- 4. FOUNTAIN MIXER WITH LOAD CELL
- 5. FORMALIN DOSING UNIT
- 6. AIR TIGHT SCREW CONVEYOR
- 7. AIR TIGHT STORAGE SILO
- 8. BAGGING OFF NET WEIGHER
- 9. CONTROL PANEL

4.0 BYPASS PROTEIN SUPPLEMENT PRODUCTION PROCESS

Protein is usually the first limiting nutrient for cattle and buffeloes fed low quality forages. All ruminants derive their protein requirement from two sources. One is rumen un-degraded feed protein that gets enzymatically digested in the abomasums and small intestine and another source of protein is rumen microbes. The microbial fermentation of soluble protein in the rumen is an unavoidable consequence of digestion and under many circumstances; it is a wasteful process because high quality proteins are broken down to ammonia, excess converted to urea in the liver and excreted through urine. The solubility of proteins change when subjected to special chemical treatment, advantage of which is to protect good quality proteins from rumen degradation (Fig. 4.1). Number of chemicals like acetaldehyde, formaldehyde, glutaraldehyde, ethanol, tannic acid, acetic acid, sodium hydroxide etc. have been tried to protect proteins. Amongst all, aldehyde suggested for protection of protein, formaldehyde has been extensively used for production of bypass protein feed.

Usually, protein meals are fed to ruminants in India, which have variable degree of naturally rumen protected proteins. Protein meals, particularly rumen escape proteins, play a very important role as excellent protein supplement, in livestock feeding. When these meals are fed as such to ruminants, about 70 per cent of the protein is broken down to ammonia by the rumen microbes in the rumen and a significant portion is converted to urea in liver and excreted in the form of urea through urine. However, if these protein

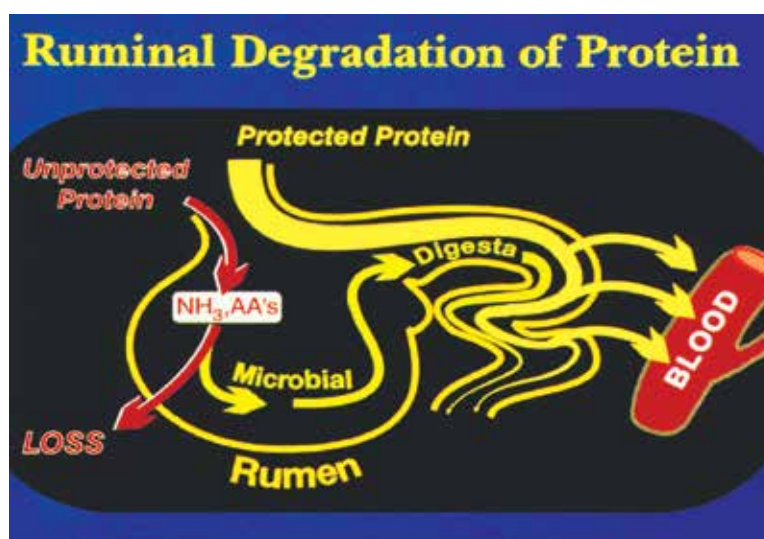


Fig. 4.1 Ruminal degradation of protein

meals are subjected to suitable chemical treatment, then their efficiency of utilization can be significantly improved. When chemically treated protein meals replace untreated one, then due to less degradability of the protein in the rumen, excessive loss of both nitrogen and energy could be avoided, resulting in an increased energy and nitrogen balance, leading to increase in milk yield and milk constituents.

Bypass protein feed is a new generation cattle feed in India and is manufactured by a special chemical treatment, developed by the National Dairy Development Board (NDDB). Bypass protein feed contains a large percentage of solvent extracted protein meals in treated form, grain by-products, whole grains, molasses, minerals and vitamins. The special feature of the formulation is that more than 70 per cent of the total protein in the feed is rumen undegradable protein.

Characteristics considered desirable for protected protein supplements

- High level of crude protein.
- Optimal essential amino acids profile.
- About 70-75 per cent of the protein to be in a rumen un-degradable form (UDP).
- Approximately 80 per cent of the rumen un-degradable protein to be digestible in the small intestine.

4.1 Nutritional properties of formaldehyde treated protein meals

Usually, rumen degradability of crude protein from protein meals is in the range of 50-75 per cent (Table 4.1). As a result, net availability of amino acids for milk production is low. If these protein meals are given suitable chemical treatment to reduce rumen degradability of protein to 25-30 per cent, net availability of amino acids could be increased for milk synthesis. The amount of formaldehyde required to

optimally protect protein in different protein meals, without decreasing the digestibility of protein and essential amino acids is very important. If excess formaldehyde is used to protect protein, then the complexes formed between formaldehyde and amino group of protein are acid resistant (Ashes *et al.* 1984) leading to reduce protein digestibility and bio-availability of essential amino acids at intestinal level. In effect, the protein will be “over protected” from ruminal degradation and metabolism.

Protein meal	CP (per cent)	UDP (per cent)
Rapeseed meal	37.0	34.0
Sunflower meal	28.0	31.0
Soyabean meal	46.0	36.0
Groundnut meal	39.0	33.0
Guar meal	48.0	39.0
Cottonseed meal	38.0	51.0

4.2 Production process for bypass protein feed supplement

- Load the protein meal under the treatment on to the hopper and transfer via auger to the vibro feeder. Then grind it to 2-3 mm particle size by the hammer mill and transfer to the fountain mixer via auger.
- When half (50 per cent) of protein meal is transferred to the mixer, operate the formaldehyde pump to discharge liquid formaldehyde having viscosity and specific gravity similar to water.
- Mix protein meal with desired level of formaldehyde (37-40 per cent w/v) for 15-30 minutes.



Fig. 4.2 Bypass protein feed manufacturing plant

- Simultaneously, the remaining half of protein meal continues to pour in the mixer, to avoid formation of formaldehyde lumps at the bottom of the mixer.
- After receiving complete protein meal in the mixer and desired quantity of formaldehyde, allow the protein meal in the mixer for homogeneous mixing for another 15 minutes.
- Discharge the treated protein meal to air tight silo and store it for a period of 8 days.
- After 3 days of incubation, treated meal can be transferred to HDPE laminated bags, for storage up to 6 days (Fig. 4.2).
- Treated protein meal(s) can be fed either directly as top feed or can be incorporated in compounded cattle feed @ 25 per cent.

Specifications for packaging bags for bypass protein supplement

The packing material should be of standard quality and free from contaminants. For packaging of formaldehyde treated protein meals, 25 kg HDPE woven sack laminated bag can be used with following dimensions:

Size : 25 kg HDPE laminated
 Length : 30 inches
 Width : 22 inches

4.3 Operational and safety guidelines for bypass protein plant

Operation of protein mixer:

1. Turn the dust collector on.
2. Put on the dust mask.
3. Turn on the mixer and load mixer with feed.
4. Close the hatch and turn off the mixer.
5. Turn off the dust collector.
6. Put on formaldehyde mask, gloves and face shield.
7. Fill the formaldehyde container.
8. Turn the mixer on.
9. Calibrate the pump. This should be done only for the first run of the day and need not be repeated thereafter.
10. Turn on pump for the required time schedule.
11. Continue to run the mixer for five minutes after the pump is switched off.
12. Fit bulk bags to auger. Take care to use formaldehyde masks while bagging.
13. Turn on out feed auger.
14. When mixer is empty, tie the bag.
15. Turn off mixer and auger.

Protective clothing and equipment:

1. Wear a laboratory coat and gloves when handling formaldehyde.
2. Face shield or goggles to protect the face and eyes from splashes should be worn when mixing or handling formaldehyde.
3. Gumboots and an impervious apron should be worn when handling tanks or drums of formaldehyde.

4. Apply a good quality barrier cream on hands and forearms to prevent dermatitis and general skin irritation.
5. Respiratory equipment must be used if formaldehyde is handled without adequate ventilation and there is a risk of inhalation.

Using formaldehyde

1. Keep the emergency face/ eye wash and shower clear from obstruction.
2. Wash your hands, arms, face and neck thoroughly after work and before eating.
3. Do not leave any containers of formalin in the sun or exposed to high temperature.
4. To prevent spillage always move large tanks and drums with the lids firmly on. Never try to move these items by yourself.
5. Always handle formaldehyde near a reliable supply of unlimited water to dilute spills etc.
6. Maintain a high standard of laboratory housekeeping.
7. Only use specimen containers with airtight lids and proper seals. Use bubble wrap under lids of large tanks to improve the seal.
8. Formaldehyde should be stored in a cool, well-ventilated location out of direct sunlight and away from other sources of heat and ignition.

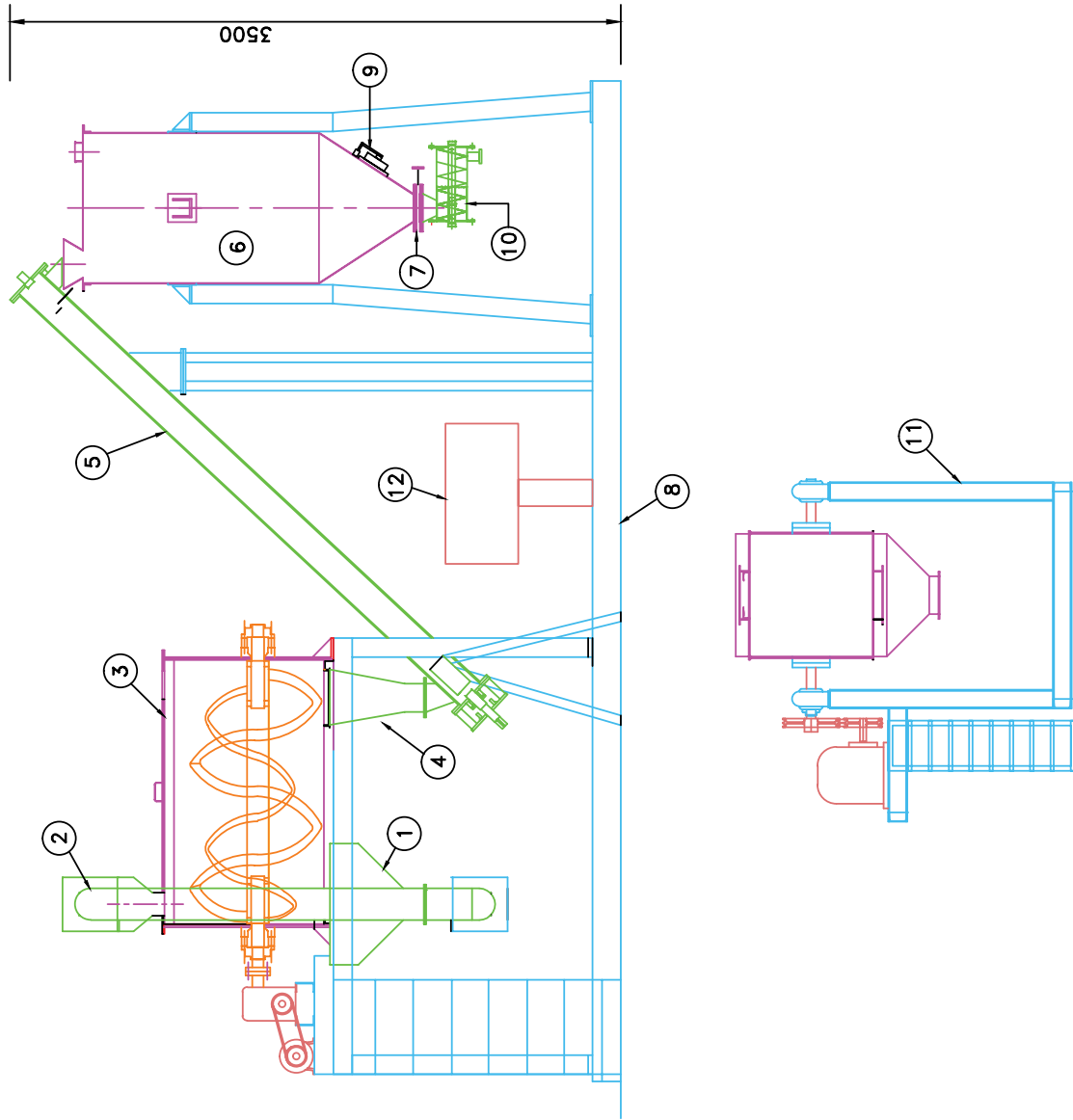
4.4 Operational health and safety aspects

Chemical treatment of protein meal(s) is carried out in a specially designed airtight plant, to minimise risk to workers operating the plant. Moreover, workers at the plant are advised to wear gloves, masks and safety glasses. Food and Drug Administration of US (US-FDA) approved use of formaldehyde as a feed additive to protect proteins from ruminal degradation, to preserve silages, to maintain animal feeds and feed ingredients free from Salmonella and to control fungi. Chemical used in treatment of protein meals is at very-very low levels and it poses no health risk to animals and the consumers. Therefore, its production and use does not pose any risk to animals and human beings.

4.5 Benefits of feeding bypass protein feed

- Increase in efficiency of utilization of proteins.
- Increase in availability of essential amino acids.
- Increase the supply of limiting amino acids like lysine and methionine to the small intestine.
- Improvement in milk production.
- Improvement in fat and SNF per cent.
- Better growth in young animals.
- Easier to meet the requirement of high yielding animals.
- Improvement in reproduction efficiency.
- Better resistance against diseases.
- Helps in increasing net daily income.
- Helps to control Salmonella and reduce mould growth in feedstuffs.

MINERAL MIXTURE PLANT LAYOUT (CAPACITY 12 MT / DAY)



LEGENED:--

- 1 INTAKE HOPPER
- 2 INTAKE INCLINED SCREW CONVEYOR
- 3 RIBBON MIXER
- 4 DISCHARGE HOPPER
- 5 SILO INCLINED SCREW CONVEYOR
- 6 SILO
- 7 SILO DISCHARGE SLIDE VALVE
- 8 SKID
- 9 E.M.HAMMER
- 10 SCREW BIN DISCHARGER
- 11 BALL MILL
- 12 CONTROL PANEL

5.0 MINERAL MIXTURE PRODUCTION PROCESS

Dairy cattle and buffaloes require a number of dietary mineral elements for normal body maintenance, growth and reproduction. Minerals that are required in relatively large amounts are called major or macro elements. Those needed in small amounts are classified as micro, minor or trace minerals. The major minerals include calcium, phosphorus, magnesium, potassium, sodium, chlorine and sulphur. Among those needed in trace amounts are iron, zinc, manganese, copper, iodine, cobalt and selenium. Deficiency of minerals in the ration of animals impairs metabolic functions, which affects the growth in young calves and milk production and reproduction efficiency in adult animals. Supplementation of bio-available minerals through mineral mixture is of paramount importance, as minerals are nowhere synthesized in animal's body.

Mineral mixture plants in different parts of the country produce mineral mixture using a ribbon mixer, in which all macro and micro mineral salts are dumped, without prior grinding. Mineral mixture contains mineral salts as high as 66 per cent and as low as 0.06 per cent. If all the mineral salts are dumped as such without prior grinding and mixing using proper diluents then the stability and uniform mixing is a problem. As a result, animals don't get various minerals in a desired proportion, when such mineral mixtures are supplemented in the ration of animals. Moreover, existing mineral mixture plants don't use mineral salts which are in dried or as monohydrates. These salts when mixed in a particular proportion and stored for further use, lead to lump formation.



Fig. 5.1 Mineral mixture manufacturing plant

Therefore, a mineral mixture plant (Fig. 5.1) has been designed in which dried/monohydrate salts are crushed and mixed to a uniform particle size, using proper diluents, in a separate device, called ball mill. This is called pre-mix, which is taken in the ribbon mixer, along with other macro mineral salts for uniform mixing and distribution. The resultant mineral mixture thus produced is a free flowing powder, which contains all mineral salts in desired proportion and stable form.

Table 5.1 Mineral salts with active element		
Element	Mineral salt	% (Min.)
Calcium & Phosphorus	Di-calcium phosphate (DCP)	Ca: 23.0%; P: 18.0%
Calcium	Calcite powder	Ca:37.0%
Magnesium	Magnesium oxide	Mg: 52.0%
Sulphur	Sodium thiosulphate	S: 39.0%
Copper	Copper sulphate (Dried/ monohydrate)	Cu: 24.0%
Zinc	Zinc sulphate (Dried)	Zn: 33.0%
Manganese	Manganese sulphate (Monohydrate)	Mn: 31.0%
Iodine	Potassium iodide	I: 75.0%
Cobalt	Cobalt sulphate (Prefer dried)	Co: 20.0%
Iron	Ferrous sulphate (Dried)	Fe: 30.0%
Chromium	Chromium chelate (trivalent)	Cr: 4.0%

5.1 Mineral mixture production process

Mineral mixture is manufactured using di-calcium phosphate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) of rock phosphate origin and dried/monohydrate mineral salts (Table 5.1). Dried/monohydrate mineral salts are crushed and mixed to a uniform particle size, using proper diluents, in a separate device, called ball mill. This trace minerals pre-mix is taken in the ribbon mixer, along with DCP and few other mineral salts, for proper dispersion and uniform mixing. The resultant mineral mixture thus produced contains all mineral elements in desired proportion and stable form. Mineral mixture should not contain any ingredient of animal origin, even in traces.

5.2 Functions of different minerals

Table 5.2 Functions of different minerals	
Minerals	Functions
Calcium	Essential for milk production Necessary for bone & teeth formation Required for contraction of muscles
Phosphorus	Essential for milk production Required in energy metabolism Required for bone & teeth formation
Magnesium	Important for the integrity of bone & teeth Involved in protein synthesis and metabolism of carbohydrates & lipids
Sulphur	Required for protein synthesis and metabolism of carbohydrates & lipids Sulphur is a part of B-complex vitamins, thiamin & biotin
Sodium & potassium	Required for maintenance of osmotic balance Required in acid- base equilibrium
Copper	Required for haemoglobin synthesis Necessary for tissue pigmentation & component of several metallo-enzymes Required for normal reproductive functions
Zinc	Spermatogenesis & the development of primary & secondary sex organs Required for normal functioning of epithelial tissue Activates vitamin A & its deficiency leads to night blindness
Manganese	Co-factor for many enzymes involved in carbohydrate metabolism Activator in the synthesis of fatty acids
Iodine	Required for the synthesis of thyroid hormone (T_3 & T_4) Necessary for reproduction & growth of animals
Cobalt	Required for the synthesis of vitamin B_{12} by the rumen microbes Essential for haemoglobin synthesis
Chromium	Component of Glucose Tolerance Factor (GTF) Required for the metabolism of carbohydrates, protein and fat Helps in improving immune status of animals

5.3 Major equipments of mineral mixture plant

The mineral mixture plant has been designed in two sections. In first section, pre-determined quantities of trace mineral salts are crushed into fine powder form in a ball mill of capacity 500 litres. In the second section, crushed mineral salts (trace elements) are thoroughly mixed with other ingredients viz. calcite powder, magnesium oxide, sodium thiosulphate and di-calcium phosphate in a ribbon mixer and conveyed through screw conveyor to a storage silo. The mineral mixture plant consists of the following major equipment.

Ball Mill

In the ball mill (Fig. 5.2), pre-determined quantities of trace mineral salts are poured in the ball mill drum for grinding. The mixture is then crushed into fine powder form. The ball mill should be of 500 litres capacity and SS 304 rotating drum with minimum 60 kg of EN-31 balls of 50 mm diameter. Approx. 120 balls are required for crushing the granule to such an extent that 90 per cent of crushed powder should pass through 212 micron IS sieve. The crushed powder shall be collected in 50 kg bags manually.



Fig. 5.2 Ball mill

Intake inclined screw conveyor

Di-calcium phosphate, magnesium oxide, calcite powder and sodium thio-sulphate along with the trace minerals pre-mix should be dumped into the hopper mounted on the conveyor to feed the ribbon mixer with the desired quantity.

Ribbon mixer

The ribbon mixer (Fig. 5.3) has a rated capacity of 500 kg. However, it is designed to handle maximum 700 kg, which is 40 per cent higher than the rated capacity. It has provision for forward and reverse rotation of the ribbon shaft for thorough mixing of various mineral salts and pre-mix, in maximum 30 minutes time. Raw materials are fed from the inlet at the top cover and after thorough mixing discharged from the bottom outlet, having a manually operated valve.



Fig. 5.3 Ribbon mixer

Silo inclined screw conveyor

The hopper of the conveyor receives the thoroughly mixed mineral mixture powder from the ribbon mixer bottom outlet. The conveyor of 2.0 MT/hr capacity transfers the powder to storage silo.

Storage silo

The SS 304 storage silo of 700 kg capacity functions as an intermediate storage till the mixed mineral mixture powder is packed in bags. The silo (Fig. 5.4) has a top inlet and a bottom outlet, provided with manually operated slide gate valve and motor operated bin discharger.



Fig. 5.4 Storage silo

5.4 Start up & shut down procedure

Ball mill

1. Check the correct level of oil in the gear box.
2. Check the correct direction of rotation of the ball mill drum.
3. Open anyone hand hole cover. Ensure that the hand hole cover on the other side is fully tight.
4. Remove the cover, place 120 nos. EN-31, 50 mm diameter balls inside the drum.
5. Load the drum with trace element pre-mix (as per the formulation).
6. Close the hand hole cover tight and close the ball valve mounted on the cover.
7. Close the hinge cover of the SS shroud.
8. Now switch on the ball mill. Ensure that it rotates clockwise if viewed from the motor side.
9. Initially operate for an hour, stop the switch and open the hinge cover of the shroud.
10. Slowly open the ball valve of the hand hole and see if any pressure releases from the drum. Sometimes the ball valve gets choked with the fine mineral mixture powder. Insert a rod through the ball valve opening and ensure that there is no choking in the ball valve and that all the pressure buildup inside the drum has been released through valve.
11. Open any one hand hole cover by loosening the opposite side nuts and then remove the cover from the drum.
12. Take a sample and see the fineness of the powder. If the desired fineness is not achieved, tighten the hand hole cover, close the ball valve and run the ball mill for one more hour. The ball mill is designed to crush the batch in maximum 4 hours time. However, depending upon the granule size available and the moisture content, the time can be standardized by hit and trial method, which could be from 1 to 2 hours.
13. Once the desired fineness is achieved, remove the hand hole as mentioned in step 10.
14. Place SS grill cover on the hand hole and tighten it. Then close the hinge cover of the shroud.

If the mineral salts of desired specifications are not taken in the ball mill then all the mineral salts will tend to form a mass, sticking to the sides of the drum and discharge of such a mass will be difficult

15. Place an empty bag fastened by hooks below the shroud discharge valve and then open the discharge flap valve.
16. For decanting, start rotating the drum by putting the switch ON.
17. Once the total crushed powder is collected in the bags, stop ball mill and bring the grilled hand hole position on top.
18. Remove the grilled cover, re-charge the drum with fresh ingredients and close the hand hole cover.
19. Repeat the process till the complete batch is crushed to the desired fineness.
20. Once the batch is over and the ball mill is not required to operate for quite some time, ensure that the drum is completely emptied and the hand hole cover is closed.
21. Switch off the main switch.

Mineral mixture plant

1. Check the correct level of oil in the gearbox of ribbon mixer, intake inclined screw conveyor, silo inclined screw conveyor and bin discharger.
2. Check the correct direction of rotation of the ribbon mixer and screw conveyors.
3. Manually close the ribbon mixer bottom outlet.
4. Put ON the intake inclined screw conveyor and ribbon mixer. In the auto mode, the ribbon mixer will run in forward and reverse direction for a pre-set time.
5. Start dumping all ingredients in the hopper of intake inclined screw conveyor. For dumping of ingredients following sequence should be maintained:
 - First load DCP in the intake hopper.
 - Then load the remaining ingredients (Pre-mix, MgO, calcite powder, sodium thio-sulphate) and,
 - At the end, load remaining DCP in the hopper.
6. In the first batch, add approx. 25 kg of DCP extra and load in the intake hopper at the end. This extra DCP will remain in the Intake screw conveyor. The net batch size for the ribbon mixer shall therefore be of 500 kg.
7. From second batch onwards, load the material as per the Sr. No. 5.
8. Run the ribbon mixer in auto mode for 15 minutes. The mixer is set for forward and reverse rotation in auto mode and will mix all the ingredients thoroughly.
9. Close the discharge valve of the storage silo and start the silo inclined screw conveyor to transfer mineral mixture powder from ribbon mixer to silo.
10. Put the ribbon mixer in the forward rotation mode and open the bottom discharge valve of the ribbon mixer.
11. Mineral mixture will start pouring into the small hopper of silo inclined screw conveyor and convey to the storage silo.
12. At the end when material stop coming out from the outlet valve, quench the ribbon mixer by reverse and forward operation for some time, so that all the powder drops into the hopper of silo inclined screw conveyor.
13. Close the bottom outlet valve of the ribbon mixer.
14. With a time lapse, stop the silo inclined screw conveyor.
15. Repeat the procedure for next batch.

Cleaning procedure

If the plant is not in operation for a longer period, it is advisable to completely unload the material.

1. The ribbon mixer can be emptied completely by quenching in forward and reverse movement.
2. In the intake feed screw conveyor and silo inclined screw conveyor, a hinge door has been provided at the lower most portion of the conveyor. By partially opening the hinge door, the accumulated material in the screw conveyor can be collected in the bag. Initially the material will fall down due to gravity; entire material can be removed by quenching the screw in the forward direction.
3. Silo is an intermediate storage facility before bagging the entire batch. However, it should not be used as storage for longer period. The mineral mixture has tendency to absorb moisture if it is exposed to atmosphere for long time. If the mineral mixture remains in silo for long, it might absorb atmospheric moisture and chances of material choking the silo increases. Hence, mineral mixture should be transferred to bags immediately and not stored in silo for long time.

Table 5.3 Technical details of mineral mixture plant	
Ribbon mixer	Capacity –500 kg mineral mixture/batch Bearing both end- UCF 212 Motor – 12.5HP, 3 Phase, 1455 rpm, H X F 160ml, B4 – Crompton/ABB make Gear box – AS-60-PP-160, Ratio- 18:55, mounting B5 X B3, Bonfiglioli make Gear box output to Ribbon mixer shaft –Flexible coupling size- FC 9"
Intake inclined screw conveyor	Capacity –2MT/hr. Bearing both end – UCF 208 Thrust bearing on bottom – 51408 ZKL Motor – 5HP, 3 Phase, 1425 rpm, Crompton /ABB make Gear box – AS-45-PP-112, Ratio- 15:83, Mounting B5 x B3, Bonfiglioli make Gearbox output to conveyor shaft – Coupling – Chain type-3/4", 18 teeth
Silo inclined screw conveyor	Capacity –2MT/hr Bearing both end – UCF 208 Thrust bearing on bottom – 51408 ZKL Motor – - 5HP, 3 Phase, 1425 rpm, Crompton/ ABB make Gear box –AS-45-PP-112, Ratio- 15:83, mounting position-B3, Bonfiglioli make Gearbox output to Intake screw conveyor shaft – Chain type-3/4", 18 teeth
Bin discharger	Capacity–700kg/hr Bearing both end – UCF 204 Motor –2HP, 3 Phase, 1421 rpm, Crompton/ ABB make Gear box – AS-25-PP-90, Ratio- 11:51, mounting position-B3, Bonfiglioli make Gearbox output to Bin discharger shaft – Chain type-1/2", 18 teeth
Ball mill	Capacity – 500 litre (200 kg trace mineral salts) Bearing on both end shaft – 22216K Pedestal Size – S 516 Sleeve size – H 316 Motor –7.5HP, 3 Phase, 1430 rpm, flange mounted- ABB make Gear box – AS-60-PP-132, Ratio- 25:47, mounting position-B3, Bonfiglioli make Gear box output to Ball mill shaft – Chain type-3/4", 22 teeth

Table 5.4 Maintenance schedule for mineral mixture plant

Equipment	Description and schedule
Intake dumping hopper with magnet grill	Clean the magnet grills everyday (remove iron particles).
Intake screw conveyer	Check gear box oil level every 15 day. Tight all drives bolts and nut every week. Greasing all bearings every 20 days. Open bottom lead and remove material at the end of production ever day.
Ribbon blender	Clean filter bag of blender every day. Greasing both end bearings every 20 days. Tight graphite gland packing every week. Replace gland packing after 3 to 4 months if adjustment is no more. Tight every drives nut and bolts every week. Check oil of gear box every 20 days.
Manually operated disc valve	Check nut & bolts and put oil drops in both the bush if find operating hard.
Silo feed conveyer	Check gear box oil every 15 days. Tight all drives bolts and nut every week. Greasing all bearings every 20 days. Open bottom lead and remove material at the end of production every day.
Storage silo	Check nut bolts and cable connection of vibrator every week. Clean the filter bags on top of silo every day.
Discharge conveyer	Check gear box oil every 15 days. Tight all drives bolts and nut every week. Greasing all bearings every 20 days. Open bottom lead and remove material at The end of production every day.
Ball mill	Check gear box oil every 15 days. Tight all drives bolts and nuts every week. Greasing all bearings every 20 days.
Motor control centre	Clean dust in inside panel with blower. Tight the terminals if found loose.

PART - II

COMMONLY USED ANALYTICAL PROCEDURES

6.0 GOOD LABORATORY PRACTICES

- All the apparatus used should be scrupulously washed and kept clean.
- If a grease film persists even after cleaning with a detergent, prepare chromic acid by adding 100 ml conc. H_2SO_4 to approximately 3.5 ml of saturated potassium dichromate solution. The mixture can be reused till it becomes greenish. The dichromate will strongly adhere to the glass surface. Wash it with ordinary water and finally with distilled water.
- Do not add water to acids as it leads to spurting.
- Use fume cupboards to protect against any types of fumes.
- If you happen to suck the acid into your mouth, wash repeatedly with copious amount of water.
- The last drop in the pipette should not be blown off, as it is not counted in the calibration of the pipette.
- As a general rule, calibrated glasswares viz. pipettes, burettes, measuring cylinders should not be heated or cooled rapidly as it will lead to change in volume.
- Animal nutrition laboratory (Fig. 6.1) should be well ventilated and provided with exhaust fans for effective removal of fumes.
- Use protective materials like apron, goggles, gloves etc. depending on the need. This will protect against artistic holes in your clothes.



Fig. 6.1 Animal nutrition laboratory

In case of fire victims, put off fire by covering with blanket or gunny jute cloth and rolling the person on the floor. To put out fire and to cool the affected skin, water should be applied in large amounts. Formation of blisters due to water application will enhance the curing.

- Know the location and operation of the fire extinguisher.
- Never try to carry out unauthorized or unassigned adventures. Violation of this may cause unexpected material damage and physical injury.
- Label your reagent bottles, standards and all solutions immediately after preparation. Read labels carefully before using any of the reagents.
- Do not move the reagent bottles to your bench from the side-shelf. Return them to their proper places on the shelf after use.
- In any laboratory, remember that individual security alone makes collective security possible.
- There should be restricted entry into the laboratory. The analyst has to restrict the number of entries into the laboratory. Outsiders except visitors should not be allowed inside laboratory for chitchatting and other discussions. All personal communications shall be outside the laboratory.

6.1 Laboratory safety

Everyone working in the laboratory should be cognizant of the potential hazards they face while working there. Fires with organic solvents, acid and base burns, toxic fumes and vapors are common hazards in almost any laboratory. Generally, laboratory safety is a matter of common sense, but there are several rules that must be followed. Commonly used laboratory safety signs have been shown in Fig. 6.2.

The following material is provided as a brief summary and guide to laboratory safety. It does not replace assigned reading material, but gives an overview of some important points.

Laboratory neatness

Clean and neat work areas avoid risk of damage to clothing and books and injury from spilled chemicals. Neatness also reduces fire hazard.

Working with glassware

Remove frozen glass stoppers with proper equipment. Broken or chipped glassware should be discarded. Properly support glassware with ring-stands and clamps when heating and use cork rings with round-bottom flasks.

Working with glass tubing

Do not touch heated glass until it has time to cool. Hot glass looks just like cool glass. To remove stoppers from glass tubing or thermometers, grasp tubing close to stopper and push gently with twisting. Use water or glycerine for lubrication.

Laboratory dress

Aprons protect clothing from corrosive or staining chemicals. Gloves protect hands from corrosive chemicals. Handle hot objects with insulated gloves. Do not wear open-toe shoes that allow spilled chemicals or broken glass to come in contact with your feet.

Working with test tubes

Gently heat solids or liquids in a test tube near the liquid or solid surface; Be prepared to remove the tube from heat quickly to prevent eruption. Never point a test tube or reaction vessel at another person. For safety and neatness, place test tubes in a rack.

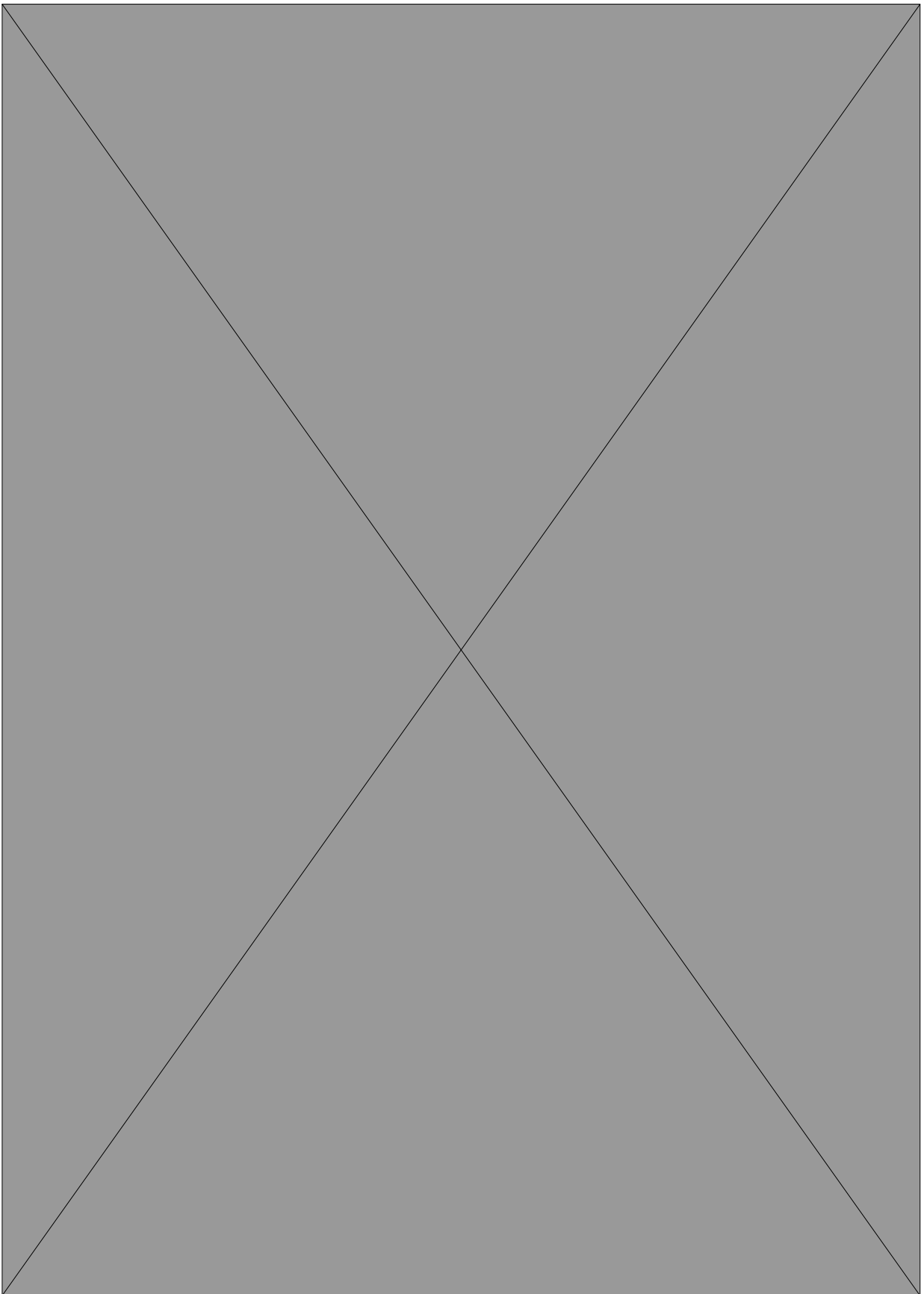


Fig. 6.2 Commonly used laboratory safety signs

Chemicals in the eye

Rapid treatment is vital. Run large volumes of water over eyeball until medical help is available. Wash with large volumes of water for at least 15 minutes. Alkaline materials in the eye are extremely hazardous. Know the location of the emergency eyewash station.

Fire on clothing

Do not run or fan flames. Smother fire by wrapping victim in fire blanket or laboratory coat and use the shower or a carbon dioxide fire extinguisher.

Using a fire extinguisher

- Know its location
- Remove from mounting
- Pull pin
- Squeeze lever
- Discharge at base of flame
- Report use and recharge
- Use dry send to extinguish burning metals

Unauthorized experiments

Always work under instructor's or lab technician's supervision in the laboratory.

Eye protection

Normal eye glasses are usually not adequate. Do not wear contact lenses in the laboratory. Eye protection is especially important when working with corrosive materials and vacuum and high pressure apparatus.

Acid/ alkali spills

For acid spills, use solid sodium bicarbonate followed by water. For alkali spills, wash with water followed by dilute acetic acid.

Handling flammable liquids

Flammable liquids should always be stored in an approved storage cabinet. Extinguish all flames in the area where flammable solvents are used, as vapours may travel to ignition source and flash back.

Types of fire extinguishers:

- A – For ordinary combustibles; wood, paper and cloth
- B – For flammable liquids, oil, grease and gasoline
- C – For use on live electrical equipment.

Handling mercury spills are very hazardous. Droplets should be picked up by suction and a mercury spill kit used to complete clean up. Notify lab technician immediately when mercury spills occur.

Protection from toxic gases

Emergency air masks should be used. However, because our lab is not equipped with such masks, clear the area where gases are, and notify the lab technician.

Waste disposal

Hot glassware or reactive chemicals should be discarded in a non-metallic container separate from paper and other flammable waste. Test-tube quantities of hazardous liquid can be

flushed down the sink with plenty of water. Contact lab technician for disposal of large quantities of hazardous materials.

Labelling chemicals

All chemicals should be clearly labelled. Do not use materials from unlabeled containers. Avoid contamination. Never return reagents to their container. Clearly label chemicals as you work.

Carrying chemicals

Carry long apparatus such as tubing or burettes, in an upright position close to the body. Grasp bottles firmly with both hands and hold them close to the body. Do not carry bottles by the neck. Use a bottle carrier when transporting chemicals any distance. Always arrange chemicals in chemical rack (Fig. 6.3).



Fig. 6.3 Chemical rack

Transferring liquids

Remember, acid or water do not pipette by mouth, use a bulb. Use gloves when pouring corrosive liquids. Use a funnel when filling a bottle or flask and prevent an air block by raising the funnel. Pour hazardous liquids over a sink.

Gas cylinders

Protect cylinder valve with cap. Fasten cylinders securely. Transport cylinders on a hand truck, don't roll. Do not drop cylinders. Mark cylinders when empty.

6.2 Laboratory safety techniques

Pipette

Do not pipette hazardous liquids by using mouth suction to fill pipette. Use pipette fillers or rubber tubing connected through trap to vacuum line for this purpose.

Wet oxidation

This technique is among the most hazardous uses of acids but can be performed safely. Observe precautions for particular acid used and rigorously follow directions given in specific method being used.

Safe handling of acids

Use effective acid-resistant fume removal device whenever heating acids or performing reactions, which liberate acid fumes. In diluting, always add acid to water unless otherwise directed in method. Keep acids off skin and protect eyes from spattering. If acids are spilled on skin, wash immediately with large amounts of water.

Acetic acid and acetic anhydride

React vigorously or explosively with CrO_3 and other strong oxidizers. Wear face shield and heavy rubber gloves when using.

Chromic and perchromic acid

Can react explosively with acetic anhydride, acetic acid, ethyl acetate, isoamyl alcohol and benzaldehyde. Less hazardous with ethylene glycol, glycerol and methanol. Conduct reactions behind safety barrier. Wear face shield and heavy rubber gloves.

Nitric acid

Reacts vigorously and explosively with aniline, H_2S , flammable solvents, hydrazine and metal powders (especially, Zn, Al and Mg). Gaseous nitrogen oxides from HNO_3 can cause severe lung damage. Copious fumes are evolved when concentrated HNO_3 and concentrated HCl are mixed. Avoid premixing. Use effective fume removal device when fumes are generated. Handle with disposable polyvinyl chloride gloves.

Oxalic acid

Forms explosive compound with silver (Ag) and mercury (Hg). Oxalates are toxic. Avoid skin contact and ingestion.

Perchloric acid

Contact with oxidizable or combustible materials or with dehydrating or reducing agents may result in fire or explosion. Persons using this acid should thoroughly familiar with its hazards. Safety practices should include the following:

- Remove spilled HClO_4 by immediate and thorough washing with large amounts of water.
- Hoods, ducts and other devices for removing HClO_4 vapours should be made of chemically inert materials and so designed that they can be thoroughly washed with water. Exhaust systems should discharge in safe location and fan should be accessible for cleaning.
- Avoid use of organic chemicals in hoods or other fume removal devices used for HClO_4 digestions.
- Use goggles, barrier shields and other devices as necessary for personal protection; use polyvinyl chloride, not rubber, gloves.
- In wet combustions with HClO_4 , treat sample first with HNO_3 to destroy easily oxidizable organic matter unless otherwise specified. Do not evaporate to dryness.
- Contact of HClO_4 solution with strong dehydrating agents such as P_2O_5 or concentrated H_2SO_4 may result in the formation of anhydrous HClO_4 , which reacts explosively with organic matter and with reducing agents.

Sulphuric acid

Always add H_2SO_4 to H_2O . Wear face shield and heavy rubber gloves to protect against splashes.

Fuming acids

Prepare and use with effective fume removal device. Wear acid-resistant gloves and eye protection.

Safe handling of alkalis

Alkalis can burn skin, eye and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali liquids. Use effective fume removal device or gas mask to protect respiratory tract against alkali dusts and vapours.

Ammonia

Extremely caustic liquid and gas. Wear skin, eye and respiratory protection when handling in anhydrous or gaseous state. NH_3 vapours are flammable. React vigorously with strong oxidizing agents, halogens and strong acids.

Ammonium hydroxide

Caustic liquid. Forms explosive compounds with many heavy metals such as Ag, Pb, Zn and their salts, especially halide salts.

Sodium and potassium hydroxides

Extremely caustic. Can cause severe burns. Protect skin and eyes when working with these alkalis as solids or concentrated solutions. Add pellets to H_2O , not vice versa.

Flammable solvents

Do not let vapours concentrate to flammable level in work area. It is nearly impossible to eliminate all chance of sparks from static electricity even if electric equipment is grounded. Use effective fume removal device to remove these vapours when released.

Toxic solvents

Vapours from some volatile solvents are highly toxic. Several of these solvents are readily absorbed through skin. Use effective fume removal device to remove vapours of these solvents as they are liberated.

6.3 Safe handling of special chemical hazards

Acetonitrile

Toxic. Avoid contact with skin and eyes. Use effective fume removal device.

Benzene

Toxic. Highly flammable. Avoid contact with skin. Do not breathe vapours. Use effective fume removal device. Decomposes violently in presence of strong oxidizing agents. Reacts violently with Cl_2 . Considered to be carcinogenic.

Acetone

Highly flammable. Forms explosive peroxides with oxidizing agents. Use effective fume removal device.

Bromine and chlorine

Hazardous with NH_3 , H_2 , petroleum gases, turpentine, benzene and metal powders. Extremely corrosive. Use effective fume removal device. Protect skin against exposure.

Carbon tetrachloride

Reacts violently with alkali metals. Toxic. Fumes may decompose to phosgene when heated strongly. Use effective fume removal device.

Chloroform

Can be harmful if inhaled. Forms phosgene when heated to decomposition. Use effective fume removal device. Can react explosively with Al, Li, Mg, Na, K, N_2O_4 and NaOH plus methanol.

Cyclohexane

Highly flammable. Use effective fume removal device. Can react vigorously with strong oxidizing agents.

Diethyl ether

Store protected from light. Extremely flammable. Unstable peroxides can form upon long standing or exposure to sunlight in bottles. Can react explosively when in contact with Cl_2 , O_3 , Li, Al, H_4 or strong oxidizing agents. Use effective fume removal device. Avoid static electricity.

Ethanol

Flammable. Use effective fume removal device when heating or evaporating.

Hydrogen sulfides

Hazardous with oxidizing gases, fuming HNO_3 and Na_2O_2 . Forms explosive mixtures with air. Toxic. Use effective fume removal device.

Hexane

Highly flammable. Use effective fume removal device.

Methanol

Flammable. Toxic. Avoid contact with eyes. Avoid breathing vapours. Use effective fume removal device. Can react vigorously with NaOH plus CHCl_3 , and KOH plus CHCl_3 or HClO_4 .

Oxidizers

(Perchlorates, peroxides, permanganates, persulfates, perborates, nitrates, chlorates, chlorites, bromates, iodates, concentrated H_2SO_4 , Concentrated HNO_3 , CrO_3 .) Can react violently with most metal powders, NH_3 , and ammonium salts, P, many finely divided organic compounds, flammable liquids, acids and S. Use exactly as specified in method. Handle in effective fume removal device from behind explosion-resistant barrier. Use face shield.

Peroxides

Hydrogen Peroxide: 30% strength is hazardous; can cause severe burns. Drying H_2O_2 on organic material such as paper or cloth can lead to spontaneous combustion. Cu, Fe, Cr, other metals and their salts cause rapid catalytic decomposition of H_2O_2 . Hazardous with flammable liquids, aniline and nitrobenzene. Since it slowly decompose with evolution of O_2 , provide stored H_2O_2 with vent caps. Wear gloves and eye protection when handling.

Ether peroxides -These peroxides form in diethyl ether, dioxane and other ethers during storage. They are explosive and must be destroyed chemically before distillation or evaporation. Exposure to light influences peroxide formation in ethers. Filtration through activated alumina is reported to be effective in removing peroxides. Store over sodium ribbon to retard peroxide formation.

Petroleum ether

Extremely flammable. Use effective fume removal device. Avoid static electricity.

Silver nitrate (AgNO_3)

Powerful oxidizing agent; strongly corrosive. Dust or solid form is hazardous to eyes. Handle as noted for oxidizers.

Permanganate

Moderately toxic. Readily soluble in water. Strong oxidizing agent. May form explosive mixture with H_2SO_4 or HClO_4 . When using with strong acids to destroy organic matter, perform reaction behind safety barrier.

Mycotoxins

Mycotoxins should be handled as very toxic substances. Perform manipulations under hood whenever possible. All mycotoxins are potential health hazard in one way or the other, Aflatoxin B_1 is a highly carcinogenic substance and shall therefore be handled very carefully. Do not transfer dry aflatoxin for weighing or other purposes unless facilities (e.g., glove box) are available to prevent dissemination of aflatoxin to surroundings due to electrostatic charge on particles. Rinse all glassware exposed to aflatoxin carefully with chloroform, then with solution of NaOCl bleach (5% v/v solution in water) and then wash thoroughly. Swab accidental spills of aflatoxins with the solution of NaOCl bleach.

6.4 Washing and cleaning of laboratory glassware

General rules

1. Always clean your apparatus immediately after each use. It is much easier to clean the glassware before residues in them become dry and hard. If the glassware is dirty and cannot be washed immediately, put it in water to soak.
2. Handle glassware carefully while cleaning it. Most breakage occurs at this time.
3. Quality water produced by water purification system (Fig. 6.4) may be used for laboratory work and rinsing of glassware.
4. Rinse off all soap or detergent residue after washing glassware to prevent any possible contamination later. Most pieces of laboratory glassware can be cleaned by washing and brushing with a detergent or a special laboratory cleaning product calledalconox, teepol, lab wash, lab clean etc. After they have been thoroughly cleaned, they are rinsed with tap water and finally with deionised water. If the surface is clean, the water will wet the surface uniformly. On soiled glass the water stands in droplets.



Fig. 6.4 Water purification system

Cleaning volumetric glassware

1. Always rinse volumetric glassware three times with deionised water after you have emptied and drained it. This prevents solutions from drying on the glassware, causing difficulty in cleaning.
2. Dry volumetric glassware at room temperature, never in a hot oven. Expansion and contraction may change the calibration.
3. The glass surface should wet evenly. Sporting is caused by grease and dirt.
4. Removing grease: Rinse and scrub with hot detergent solution followed by adequate deionised water rinses. Removing dirt: Fill or rinse with an acid solution. Allow to stand for several hours, if necessary. Follow with multiple deionised water rinses.

Cleaning pipettes

Pipettes may be cleaned with a warm solution of detergent or with cleaning solution. Draw in sufficient liquid to fill the bulb to about one-third of its capacity. While holding it nearly horizontal, carefully rotate the pipette so that all interior surfaces are covered. Drain inverted and rinse thoroughly with deionised water. Inspect for water breaks and repeat the cleaning cycle as often as necessary.

Cleaning burettes

Thoroughly clean the tube with detergent and a long brush. Allow the washings to exit through the burette tip.

Chromic acid wash

Follow all normal safety precautions when using concentrated acids and acid solutions. Acids can severely burn the skin. Dispose of all acids properly. A chromic acid wash has hydrolytic and oxidative capabilities for the chemical decomposition of biological molecules. The acid may also dissolve mineral deposits. To prepare a chromic acid wash, mix 20 g of sodium or potassium chromate with sufficient distilled water to make a paste of chromate salt. Add 300 ml of concentrated sulfuric acid. Increase the proportions to make larger amounts. Use the mixture until it turns green in color. It may be necessary to let heavily soiled items soak overnight.

Mineral deposits

Soak glassware in concentrated sulfuric, hydrochloric or nitric acid. Follow all precautions for the handling of these solutions. Hydrochloric acid diluted to 1 M in water is often used to remove mineral scales built up in water. Nitric acid is useful for cleaning stainless steel and removing rust strains.

6.5 Preparation of standard solutions

Solutions of accurately known strength are called standard solutions. A standard solution contains a known weight of reagent in a definite volume of solution. Molecular weight and atomic weight of commonly used chemicals has been shown in Table 6.1.

Molar solution

Molar solution is one, which contains one molecular weight of the reagent in one litre of the solution. Molarity is expressed as M.

Normal solution

Normal solution is one, which contains one equivalent weight of the reagent in one litre of the solution. Normality is expressed as N.

Equivalent weight of acid = Molecular weight/ No. of replaceable H ions

Name	Formula	Mol. wt. (g/mol)	Eq. wt. (g/mol)
Hydrochloric acid	HCl	36.5	36.5
Nitric acid	HNO ₃	63	63
Potassium hydroxide	KOH	56	56
Calcium hydroxide	Ca(OH) ₂	74	37
Potassium dichromate	K ₂ Cr ₂ O ₇	294	49
Sodium thiosulphate	Na ₂ S ₂ O ₃ ·5H ₂ O	248	248
Sodium chloride	NaCl	58.5	58.5
Potassium chloride	KCl	74.5	74.5
Iodine	I ₂	254	127

Most of the synthetic dyes in generally used as indicators are organic substances of complex structure. Among the most reliable of these indicators are methyl red and phenolphthalein (Table 6.2).

Indicator	pH range	End point	Preparation
Methyl red	4.4 to 6.3	Pink in acidic medium and colourless in basic medium	For preparing a stock solution, 0.2 g of dye is dissolved in 100 ml of alcohol and filtered
Methyl orange	2.9 to 4.0	Orange in acidic medium and colourless in basic medium	For preparing a stock solution, 0.1 g of dye is dissolved in 100 ml of distilled water, filtered and used
Phenolphthalein	8.3 to 10.0	Pink in basic medium and colourless in acidic medium	For preparing a stock solution, 0.2 g of phenolphthalein is dissolved in 110 ml of alcohol and 90 ml of distilled water

There are a few standard solutions which are used for analysis of feed stuffs:

1. N/10 H₂SO₄
2. N/10 NaOH
3. N/10 KMnO₄
4. 0.256 N (1.25% (w/v)) H₂SO₄
5. 0.313 N (1.25% w/v) NaOH
6. 40 per cent NaCl (w/v)
7. 3 per cent KNO₃ (w/v)
8. 20 per cent ammonium molybdate (w/v)
9. 50 per cent HCl (w/v)

Certain primary standard solutions are also required for standardization of the above solutions. These are:

1. N/10 Na₂CO₃
2. N/10 (COOH)₂· 2H₂O

Preparation of N/10 H₂SO₄

Equivalent weight of H₂SO₄ = 49 g
Specific gravity = 1.84 g/ml
So, volume of 49 g H₂SO₄ = 26.6 ml

Concentrated H₂SO₄ (reagent grade) is about 97 per cent pure.

Therefore, actual amount of concentrated H₂SO₄ required for 1.0 litre of N/10 H₂SO₄ solution =

$$\frac{100}{97} \times 26.6 = 27.42 \text{ ml}$$

Thus, for 1.0 litre of N/10 H₂SO₄ solution, 27.42 ml of concentrated H₂SO₄ is required.

Procedure

Take 27.42 ml sulphuric acid in a beaker half-filled with distilled water. Transfer the contents and washings to a volumetric flask (1 litre) and make volume up to the mark. Shake well and titrate this solution with 10 ml of 0.1 N Na₂CO₃ using mixed / methyl orange as an indicator. Repeat the titration to get at least three concordant readings.

Standardization

Suppose 10 ml of 0.1 N Na₂CO₃ = 9.5 ml of H₂SO₄

$$V_1 N_1 = V_2 N_2$$

$$10 \times 0.1 \text{ N} = 9.5 \times N_2$$

$$N_2 = 0.10526$$

To prepare 1 litre N/10 H₂SO₄, the volume of 0.10526 N acid required is $1000 \times 0.1/0.10526 = 950$ ml. Take 950 ml of 0.10526 N acid and dilute it to one litre. Check it again with N/10 Na₂CO₃ for three times. It must neutralize equal volume of N/10 Na₂CO₃ solution. Label it as 0.1 N H₂SO₄.

Precautions

Add H₂SO₄ with the help of a burette.

Never add water to an acid.

Preparation of N/10 NaOH solution

Molecular weight of NaOH = 40
Acidity (number of replaceable OH group) = 1
Equivalent weight of NaOH = 40

Therefore, 4 g of NaOH dissolved in one litre of solution will give N/10 NaOH solution.

Procedure

Weigh quickly 4 g NaOH in a beaker (as it is hygroscopic) and dissolve it in distilled water (preferably CO₂-free). Transfer the contents and the washings to a volumetric flask (1 litre). Cool and then make volume up to the mark. Shake well and standardize this solution against N/10 oxalic acid using phenolphthalein as an indicator. Label it as 0.1 N NaOH solution.

Preparation of N/10 KMnO₄ solution

Dissolve 3.2 g KMnO₄ in one litre of distilled water. Boil it for 10-15 minutes and then allow to stand for few days and then filter it through glass wool.

Take 10 ml of N/10 oxalic acid in a beaker. Add 5 ml dilute sulphuric acid, warm it to 60-70°C and titrate against KMnO_4 from the burette till a light pinkish colour appears. Take three concordant readings.

Suppose 10 ml 0.1 N oxalic acid = 9.75 ml of KMnO_4

$$V_1 N_1 = V_2 N_2$$

$$10 \times 0.1 \text{ N} = 9.75 \times N_2$$

$$N_2 = \frac{10 \times 0.1 \text{ N}}{9.75} = 0.10256$$

To prepare 1000 ml 0.1 N KMnO_4 solution the volume of KMnO_4 will be taken.

$$\frac{100 \times 9.75 \times 0.1}{10 \times 0.1}$$

Now take 975 ml of prepared KMnO_4 solution and make it 1000 ml by adding distilled water.

Note:

Ordinary or even pure distilled water contains traces of organic matter which reduces the KMnO_4 solutions. That is why the solution is boiled and kept for some time before standardization.

In the absence of sufficient amount of dilute H_2SO_4 or due to the rapid addition of KMnO_4 in titration flask, brown turbidity (manganous oxide) may appear.

Preparation of N/10 Na_2CO_3 solution

Molecular weight of Na_2CO_3 = 106

$\text{Na}_2\text{CO}_3 + 2 \text{HCl} = 2 \text{NaCl} + \text{H}_2\text{O} + \text{CO}_2$

So, acidity of Na_2CO_3 = 2

Equivalent weight of Na_2CO_3 = 53

Therefore, 5.3 g Na_2CO_3 is required for each litre of solution to make N/10 Na_2CO_3 . Na_2CO_3 is hygroscopic, therefore, it must be made perfectly anhydrous before it is weighed out. Quantity of acid/ alkali required for preparation of different molar/normal solutions has been shown in Table 6.3.

Table 6.3 Acid/ alkali required for preparation of different normal solutions				
Normality	95% NaOH (g/l), M=N	36% HCl (ml/l), M=N	98% H_2SO_4 (ml/l), M=2N	<68% HNO_3 (ml/l), M=N
0.1 N	4.2	8.5	2.7	6.5
0.5 N	21.0	42.6	13.7	32.8
1.0 N	42.1	85.3	27.4	65.7
1.5 N	63.1	127.9	41.1	98.5
2.0 N	84.2	170.6	54.8	131.4
2.5 N	105.2	213.2	68.5	164.2
5.0 N	210.5	426.5	137	328.5
10.0 N	421	853	274	657

M = Molar; N = Normal

Procedure

Take 6-7 g of Na_2CO_3 (A.R.) in a nickel crucible and heat it in a hot air oven at about 100°C for few hours so as to drive out any moisture and to convert any moisture and to convert any preformed NaHCO_3 to Na_2CO_3 . Cool in a desiccator and weigh exactly 5.3 g dried salt and dissolve it in a little quantity of freshly boiled distilled water. Transfer it to one litre measuring flask and make volume up to the mark. Shake well and label it as 0.1 N Na_2CO_3 solution.

Preparation of N/10 oxalic acid

Oxalic acid $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$ is to be dissolved in one litre of distilled water to get N/10 oxalic acid solution.

Procedure

Weigh accurately 6.3 g $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$ and transfer it to a volumetric flask (1 litre), half-filled with distilled water. Shake well and make the volume up to the mark. Label it as N/10 oxalic acid solution.

Note: If anhydrous oxalic acid $(\text{COOH})_2$ is available then dissolve 4.5 g of the acid in one litre of distilled water to get 0.1 N oxalic acid solution.

Preparation of standardized 0.313 N (1.25%) NaOH solution

Add 13.16 g of NaOH (95% NaOH) in one litre distilled water and shake well. Standardize this solution against known concentration of oxalic acid solution using phenolphthalein as an indicator.

Preparation of standardized 0.256 N (1.25per cent (w/v) H_2SO_4 solution

To prepare 1.25 per cent (w/v) H_2SO_4 solution, 12.5 g of H_2SO_4 (100 per cent) is to be added to distilled water to make the volume 1000 ml.

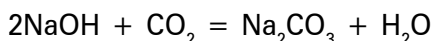
$$\text{Volume of } \text{H}_2\text{SO}_4 \text{ be taken} = \frac{12.5 \times 100}{1.84 \times 97} = 7 \text{ ml}$$

Procedure

Add 7.0 ml concentrated H_2SO_4 (specific gravity 1.84 g/ml and 97per cent concentration) in a 1000 cc volumetric flask half-filled with distilled water. Shake well and add distilled water to make volume up to the mark. Standardize this solution against known concentration of Na_2CO_3 using mixed/methyl orange indicator.

Precaution and preservation of standard solutions

The bottle must be kept tightly stoppered to prevent evaporation of solvent. Some solutions must be protected from atmospheric gases. For example, sodium hydroxide solution is affected by atmospheric CO_2 .



But never put a glass stopper on NaOH solution container because NaOH will react with air and glass between stopper and neck of volumetric flask. It will fix permanently and you cannot remove the glass stopper from volumetric flask. KMnO_4 solution should be preserved in colour (amber) bottles. The container should be shaken well before the withdrawal of a portion of solution to ensure uniform composition of the solution.

7.0 PROXIMATE ANALYSIS

The proximate analysis is a scheme for routine description of animal feed stuffs devised in 1865 by Henneberg and Stohmann at Weende's Experiment Station in Germany. It is often referred to as the Weende's System of Analysis and is principally devised to separate carbohydrates into two broad classifications; crude fibre and nitrogen free extract (NFE). The system consists of determinations of water, ash, crude fat (ether extract), crude protein and crude fibre. As indicated, NFE is a component of the system, but it is measured by difference rather than by analysis.

Proximate analysis allows us to make legitimate comparisons of feeds on the basis of specific nutrients. This makes it possible to know how much better one feed is than another in terms of specific nutrients.

7.1 Determination of moisture

Apparatus

Aluminium moisture cup with lid, hot air oven, metal tongs, desiccator, analytical balance.

Procedure

- Dry aluminium cup in oven at 100°C for 15 to 30 minutes.
- Cool in a desiccator, weigh and record its weight.
- Weigh accurately about 5 g of previously ground sample in an aluminium cup and record its weight.
- Place the cup with sample in hot air oven (Fig. 7.1) maintained at $105 \pm 2^\circ\text{C}$ and dry for at least 2 h.
- Cool in a desiccator and weigh.
- Repeat the process of heating, cooling and weighing until the difference between two successive weighings is less than one mg.



Fig. 7.1 Hot air oven

Calculation

$$\text{Moisture, per cent by mass} = \frac{100 (M_1 - M_2)}{M_1 - M}$$

Where,

M_1 = mass in g of the cup with the material before drying

M_2 = mass in g of the cup with the material after drying and

M = mass in g of the empty cup

Reference: IS 2052-2012, Indian Standards Institution, Specification for compounded feeds for cattle, Fifth revision.

7.2 Determination of total ash

Principle

Principle involved is that when a known weight of feed is ignited to ash, the weight of ash thus obtained is expressed in terms of percentage.

Apparatus

- Silica crucible
- Tongs
- Weighing balance
- Electrical bunsen burner
- Muffle furnace
- Desiccator
- Asbestos sheet



Fig. 7.2 Muffle furnace

Procedure

1. Find out the weight of a clean dry crucible.
2. Place about 2 g of sample and weigh this to find out accurate weight of the sample taken.
3. Carefully place the weighed crucible over electric burner. The crucible should be partially opened.
4. The sample will get charred with initial expulsion of smoke.
5. Place the crucible in a muffle furnace (Fig. 7.2) and heat to 600°C. Keep it for 2 hours. At this temperature all organic matter will be burnt leaving behind minerals.
6. Remove the crucible from the furnace carefully and cool it in a desiccator to room temperature and weigh again.

Calculation

$$\text{Ash content (\%)} = \frac{(Z - X) / (Y - X)}{1} \times 100$$

Weight of empty crucible	-	X g
Weight of crucible + sample	-	Y g
After complete ashing, Weight of crucible + ash	-	Z g

What is obtained after complete combustion of a sample is total ash. It comprises of two portions: The portion that is soluble in dilute acids contains all essential minerals and that is the useful portion of the ash. Other portion, insoluble in dilute acids consists of mainly sand and silica. For the most part, it represents impurity or adulteration.

Reference: AOAC Official Method 942.05

7.3 Calculation of organic matter (OM)

Fresh sample:

$$\% \text{ organic matter} = 100 - (\% \text{ moisture} + \% \text{ of Total ash})$$

Dried sample:

$$\% \text{ Organic matter} = 100 - \% \text{ Total ash}$$

Precaution: The ash is highly hygroscopic and thus weighing should be done quickly.

7.4 Calculation of nitrogen free extract (NFE)

This includes all the nutrients which are not assessed by the prior methods of proximate analysis, and consists mainly of digestible carbohydrates, vitamins and other non-nitrogen soluble organic compounds. Since the result is obtained by subtracting the percentages calculated for each nutrient from 100, any errors in evaluation will be reflected in the final calculation for NFE as well.

$$\left(\text{NFE \%} = 100 - (\text{Moisture \%} + \text{Crude protein \%} + \text{Crude fat \%} + \text{Crude fibre \%} + \text{Ash \%}) \right)$$

Reference: AOAC Official Method 942.05

7.5 Determination of acid insoluble ash

Reagent

Dilute hydrochloric acid – approximately 5 N, prepared from concentrated hydrochloric acid.

Procedure

Weigh accurately 2 g of the dried material in a porcelain, silica or platinum dish. Ignite with a burner for about one hour. Complete the ignition by keeping in a muffle furnace at $550 \pm 20^\circ\text{C}$ until grey ash results. Moisten with concentrated hydrochloric acid and evaporate to dryness. Keep in an oven maintained at $135 \pm 2^\circ\text{C}$ for about 3 hours. Cool and add 25 ml of dilute hydrochloric acid, cover with a watch glass and heat in a water bath for 10 minutes. Cool and filter through Whatman filter paper No. 42 or its equivalent. Wash chlorides as tested with silver nitrate solution and return the filter paper and residue to the dish. Ignite it in a muffle furnace at $550 \pm 20^\circ\text{C}$ for one hour. Cool in a desiccator and weigh. Ignite the dish again for 30 minutes, cool and weigh. Repeat this process till the difference between two successive weighing is less than one milligram. Note the lowest mass.

Calculation

$$\text{Acid insoluble ash (on moisture-free basis), per cent by mass} = \frac{100 (M_2 - M)}{M_1 - M}$$

Where,

$$\begin{aligned} M_2 &= \text{the lowest mass in g of the dish with the acid insoluble ash} \\ M &= \text{mass in g of the empty dish, and} \\ M_1 &= \text{mass in g of the dish with dried material} \end{aligned}$$

Reference: IS:7874 (Part I) – 1975, Indian Standards Institution, Methods of test for animal feeds and feeding stuffs.

7.6 Determination of crude protein content – Kjeldahl method

Principle

Digestion of organic matter with sulfuric acid in the presence of a catalyst, Rendering the reaction product alkaline then distillation and titration of the liberated ammonia, Calculation of the nitrogen content, Multiplication of the result by the conventional factor 6.25 to obtain the crude protein content.

Reagents and materials

- Potassium sulfate
- Copper (II) oxide (CuO)
- Copper (II) sulfate pentahydrate (CuSO₄·5H₂O)
- Sulfuric acid - 18 mol/l, = 1.84 g/ml
- Paraffin wax
- Saccharose
- Acetanilide, with melting point 114°C, nitrogen content 103.6 g/kg.
- Tryptophan, with melting point 282°C, nitrogen content 137.2 g/kg. Before use, dry the tryptophan.
- Sodium hydroxide solution, (NaOH) = 33% (m/m).
- Boric acid = 40 g/l.
- Sodium hydroxide - 0.1 mol/l or (NaOH) = 0.25 mol/l.
- Sulfuric acid - 0.05 mol/l or (H₂SO₄) = 0.125 mol/l.
- Mixed indicator, neutral point at pH 4.4 to 5.8.
- Dissolve 2 g of methyl red and 1 g of methylene blue in 1000 ml of ethanol (w(C₂H₅OH) = 95% (v/v).

Apparatus

- Analytical balance (Fig. 7.3)
- Digestion, distillation and titration apparatus

Procedure

Weigh, to the nearest 1 mg, a mass of the test sample chosen according to the expected nitrogen content so that the test portion contains between 0.005 g and 0.2 g of nitrogen and, preferably more than 0.02 g.

Note: The mass of the test portion of homogeneous air-dry samples should be between 0.5 g and 2.0 g. The mass of the test portion of wet and/ or inhomogeneous samples should be between 2.5 g and 5.0 g.

Digestion of organic matter

Transfer the test portion quantitatively into a Kjeldahl digestion flask of suitable size (usually 800 ml).

Add 15 g of potassium sulfate.

Add an appropriate quantity of catalyst as follows: 0.3 g of copper (II) oxide or 0.9 g to 1.2 g of copper (II) sulfate pentahydrate.



Fig. 7.3 Analytical balance

Add 25 ml of sulfuric acid (18 mol/l) for the first gram of dry matter of the test portion and 6 to 12 ml for each additional gram of dry matter. Mix thoroughly, ensuring complete wetting of the test portion.

Heat the flask moderately at first to prevent foam from rising into the neck of the flask or escaping from the flask.

Heat moderately, swirling from time to time, until the mass has carbonized and the foam has disappeared. Then heat more intensively until the liquid is boiling steadily.

Avoid overheating of the walls of the flask not in contact with liquid.

After the liquid has become clear with a light green-blue colour, heat for 2 h.

Leave to cool. If the digest starts to solidify, add some water and mix by swirling.

Distillation of ammonia

Carefully add 250 to 350 ml of water to dissolve the sulfates completely. If necessary, facilitate dissolving by heating the flask in warm water. Mix by swirling and allow cooling.

Add a few boiling aids.

Pipette into the collecting flask of the distillation apparatus 25 ml of the sulfuric acid (0.05 mol/l), choosing the concentration according to the expected nitrogen content of the test portion. Add 100 to 150 ml of water. Add a few drops of the mixed indicator. Proceed as follows:

Immerse the end of the condenser in the liquid contained in the collecting flask, to a depth of at least 1 cm.

Slowly pour 100 ml of sodium hydroxide solution (33 per cent) into the digestion flask along the wall.

Immediately connect the flask to the distillation apparatus.

Heat the flask in such a manner that approximately 150 ml of distillate is collected in 30 min. At the end of this time, check the pH of the distillate at the tip of the condenser using litmus paper. If the reaction is alkaline, continue distillation.

Alternatively, transfer into the collecting flask 100 to 250 ml of boric acid. Add a few drops of mixed indicator.

Titration

If sulfuric acid is used as the collecting liquid, titrate in the collecting flask, the excess sulfuric acid with sodium hydroxide solution 0.1 mol/l or 0.25 mol/l as appropriate, until the endpoint is indicated by the pH meter or until the color changes from violet to green.

If boric acid is used as the collecting liquid, titrate the ammonia with sulfuric acid 0.05 mol/l or 0.125 mol/l as appropriate, until the endpoint is indicated by the pH-meter or the color changes from green to violet (Fig. 7.4).

Blank test

Perform a blank test using about 1 g of saccharose in place of the test portion.



Fig. 7.4 Titration

Check test

Perform a check test by determining the nitrogen content of acetanilide or tryptophan after addition of 1 g of saccharose.

Calculation and expression of results

Distillate collected in sulfuric acid:

$$W_{N1} = \frac{(V_0 - V_1) \times C_1 \times M}{m}$$

Where,

W_{N1} is the nitrogen content, in grams per kilogram, of the test sample.

V_0 is the volume, in milliliters, of the sodium hydroxide solution required for the blank test.

V_1 is the volume, in ml, of the sodium hydroxide solution required for the determination.

C_1 is the concentration, in moles per litre, of the sodium hydroxide solution used for the titrations;

M is the molar mass, in grams per mole, of nitrogen ($M = 14$ g/mol);

C_2 is the concentration, in moles per litre, of the sulfuric acid (4.9.2) used for the titrations;

m is the mass, in grams, of the test portion.

Distillate collected in boric acid:

$$W_{N2} = \frac{2(V_3 - V_2) \times C_2 \times M}{m}$$

W_{N2} is the nitrogen content, in grams per kilogram, of the test sample.

V_2 is the volume, in ml, of the sulfuric acid required for the blank test.

V_3 is the volume, in ml, of the sulfuric acid required for the determination.

M is the molar mass, in grams per mole, of nitrogen ($M = 14$ g/mol);

m is the mass, in grams, of the test portion.

Calculation of crude protein content

Calculate the crude protein content of the test sample by the equation.

$$Wp = 6.25 \times w_N$$

Where,

Wp is the crude protein content, in grams per kilogram, of the test sample;

Wn is the nitrogen content, in grams per kilogram, of the test sample (either w_{N1} or w_{N2});

Reference: IS 14825 : 2000, ISO 5983 : 1997

7.7 Determination of crude protein – Automated Kjeldahl method

Digestion

Operation

- Weigh 0.5 g of sample (or 5 ml of biological samples, e.g. milk, urine) into the digestion tubes. Note that if there are fewer than 20 samples per rack, then empty tubes must be placed in unused positions in the rack of digestion unit (Fig. 7.5).
- Add reagents to the tubes containing samples; for most application, it is 2 catalyst tablets and 12 ml H₂SO₄.
- The digestion block should be pre-heated to 420°C. Check by inserting thermometer in the well in the block.
- Turn on the scrubber unit and fully open the air-flow-regulating valve.
- Remove thermometer, and fit the exhaust manifold on the tubes in the rack and place the rack in the digestion unit.
- Fit the heat shields to the rack and set the timer for 1 hour.
- After 5-7 minutes turn the valve to 1-2 stops before close-off, so as to prevent acid escaping between the tubes and the manifold. During digestion, check the temperature of the scrubber unit – it should be < 60°C. A high temperature or excessive build-up of liquid in the acid trap probably indicates too high an airflow.
- When digestion is complete, increase the scrubber airflow and carefully remove the hot rack and manifold from the digestion unit and place on a heat resistant surface.
- Remove the heat shields and allow the tubes to cool.
- When no further fumes are evident at the top of the tubes, remove the manifold and replace on its stand.
- When the tubes are partly cooled, add 70 ml distilled water to each tube and mix. This step should be carried out while the tubes are still warm, so as to prevent the precipitation of salts.
- The tubes are now ready for distillation.



Fig. 7.5 Digestion unit

Distillation

Start-up procedure

- Check that the water drain valve on the back of Tecator is closed.
- Switch on the power to the 1030, computer, printer and interface.
- Check burette for bubbles and clear by operating TITRANT SWITCH. Note that when safety door is open, acid will go to and from the reservoir, and when closed, to and from the titration cell.
- Press REC-SOL 5-6 times to flush the line.



Fig. 7.6 Automatic distillation unit

- Turn on the cooling water.
- Connect test tube and shut door.
- Turn the STEAM switch ON and wait until steam is generated for 1-2 minutes.
- Turn the steam switch OFF.

Operation

- Select KJELDAHL program (should be set already).
- Set constants A to 00.00, B to 1.000 and blank to 0.00.
- Open safety door and press AUTO/RESET TO AUTO.
- Place the 1st test tube (usually containing = 70 ml D.I. water) to be distilled in position.
- Close door when the "CYCLE OVER" L.E.D. is ready. Distillation should begin (Fig. 7.6).
- Note the displayed value when "CYCLE OVER" lights up. Open door and remove tube with *hot hand* and repeat from step 4 (usually 2-3 times), until the blank value is satisfactory.
- Set the blank value which will be subtracted from the distillations and select the B value to be used for the display output from the Table 1.

Table 1

HCl concentration	0.05 M	0.1 M	0.2 M	0.5 M
RESULT	B=	B=	B=	B=
% P, f=5.7	0.399	0.799	1.597	3.993
% P, f=6.25	0.438	0.876	1.751	4.378
% P, f=6.38	0.447	0.894	1.788	44.69
% N, f=1.00	0.070	0.140	0.280	0.701
MI titrant*	1.000	1.000*	1.000	1.000

B=1.000 is the preferred setting for all analysis, and blank = .06

NOTE: If the door is closed before the "CYCLE OVER" light comes on, open the door and wait until the light is steady, then close the door and start the distillation.

Shut down procedure

- Remove tube and clean plastic cover behind safety door and tube holder foot.
- Turn off power to the 1030, computer, the interface and printer.
- Fill the titration cell with D.I. water.
- Remove the drip tray from under test tube and clean.
- Wipe any spills from the chassis of the TECATOR with a solution of 3 per cent acetic acid.
- If the TECATOR is not to be used for 2-3 days, open the drain valve on the back of the unit.
- Turn off cooling water at tap.

Calculations

$$\% \text{ Nitrogen} = \frac{\text{Titrant (ml)} - \text{Blank (ml)} * 0.1 \text{ (N)} * 14.01}{\text{Sample Wt (mg)} * \text{DM}/100} \times 100$$

$$\% \text{ Protein} = \% \text{ Nitrogen} * 6.25$$

N = Normality of acid (mole/l); 14.01 = Atomic weight of nitrogen; 6.25 standard Kjeldahl factor

Testing recovery

1. Weigh 0.500 g ammonium iron (II) sulphate $[(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$ into a tube.
2. Add about 75 ml distilled water.
3. Using 0.1N HCl as titrant, connect to the distilling unit and distil.
4. Calculation is as follows:

$$\% \text{ Nitrogen} = \frac{(\text{Titrant} - \text{Blank}) * \text{N} * 1.401}{\text{Sample (g)}}$$

$$\% \text{ Nitrogen} = 7.145$$

The results after the calculations are done should be = 7.145 or close to it. The closer the value, higher the accuracy of nitrogen recovery.

Reference: AOAC (1997). 976.05, 16th edition.

7.8 Estimation of ether extract using Soxhlet apparatus

Principle

The crude fat present in the feed is extracted by petroleum ether using the soxhlet apparatus.

Apparatus

- Spatula
- Weighing balance
- Thimble
- Heating mantle/hot plate.
- Soxhlet apparatus

Reagent

- Petroleum ether

Procedure

Set the soxhlet apparatus in position. Take thimble and find out its weight. Transfer about 5 g of sample into the thimble and find out the correct weight of the sample by weighing the thimble with the sample. Plug the mouth of the thimble with cotton wool, to avoid the escape of material from the thimble during extraction.

Slide the thimble with the contents into the soxhlet extractor. Fix the lower end of the soxhlet extractor to the flask underneath. Then fix the condenser above the soxhlet extractor. Take care to fix the ground surface suitable for easy removal after extraction. Adjust the water circulation for efficient and uniform cooling of the condensing unit.

The soxhlet apparatus is placed over an electrical heater/ hot plate (Fig. 7.7). From the top end of the apparatus pour about 100 ml of petroleum ether and plug the mouth with cotton. Run the extraction for 6 hours till the collecting ether in the extractor is clean.



Fig 7.7 Hot plate

Dismantle the apparatus on the completion of extraction. Remove the extractor with the flask from the condenser. Remove the thimble with its contents. Place it in the oven for drying. When dried find out the weight of the thimble with the extracted residue.

Remove the soxhlet flask with the extract. Transfer it to a hot air oven (80°C) for evaporating the petroleum ether. Weigh the flask with the dried residue.

Calculation

You can find out the weight of ether extract either directly from weighing the flask with and without ether extract or indirectly by weighing the thimble with the substance before and after extraction. The loss of weight in this case will give the value for ether extract.

Direct

Weight of flask (empty)	= X g
Weight of flask + ether extract	= Y g
Weight of ether extract	= Y - X g
Percentage of ether extract (W is the weight of sample taken)	= $\frac{Y-X}{W} \times 100$

Indirect

Weight of thimble + feed sample before extraction	= X g
Weight of thimble after extraction	= Y g
Weight of the ether extract (Loss of weight represents the ether extract)	= X-Y g
Percentage of ether extract (W is the weight of sample taken).	= $\frac{X-Y}{W} \times 100$

Precaution: While placing the thimble-containing sample in the soxhlet flask, make sure that the top of the thimble is above the siphon tube. Put a cotton swab on the mouth of the condenser to avoid the loss of ether vapours.

Reference: AOAC official method 920.39, 16th Edition.

7.9 Estimation of ether extracts using Soxtec system

Introduction

The Soxtec system has been developed to make solvent extraction faster, safe and more rational. The design of the Soxtec system (Fig. 7.8) makes it possible to extract crude fat from feeds in typically 45 minutes, to process 6 samples simultaneously and to use only 20 ml of solvent per extraction. The heating system with no electricity close to the extraction unit has minimized possible explosion risks.

Equipment

- Analytical balance
- Soxtec system
- Thimbles 26 x 60 mm
- Heating oven
- Desiccator

Chemicals

- Appropriate solvent e.g. diethyl ether, petroleum ether, hexane.
- Cotton wool (defatted)
- Boiling chips (glass ball – 4-5 mm)



Fig. 7.8 Soxtec apparatus

Soxtec HT procedure

- Grind the samples thoroughly.
- Load each thimble with about 3 g (W_1) of the well mixed sample and cover with a thin layer of cotton wool.
- Dry the thimbles.
- Insert the thimbles into the Soxtec HT.
- Dry and pre-weigh (W_2) the extraction cups (with boiling chips). Add 25-50 mls of the solvent into each cup.
- Insert the cups in to the Soxtec HT.
- Extract for 15 minutes in "Boiling" position and for 30-45 minutes in "Rinsing" position.
- Evaporate the solvent.
- Release the cups and dry at 100°C for 30 minutes.
- Cool the cups in a desiccator and weigh (W_3). Calculate percentage fat/oil according to the formula.
- Fat extraction can also be done by using Soxtherm system (Fig. 7.9).



Fig. 7.9 Fat extraction by Soxtherm

$$\% \text{ Fat / oil} = \frac{(W_3 - W_2)}{W_1} \times 100$$

Reference: Tecator – Application note No. 983.06.13 AN67/83

7.10 Determination of crude fibre – Muslin cloth method

Principle

The feed sample is subjected to acid digestion followed by alkali digestion and the remaining residue is weighed and ashed. The loss of weight after ashing is the crude fibre content of the feed.

Apparatus

- Lipless beaker 600 ml capacity
- Condensing flask
- Electric heater/hot plate
- Crucible
- Muslin cloth
- Spatula
- Porcelain tile

Reagents

- 0.255 N H_2SO_4 : 7 ml of conc. H_2SO_4 is dissolved in distilled water to get one litre of solution.
- 0.313 N NaOH : 12.78 g of sodium hydroxide is dissolved in distilled water to get one litre of solution.

Procedure

Two gram of feed is accurately weighed. Accurately measure 200 ml of 0.255 N H_2SO_4 in a 600 ml lipless beaker. Place it on an electric heater and keep a suitable condensing flask (round bottom) over the beaker. See that the condensing flask fixes well over the beaker leaving no space. The condensing flask is filled with cold water. Now switch on the heater. The purpose of keeping a condensing flask filled with cold water is for condensing back the evaporating acid to the beaker. This maintains the volume of the acid without any reduction.

Digestion in acid

The beaker is heated to bring the acid (0.255 N H_2SO_4) to boiling stage. Then 2 g of substance is transferred to the boiling acid. The acid boils and the feed is digested in acid. This boiling and digestion is continued for 30 minutes. After the end of 30 minutes stop the boiling and remove the condenser.

Filtration

Set up a funnel in a large conical flask. Fix a linen cloth over the funnel. Transfer the contents from the beaker to the filtering funnel. After all the acid and acid digested residues are transferred to the linen cloth, wash the beaker with distilled water and transfer the contents to the filtering funnel. Continue the washing till the residue is made acid-free.

Test

This is tested simply by catching one or two drops of filtrate over blue litmus. If the blue litmus remains blue, that means the residue is washed free of acid. After complete washing take the filter cloth along with the residue, squeeze well to remove the water from the residue. Place the cloth over porcelain slab. Scrap gently the adhering residue from the filter cloth and keep the residue in the centre of the filter cloth.

Digestion in alkali

The acid digested residue is then subjected to alkali digestion. For this 0.313 N sodium hydroxide solution is used. As in the acid digestion, pour 200 ml of sodium hydroxide (0.313 N) into a lipless beaker (600 ml capacity). Place over the heater and fix a condensing flask over this. Bring the alkali solution to boiling stage by heating. When it starts boiling, remove the condensing flask and transfer the acid digested residue to the boiling alkali. Replace the condensing flask and continue the heating. The residue should be digested in the boiling NaOH for a period of 30 minutes. After 30 minutes, remove the condenser; transfer the contents of the beaker to a filtering funnel. The residue is washed repeatedly with distilled water till it is alkali free.

Test

This is tested by catching one or two drops of the filtrate over red litmus. If it remains red it indicates that the residue is free from alkali. When the residue is free from alkali squeeze the cloth well to dry the residue. Transfer the residue, without any loss, to a clean silica crucible.

Note: The cold water in the condensing flask should never be hot at any time. If the water is hot replace it with cold water. Before removing the condensing flask care should be taken to avoid the loss of any residue sticking to the bottom of the flask.

Drying and ashing

The crucible is placed in preheated hot air oven (110°C) over night. This is to drive off the moisture completely. After complete drying, the crucible is cooled in desiccator. It is weighed along with the residue. Heat the crucible with electrical bunsen in order to ash the residue. Continue the heating till you obtain a whitish ash. Cool the crucible to room temperature and find out the weight.

Calculation

The difference in weight of the crucible before and after ashing is reported as the crude fibre content of the feed taken.

$$\begin{aligned} \text{Weight of the sample} &= c \text{ g} \\ \text{Weight of crucible with dry residue} &= a \text{ g} \\ \text{Weight of crucible with ash} &= b \text{ g} \\ \text{Per cent crude fibre} &= \frac{a - b}{c} \times 100 \end{aligned}$$

Reference: AOAC, 962.09, 16th edition.

7.11 Determination of crude fibre using Fibertec system

Reagents

1. Sulphuric acid – 0.256 N (12.5 g of H₂SO₄ diluted to 1 litre and mixed)
2. Potassium hydroxide – 0.223 N (12.5 g of KOH dissolved in de-ionized water and diluted to 1 litre)
3. n-Octanol
4. Acetone

Procedure

1. Weigh and transfer quantitatively an exact quantity (about 1 g = W₀) of fat free sample into clean filter crucibles placed in a crucible stand.
2. When a set of crucibles is loaded with samples, hook the stand on the front of the hot extraction unit, fix the crucible holder to the crucible and transfer them to the boiling positions in the hot extraction unit.
3. Through the valves add to each sample 150 ml of 0.256 N sulphuric acid, preheated in one of the reagent heating systems.
4. Add a few drops of octanol to prevent foaming and heat to boiling.
5. Adjust heat and boil for 30 minutes.
6. Filter and, if necessary, remove sediment from the filter surface by applying reversed pressure.
7. Wash three times with hot de-ionized water from the spray device sliding on the bar above the boiling tubes. Use about 30 ml of water each time and suck as dry as possible.
8. Add to each sample 150 ml of potassium hydroxide solution preheated in the second reagent system.
9. Add a few drops of octanol and boil as above for another period of 30 minutes.
10. Filter and wash as above three times with hot water.
11. Using the crucible holder move the crucibles from the hot extraction unit to the cold extraction unit.
12. Wash three times with acetone (about 25 ml each time) and suck dry.



Fig. 7.10 Crude fibre estimation by Fibretherm

13. Remove the crucibles and transfer them to a crucible stand.
14. Dry the crucibles (in the stand) at 100°C overnight or at 130°C for 2 hours.
15. Cool the crucibles in desiccator or weigh them hot directly from the drying oven. Where the latter procedure is used, check zero of balance after each weighing and adjust weight accordingly (Weight = W_1).
16. Ash the sample in the crucibles at 500°C for at least 3 hours.
17. Cool the crucibles slowly to 100°C or room temperature depending on which weighing method you use, and weigh again (weigh = W_2).
18. Fibretherm can also be used for estimation of fibre content (Fig. 7.10).
19. Calculate the fibre content from the formula:

$$\% \text{ Crude fibre} = \frac{W_1 - W_2}{W_0} \times 100$$

20. Rinse the crucibles by blowing the ash out with compressed air or if the crucibles are dirty, wash with chromic acid.

Reference: Tecator Application note No. 1978.03.15 AN01/78

7.12 Determination of crude fibre using Ankom technology

Reagents

- a) Sulfuric acid solution – 0.255 +/-0.005N. 1.25 g H_2SO_4 /100 ml distilled water (concentrate must be checked by titration) or ANKOM crude fibre solution concentrate mixed per instructions. Available in 20 litre and 2 litre concentrate.
- b) Sodium hydroxide solution – 0.313 +/-0.005 N. 1.25 g NaOH/100 ml distilled water. NaOH needs to be free or nearly free from Na_2CO_3 (concentrate must be checked by titration) or ANKOM crude fibre solution concentrate mixed per instructions. Available in 20 litre and 2 litre concentrate.
- c) Acetone – Use grade that is free from colour and leaves no residues upon evaporation.

Apparatus

- a) Digestion apparatus
- b) Filtration device – ANKOM TECHNOLOGY – F57 Filter bags
- c) Impulse bag sealer – Requires high enough temperature to melt and seal polymer in filter bags.
- d) Desiccator



Fig. 7.11 Ankom fiber analyzer

Procedure

- a) Prepare filter bags / samples
 - Weigh F57 filter bag (W_1), record weight and tare balance. The bags have negligible moisture content and do not need to be pre-dried unless stored out of desiccant in a high moisture environment.
 - Weigh 1.0 g (± 0.05 g) of air-dried sample (W_2), ground to pass through a 1 mm screen, directly into filter bag. Weigh one blank bag and include indigestion to determine blank bag correction (C_1).
 - Seal the bag closed within 0.5 cm from the open edge using a heat sealer.
 - Spread the sample uniformly inside the filter bag. This should be done by shaking and / or lightly flicking the bag to eliminate clumping.
 - b) Extract fat from samples by placing 24 bags with sample into a 500 ml bottle with a top. Pour enough acetone into bottle to cover bags and secure top. Shake the container 10 times and allow bags to soak for 10 minutes. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry (approximately 5 minutes).
 - c) Place the 24 bags in the bag suspender trays (ANKOM Technology). Place three bags per tray; 24 bags total. Stack trays on centre post with each level rotated 120 degrees. The 9th tray remains empty and acts as a top for the 8th tray. The bag suspender weight is placed on top of the 9th tray to keep the bag suspender submerged.
 - d) Add 1900-2000 ml of ambient temperature acid (0.255 N H_2SO_4) solution to ANKOM fiber analyzer vessel (Fig. 7.11). Submerge the loaded bag suspender, set the timer for 45 minutes, turn *Agitation* and *Heat* on and start the timer. After confirming that the bag suspender is agitating, tightly seal lid. The temperature will automatically be controlled at 100°C.
- Note: Fewer bags and less solution can be used per study but a minimum of 1500 ml of solution is required in the vessel. All trays of the bag suspender must be used, with or without bags.
- e) After 45 minutes (timer will beep) turn *Heat* and *Agitation* OFF. Open the exhaust valve and release hot solution before opening lid.
WARNING – The solution in the vessel is under pressure. The exhaust valve needs to be opened to release the pressure and solution prior to opening the lid.
 - f) After the solution has been exhausted, close the exhaust valve and open the lid. Add approximately 1900-2000 ml of hot (90-100°C) rinse water turn *Agitation* ON and leave the *Heat* OFF. Close the lid but do not tighten. Agitate the bags in rise water for 3-5 minutes. Repeat hot water rinses two more times (total of three rinses).
 - g) Add 1900-2000 ml of ambient temperature base (0.313 N NaOH) solution to ANKOM fiber analyzer vessel. Set the timer for 45 minutes, turn *Agitation* and *Heat* ON, seal lid and start the timer. The temperature will automatically be controlled at 100°C.

- h) After 45 minutes (timer will beep) turn Heat and Agitation OFF. Open the exhaust valve and release hot solution before opening lid. Add approximately 1900-2000 ml of hot (90-100°C) rinse water and turn Agitator ON and leave the Heat OFF. Close the lid but do not tighten. Agitate the bags in rinse water for 3-5 minutes. Repeat hot water rinses two more times (total of three rinses).
- i) Remove filter bags from suspender and gently press out excess water. Place bags in a 250 ml beaker and add acetone to cover bags. Allow bags to soak 2-3 minutes then remove and lightly press out excess acetone.
- j) Spread bags out and let air dry. Completely dry in oven at 105°C (most ovens provide complete drying within 2-4 hours). Remove from oven and place in a desiccator until cooled to ambient temperature and weigh (W_3). Ash entire bag/sample in pre-weighed crucible for 2 hours at 550°C, cool in desiccator and weigh for Organic Matter calculation.

Calculation

$$\% \text{ CF}_{\text{OM}} \text{ (DM basis): } \frac{(W_4 - (W_1 \times C_2)) \times 100}{W_2 \times \text{DM}}$$

- Where,
- W_1 = Bag tare weight
 - W_2 = Sample weight
 - W_3 = Weight after extraction process
 - W_4 = Weight of organic matter (OM) (Loss of weight on ignition of bag and fibre residue)
 - C_2 = Ash corrected blank bag (Loss of weight on ignition of bag/original blank bag)

Reference: ANKOM Technology, USA-220 manual.

7.13 Determination of urea - Distillation method

Principle

Suspension of water of the test portion in the presence of de-colorant. Addition of Carrez I and Carrez II solutions. Stirring of the suspension, then filtration. Addition to the filtrate of 4-dimethyl-amino-benzaldehyde (DMAB) and spectrometric measurement at 420 nm of the absorbance of the solution thus obtained.

Reagents

- Active carbon - Which does not absorb urea.
- 4-Dimethyl-Amino-Benzaldehyde (4-DMAB) - Dissolve 1.6 g of 4-DMAB in 100 ml of 96 per cent (v/v) ethanol, add 10 ml of concentrated hydrochloric acid ($P_{20}=1.19$ g/ml) and mix.
- Carrez I Solution - Dissolve in water 24 g of zinc acetate dehydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) and 3 g of glacial acetic acid. Make up to 100 ml with water and mix.
- Carrez II Solution - Dissolve in water 10.6 g of potassium hexacyanoferrate (II) trihydrate (potassium ferrocyanide trihydrate) ($\text{K}_4(\text{Fe}(\text{CN})_6) \cdot 3\text{H}_2\text{O}$). Make up to 100 ml with water and mix.
- Urea - Standard solution corresponding to 1 g of urea per litre.

Apparatus

- Rotary shaker
- Spectrometer

Procedure

Weigh, to the nearest 1 mg, about 2 g of the test sample.

Preparation of the test solution

Transfer the test portion together with 1 g of the active carbon into a 500 ml volumetric flask. Add 400 ml of water, 5 ml of the Carrez I solution and 5 ml of the Carrez II solution. Mix for 30 min in a rotary shaker (Fig. 7.12). Make up to the mark with water homogenize and filter. If the filtrate is coloured, increase the quantity of active carbon.



Fig. 7.12 Rotary shaker

Colour development

Transfer, by means of a pipette, 5 ml of the clear colourless filtrate into a test tube and add, by means of a pipette, 5 ml of the 4-DMAB solution.

Mix and leave to stand for 15 min in a water bath controlled at 20°C.

Blank test

Carry out a blank test in parallel with the determination using the same procedure and the same quantities of all reagents, but omitting the test portion.

Preparation of the calibration graph

Pipette into a series of five 100 ml volumetric flasks 10, 20, 40, 50 and 80 ml of the urea standard solution. Make each flask up to the mark with water. One millilitre of the standard solution contains 100, 200, 400, 500 and 800 µg of urea, respectively.

Pipette into a series of five test tubes 5 ml of each of these solutions (one dilution per test tube). Add to each test tube, by means of a pipette, 5 ml of the 4-DMAB solution and homogenize. Transfer the solution to spectrometer cells and measure their absorbance at 420 nm, using the spectrometer against a compensation solution containing 5 ml of 4-DMAB and 5 ml of water.

Plot the calibration graph, with the absorbance value on the ordinate and corresponding concentrations of urea, in micrograms per millilitre.

Transfer the solution to a spectrometer cell and measure its absorbance at 420 nm, using the spectrometer, against the blank test.

Calculation

$$\text{Urea content, (percentage by mass)} = \frac{C}{20 \times m}$$

Where,

C = is the urea content, in micrograms per ml of the filtrate of the test solution, determined from the calibration graph.

m = is the mass, in grams, of the test portion.

Reference: IS 13399 : 1992

7.14 Determination of urea in animal feed - Colorimetric method

Apparatus

Spectrophotometer – Instrument with maximum band width 2.4 nm and 420 nm, with 1 cm cells.

Reagents

- Di-methyl amino benzaldehyde (DMAB) solution – Dissolve 16.00 g in 1 L alcohol and add 100 ml HCl. Stable 1 month. Prepare new standard curve with each new batch of reagent.
- Zinc acetate solution – Dissolve 22.0 g $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ in water add 3 ml CH_3COOH , and dilute to 100 ml.
- Potassium ferrocyanide solution – Dissolve 10.6 g $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 2\text{H}_2\text{O}$ in water and dilute to 100 ml.
- Vegetable charcoal
- Phosphate buffer solution – pH 7.0. Dissolve 3.403 g anhydrous KH_2PO_4 and 4.355 g anhydrous KH_2PO_4 separately in ca 100 ml portions freshly distilled water.
- Urea standard solutions – (1) Stock solution – 5 mg/ml. Dissolve 5.000 ± 0.001 g reagent grade urea in H_2O and dilute to 1 l with H_2O (2) Working solutions – 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg urea/5 ml. Pipette 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 ml stock solution into 250 ml volumetric flasks and dilute to volume with phosphate buffer. (3) Reference solution – Use standard solution containing 1.0 mg urea/ 5 ml as reference standard, store at $< 24^\circ\text{C}$. Stable 1 week.

Preparation of standard curve

Pipette 5 ml aliquots of working standard solutions into 20 x 150 mm (25 ml) test tubes and add 5 ml DMAB solution to each. Prepare reagent blank of 5 ml buffer solution and 5 ml DMAB solution. Shake tubes thoroughly and let stand 10 min in water bath at 25°C . Read A in 1 cm cell at 420 nm with reagent blank at zero A. Plot A against concentration urea. Plot should be straight line; if not, repeat, using new lot of DMAB.

Determination

Weigh 1.00 g ground sample into 500 ml volumetric flask. Add 1 g charcoal, ca 250 ml H_2O , 5 ml $\text{Zn}(\text{CH}_3\text{COO})_2$ solution and 5 ml $\text{K}_4\text{Fe}(\text{CN})_6$ solution. Shake mechanically 30 min and dilute to volume with water. Let stand until precipitate settles. Decant through Whatman No. 40 paper and collect clear filtrate. Pipette 5 ml filtrate into test tube, add 5 ml DMAB solution, and shake thoroughly. Include reference standard (5 ml solution and 5 ml DMAB solution) and reagent blank with each group of samples. Let stand 10 min in water bath at 25°C . Read A at 420 nm against reagent blank.

$$\% \text{ Urea} = (1.0 \times A_{\text{sample}} \times 100) / (A_{\text{standard}} \times \text{mg sample in aliquot})$$

Reference: AOAC Official Method 967.07, 16th Edition.

7.15 Determination of serum/ plasma urea

Principle

A pink colour complex is formed when urea is treated with di-acetyl monoxime and thiosemicarbazide in the presence of sulphuric acid, phosphoric acid and ferric chloride which is read at 525 nm in spectrophotometer.

Reagents

- Reagent A:**
 300 ml concentrated H_2SO_4 (95-98%)
 50 ml distilled water
 100 ml concentrated H_3PO_4 (85%)
 100 mg ferric chloride ($FeCl_3$)
 Mix well and volume it to 1 litre with distilled water.
- Reagent B:**
 500 g diacetyl monoxime (DAMO)
 10 mg thiosemicarbazide (TSC)
 Mix well with distilled water to make 100 ml solution
- Reagent C:**
 Prepare immediately before use with Reagents A & B at 2:1 proportion.
- Trichloro acetic acid (TCA) 5% (w/v) with distilled water.
- Standard urea solution (0-150 nmol)
 Working standard (50 μ g/ml): Dissolve 50 mg urea in 1 litre of distilled water.



Fig. 7.13 Centrifuging of extracts

Procedure

- Protein is precipitated out from serum/plasma 0.2 ml mixed with 1.8 ml of 5 per cent TCA.
- Centrifuge at 2000 rpm for 10 min (Fig. 7.13) and collect supernatant for determination using the protocol given below.
- Boil in water bath for 5 minutes.
- Cool to room temperature and read absorbance at 525 nm against reagent blank.
- Plot a standard curve and read concentration of the sample against absorbance.

Protocol

Reagents/ Solutions (ml)	Blank	Sample	Standard				
			1	2	3	4	5
Protein free sample	0.20	-	-	-	-	-	-
Standard	-	-	0.02	0.04	0.06	0.08	0.10
Distilled water	-	0.20	0.18	0.16	0.14	0.12	0.10
Reagent C	3.00	3.00	3.00	3.00	3.00	3.00	3.00

Calculation

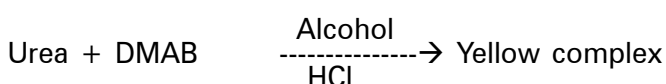
$$\text{Urea (mg/ 100 ml)} = \frac{\text{OD unknown}}{\text{OD standard}} \times \text{concentration of standard} \times 50$$

Reference: Rahmatullah, M. and Boyde, T.R.C. 1980. An improvement in determination of urea using diacetylmonoxime method with and without deproteinization. *Clin. Chem. Acta*, 107: 3-9.

7.16 Estimation of urea in milk-1,4-p-dimethylaminobenzaldehyde (DMAB) method

Principle

This method is based on the principle that urea forms a yellow complex with p-dimethyl aminobenzaldehyde in a low acidic solution at room temperature. The intensity of yellow color is measured at 425 nm.



Reagents

- Trichloro acetic acid (TCA) 24 per cent w/v: Dissolve 24 g of TCA in distilled water and make the total volume to 100 ml.
- Phosphate buffer (pH 7.0): Dissolve anhydrous potassium di-hydrogen orthophosphate (3.403 g) and anhydrous di-potassium mono hydrogen orthophosphate (4.355 g) in distilled water and make the volume to one litre.
- Diluting reagent: Mix equal volumes of 24 per cent (w/v) TCA and phosphate buffer (pH 7.0) to make the diluting reagent.
- p-dimethylaminobenzaldehyde (DMAB) reagent (1.6 per cent w/v): Dissolve p-dimethylaminobenzaldehyde (1.6 per cent w/v) in ethyl alcohol containing 10 per cent (v/v) concentrated hydrochloride acid.
- Standard urea solution (1 mg/ml): Dissolve 100 mg of urea (AR grade) in phosphate buffer (pH 7.0) and make up the volume to 100 ml.

Procedure

1. Ten ml of well mixed sample of milk is mixed with 10 ml of TCA (24 per cent w/v) to precipitate the protein and filtered through Whatman No. 42 filter paper.
2. Add five ml of filtrate with 5 ml of 1.6 per cent DMAB reagent to develop the yellow colour in a test tube.
3. Prepare reagent blank by taking 5 ml of diluting reagent and treating it with 5 ml of DMAB reagent.
4. Measure the optical density of yellow colour of the sample at 425 nm in a spectrophotometer against reagent blank.
5. From standard curve, as described below, the amount of urea in milk is calculated.

Preparation of standard curve

Prepare standard urea solution (1 mg/ml) in phosphate buffer (pH 7.0). Take different concentrations of urea solution ranging from 0.1 to 2.0 mg separately in different test tubes and the total volume made to 5 ml with diluting reagent solution. Add 5 ml of 1.6% DMAB reagent to each test tube to develop the colour. Measure the optical density of the yellow colour thus obtained at 425 nm and plot against concentration.

Reference: Indian standard methods of test dairy industry. Part II. Chemical analysis of milk. IS: 1479 (Part II). Indian Standard Institution, New Delhi.

7.17 Analysis using near infrared analyser (NIR)

Principle

The sample representing the chemical composition of the sample material is measured by NIR spectrometry. Spectral data in the near infrared are collected and transformed to constituent or parameter concentrations by calibration models developed on representative samples.

Near infrared (NIR) instrument

NIR instrument (Fig. 7.14) based on diffuse reflectance or transmittance measurement in the near infrared wavelength region of 700-2500 nm or segments of this or at selected wavelengths. The optical principle may be dispersive (e.g. grating mono-chromators) interferometric or non-thermal (e.g. light emitting diodes, laser diodes and lasers). The instrument should be provided with a diagnostic test system for testing photometric noise and reproducibility, wavelength accuracy and wavelength precision (for scanning spectrophotometers). The instrument should measure a sufficiently large sample volume or surface to eliminate any significant influence of in homogeneity derived from chemical composition or physical properties of the test sample. The sample path length (sample thickness) in transmittance



Fig. 7.14 Near infrared analyzer

measurements should be optimized according to the manufacturer's recommendation with respect to signal intensity for obtaining linearity and maximum signal/noise ratio. In reflectance measurements, a quartz window or other appropriate material to eliminate drying effects should preferably cover the interacting sample surface layer.

Sampling

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. All laboratory samples should usually be kept under conditions that will not change the composition of the sample from the time of sampling to the time of commencing the procedure.

Procedure

Preparation of test sample

The preparation of samples should be made in the same way as the preparation of the validation samples. It is necessary to apply standard conditions. Before the analysis, the sample should be taken in such a way as to obtain a sample representative of the material to be analysed.

Measurement

Follow instructions for the specific NIR instrument and associated calibrations. The prepared sample should reach a temperature within the range included in the validation.

Evaluation of result

For the validation results to be valid, they have to be within the range of the calibration model used. Results obtained on samples detected as spectral outliers cannot be regarded as reliable.

Checking instrument stability

Control sample

At least one control sample should be measured at least once per day to check instrument hardware stability and to detect a malfunction. Knowledge of the true concentration of the

analyte in the control sample is not necessary. The sample material should be stable and, as far as possible, resemble the samples to be analysed. The parameter(s) measured should be stable and, as far as possible identical to or at least biochemically close to the sample analyte. These samples are normally stable for lengthy periods but the stability should be tested in the actual cases. Shifts between control samples should be overlapped to secure uninterrupted control. The recorded day-to-day variation should be plotted in control charts and investigated for significant patterns or trends.

Instrument diagnostics

For spectrophotometers the wavelength accuracy and precision should be checked at least once per week, or more frequently if recommended by the instrument manufacturers, and the results should be compared to specifications and requirements. A similar check of the instrument noise should also be carried out weekly, or at intervals recommended by the manufacturer.

Running performance check of calibration

NIR methods should be validated continuously against reference methods to secure steady optimal performance of calibrations and observance of accuracy. The frequency of checking the NIR method should be sufficient to ensure that the method is operating under steady control with respect to systematic and random deviations from the reference method. The frequency depends on the number of samples analysed per day and the rate of changes in sample population.

The running validation should be performed on samples selected randomly from the pool of analysed samples.

It may be necessary to resort to some sampling strategy to ensure a balanced sample distribution over the entire calibration range, e.g. segmentation of concentration range and random selection of test samples within each segment or to ensure that samples with a commercially important range are covered.

The number of samples for the running validation should be sufficient for the statistics used to check the performance. For a solid validation at least 20 samples are needed (to expect a normal distribution of variance). One can fill in the results of the independent validation set for starting the running validation. To continue about 5 to 10 samples every week is quite sufficient to monitor the performance properly. Using less samples it is hard to make the right decision in case one of the results is outside the control limits.

An attempt to force the results within the limits by frequent adjustments of the calibration will not improve the situation in practice. The standard errors of prediction (SEP) should instead be re-evaluated using the latest results.

If the calibration equations after a period of stability begin to move out of control, the calibration should be upgraded. Before this is done, an evaluation should be made of whether the changes could be due to changes in reference analyses, unintended changes in measuring conditions (e.g. caused by a new operator), instrument drift or malfunction etc. In some cases a simple adjustment of the constant term (bias) in the calibration equation may be sufficient. In other cases it may be necessary to run a complete re-calibration procedure, where the complete or a part of the basic calibration set is expanded to include samples from the running validation, and perhaps additional samples selected for this purpose.

Considering that the reference analyses are in statistical control and the measuring conditions and instrument performance are unchanged, significant biases or increased SEP values can be due to changes in the chemical, biological or physical properties of the samples compared to the underlying calibration etc.

8.0 ANALYSIS OF FIBRE FRACTIONS

P.J. Van Soest and associates, working at the USDA Station at Beltsville, Maryland, developed a rapid technique of separating feed carbohydrates on the basis of nutritional availability to ruminants and rumen bacteria. Essentially, the method divides feeds into two fractions; 1) plant cell contents, a highly digestible fraction consisting of sugars, starches, soluble protein, pectin and lipids; 2) plant cell wall constituents, a fraction of variable digestibility consisting of insoluble protein, hemi-cellulose, cellulose, lignin and bound nitrogen. The method involves boiling a sample in a neutral detergent solution. The soluble fraction is termed NDS - Neutral Detergent Soluble (cell contents), while the fibrous residue is called NDF - Neutral Detergent Fibre (cell wall constituents). Unlike crude fibre and NFE, both NDS and NDF accurately predict the proportions of more or less digestible fractions, respectively, found in a wide variety of feedstuffs.

The Van Soest (or detergent) scheme has been further refined with the addition of Acid Detergent Fibre (ADF) analysis, which breaks down NDF into a soluble fraction containing primarily hemi-cellulose and some insoluble protein and an insoluble fraction containing cellulose, lignin and bound nitrogen. Furthermore, the content of lignin in acid detergent fibre can be determined by either treating the fibre with H_2SO_4 to dissolve the cellulose or by oxidation with permanganate to degrade the lignin. This is quite important because lignin has been shown to be a major factor influencing the digestibility of forages.

A general scheme of the detergent analysis			
Feed samples boiled with neutral detergent solution (pH 7.0)			
NDF (Plant cell wall) Pectins, Hemi-cellulose, Cellulose, Lignin		NDS (Cell contents) Soluble carbohydrate, Starch, Organic acids, Proteins	
Boiled with acid detergent solution			
Acid detergent fibre (ADF) Cellulose and Lignin		Acid detergent soluble Hemi-cellulose	
KMnO ₄ treatment		H ₂ SO ₄ Treatment	
Lignin lost by oxidation	Cellulose + some minerals	Cellulose lost	Lignin + Some minerals
Ashing 500°C			
Cellulose lost	Ash	Lignin lost	Ash

8.1 Determination of neutral detergent fibre (NDF)

Apparatus

- 1) Refluxing apparatus: Tall pyrex or corning beakers (spout less) of about 500 ml capacity round bottom flask as condenser
- 2) Sintered glass crucibles with coarse porosity (Grade 1) of about 50 ml capacity
- 3) Electronic balance
- 4) Vacuum pump
- 5) Hot plate
- 6) Wash bottle
- 7) Hot air oven and muffle furnace

Reagents

- Neutral detergent solution:

Distilled water	1 litre
Sodium lauryl sulphate	30 g
Disodium ethylene di-aminetetraacetate (EDTA) dehydrate	18.61 g
Sodium borate decahydrate	6.81 g
Disodium hydrogen phosphate (anhydrous)	4.56 g
2-ethoxyethanol (ethylene glycol monoethyl ether)	10 ml

Put EDTA and $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ together in a large beaker, add some of the distilled water and heat until dissolved, then add to solution containing di-sodium lauryl sulphate and 2-ethoxy ethanol. Put Na_2HPO_4 in a beaker, add some of the distilled water and heat until dissolved, then add to solution containing other ingredients. Check pH range 6.9 to 7.1. If solution is properly made, pH adjustment is rarely required.

- Decahydronaphthalene (Decalin) – Reagent grade.
- Acetone – Use grade that is free from color and which leaves no residues upon and evaporation.
- Sodium sulphite (anhydrous)

Procedure

- Take 0.5 to 1.0 g air dry sample ground to pass 20 to 30 meshes (1mm) into a beaker of the refluxing apparatus.
- Add in order 100 ml (preheated) neutral detergent solution, 2 ml. of decalin and 0.5 g sodium sulphite with a calibrated scoop and reflux for 60 minutes, time starting from the onset of boiling.
- Filter off the reagent, wash thrice with hot distilled water under vacuum, remove vacuum, break up mat and wash crucible with hot water.
- Wash twice with acetone in same manner and suck dry. Dry crucible at 100°C for 8 hrs or overnight and weigh it.
- Report yield of recovered NDF as per cent of cell wall constituents. Estimate cell soluble material by subtracting this value from 100.
- Ash residues in the crucible for 3hrs at $500\text{-}550^\circ\text{C}$ and weigh. Report ash content as ash insoluble in neutral detergent.

Observations

Empty wt. of crucible	=	----- g
Wt. of dry sample	=	----- g
Wt. of crucible + cell wall constituent	=	----- g
Wt. of crucible + Ash	=	----- g

Calculation

- Cell wall constituents (%) (NDF) =
$$\frac{(\text{wt. of crucible} + \text{cell wall constituents}) - (\text{wt. of crucible})}{\text{Wt. of dry sample}} \times 100$$
- Cell contents (%) = $100 - \text{cell wall constituents}$
- Insoluble ash in neutral detergent (%) = $\frac{\text{Wt. of crucible} + \text{Ash} - \text{Wt. of crucible}}{\text{Wt. of dry sample}} \times 100$

Reference: Goering and Van Soest, 1970.

8.2 Determination of neutral detergent fibre (NDF) - Fibertec system

Apparatus

- Fibertec system
- Sintered glass crucible (Porosity 2: 40-90 microns)
- Glass rod
- Enamel tray
- Muffle furnace

Procedure

1. Record the weight of a sintered glass crucible
2. Record the weight of the crucible along with approximately 1 g of feed sample.
3. Attach the crucible in a Fibertec system and reflux the sample for one hour with 100ml of neutral detergent solution.

Composition of neutral detergent solution (g per litre of distilled water)

- | | |
|---|-------|
| • Sodium lauryl sulphate | 30 |
| • Disodium ethylene diamine tetra-acetate | 18.61 |
| • Sodium borate decahydrate | 6.81 |
| • Disodium hydrogen phosphate (anhydrous) | 4.56 |
| • Ethoxy ethanol | 10 ml |

Then 2 ml of decahydrogenaphthalene and 0.5 g of sodium sulphite are to be added and heated to boiling in 5-10 minutes. Adjust boiling to an even level and refluxed for 60 minutes from the onset of boiling. Apply vacuum to drain the fluid. The sample is then rinsed with minimum of hot water. Break the mat formed in the bottom of the crucible by applying pressure and filled the crucible with hot water. Filter the liquid by applying vacuum. Wash the residue twice with acetone in the same manner and suck dry. Dry the crucibles at 100°C overnight and weigh.

Calculation

$$\% \text{ NDF} = \frac{\text{Weight of crucible with NDF residue} - \text{weight of empty crucible}}{\text{Weight of substance}} \times 100$$

Reference: Goering, H.K. and P.J. Van Soest, 1970. Forage fibre analyses. Agric. Handbook No.379., ARS, USDA.

8.3 Neutral detergent fibre (NDF) and neutral detergent insoluble nitrogen (NDIN) estimation using Ankom 220 Fiber analyzer

Reagents

- Neutral detergent solution (NDS – Add 30 g sodium lauryl sulfate, USP; 18.61 g ethylene diamine tetra acetic disodium salt, dehydrate; 6.81 g sodium tetra borate decahydrate; 4.56 g sodium phosphate dibasic, anhydrous and 10.0 ml triethylene glycol in 1 litre distilled water. Agitate and heat to facilitate solubility. Check pH range to 6.9 to 7.1.
- α - amylase
- Sodium sulfate – Na_2SO_3 anhydrous
- Acetone

Equipment

- ANKOM 220 Fiber analyzer
- Tecator – nitrogen analyzer
- Filtration device – ANKOM –F57 filter bags
- Impulse bag sealer

Procedure

- Weigh filter bag (W_1).
- Weigh 0.5 g of air-dried sample (W_2), ground to pass through 1 mm screen, directly into filter bag. Weigh one blank bag and include in the digestion to determine blank bag correction (C_1).
- Seal the bags closed within 0.5 cm from the open edge using the heat sealer.
- Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.
- A maximum of 24 bags may be placed in the bag suspender. Place three bags per tray and then stack trays on centre post with each level rotated 120 degrees. The weight is placed on top of the empty 9th tray to keep the bag suspender submerged.
- Add 2000 ml of ambient temperature neutral detergent solution into ANKOM fiber analyzer vessel. Place bag suspender with samples into the solution in vessel. Turn *Agitate* and *Heat* ON and confirm that bag suspender is agitating properly. Set timer for 75 minutes and push *Start*. Close and seal lid of vessel.
- After 75 min. have elapsed turn *Agitate* and *Heat* OFF, open the drain valve and exhaust hot solution.
- After the solution has been exhausted, close valve and open the lid. Add approx. 2000 ml of hot (90-100°C) water. Turn *Agitate* ON and leave *Heat* OFF and rinse for 3-5 min. Exhaust water and repeat rinsing for at least three times.
- Remove filter bags, place in beaker and cover with acetone. Allow bags to soak 3 min.
- Spread bags out and allow acetone to evaporate. Complete drying in oven at 105°C for at least 2 hours.
- Remove bags and cool and weigh bags (W_3).
- After the bags have been dried and weighed, transfer them into “Tecator” test tube and proceed with the digestion and distillation as indicated by the Kjeldahl protein method.
- NDIN is the nitrogen value as a per cent of total nitrogen in feed on a dry matter basis.

Calculation

$$\text{NDF (as-is basis)} = \frac{(W_3 - (W_1 \times C_1)) \times 100}{W_2}$$

Where,

W_1 = Bag weight

W_2 = Sample weight

W_3 = Weight after extraction process

C_1 = Blank bag correction (final oven dried weight/ original blank bag weight)

Reference: ANKOM Technology, USA-220 Manual.

8.4 Determination of acid detergent fibre (ADF)

Apparatus

- Refluxing apparatus
- Tall pyrex or corning beakers (spout less) of about 500 ml capacity
- Round bottom flask as condenser
- Sintered glass crucibles with coarse porosity (Grade 1) of about 50 ml capacity
- Electronic balance
- Vacuum pump
- Hot plate
- Wash bottle
- Hot air oven and muffle furnace

Reagents

- a) Sulfuric acid (H_2SO_4)
Reagent grade, standardized to 1 N (100% assay) is 49.04 g dissolved in 1000 ml.
- b) Cetyltrimethylammonium bromide (CTAB) Technical grade – 20 g. Weigh sulphuric acid and make up to volume with distilled water. Check normality by titration before addition of detergent. Then add CTAB and stir.
- c) Decalin (reagent grade)
- d) Acetone – Use grade that is free from colour and leaves no residue upon evaporation.
- e) n-hexane (technical grade)

Procedure

- i) Weigh 1 g air dry sample ground to pass 20 to 30 mesh (1mm.) screen or approximate equivalent of wet material in a beaker suitable for refluxing.
- ii) Add 100 ml. cold (room temperature) acid-detergent solution and 2 ml decalin. Heat to boiling in 5 to 10 minutes. Reduce heat as boiling begins, to avoid foaming. Reflux 60 minutes from onset of boiling; adjust boiling to a slow, even level.
- iii) Filter on a previously weighed crucible. Wash with hot distilled water 3-4 times. Repeat wash and acetone twice or until it removes no more color and suck dry.
- iv) Optional wash with hexane. Hexane should be added while crucible still contains some acetone (Hexane can be omitted if lumping is not a problem in lignin analysis). Suck the acid detergent fibre free of hexane and dry at 100°C for 8 hrs or overnight and weight after cooling of crucible in desiccator.

Observations

Empty wt. of crucible	=	----- g
Wt. of dry sample	=	----- g
Wt. of crucible + fibre	=	----- g

Calculation

Acid detergent fibre per cent on dry matter basis =

$$\frac{(\text{Wt. of crucible + fibre}) - \text{Empty weight of crucible}}{\text{Wt. of dry sample}} \times 100$$

Reference: Goering and Van Soest, 1970.

8.5 Determination of acid detergent fibre (ADF) - Fibertec system

Apparatus

- Fibertec system
- Sintered glass crucible (Porosity 2, 40-90 microns)
- Glass rod
- Enamel tray
- Muffle furnace

The residue left after NDF determination should be utilized for ADF determination. The acid detergent solution should contain:

H ₂ SO ₄ reagent grade standard to 1 N	49.04 gL ⁻¹
Cetyltrimethylammonium bromide	20.00

To the NDF residue, 100 ml of the acid detergent solution and 2 ml of deca hydrogen naphthalene are to be added in that order and heated to boiling in 5 to 10 minutes. Reduce the heat reduced as boiling begin, to avoid foaming and reflex for 60 minutes from the onset of boiling and adjust boiling to a slow even level. After completion of refluxing, drain the fluid by applying solution and wash twice with hot water and finally wash dry with acetone. Dry the residue at 100°C overnight.

Calculation

$$\% \text{ ADF} = \frac{\text{Wt. of crucible with ADF residue} - \text{Wt. of empty crucible}}{\text{Wt. of substance}} \times 100$$

Reference: Goering, H.K. and P.J. Van Soest, 1970. Forage fibre analyses. Agric. Handbook No. 379, ARS, USDA.

8.6 Acid detergent fibre (ADF) and acid detergent insoluble nitrogen (ADIN) estimations using Ankom 220 fiber analyzer

Reagents

- Acid detergent solution (ADS – Add 20 g cetyltrimethyl ammonium bromide (CTAB) to 1 litre 1.00 N H₂SO₄ previously standardized). Agitate and heat to aid solution.
- Acetone

Equipment

- ANKOM 220 fiber analyzer
- Tecator – nitrogen analyzer
- Filtration device – ANKOM –F57 filter bags
- Impulse bag sealer
- Desiccator

Procedure

- Weigh filter bag (W₁).
- Weigh 0.5 g of air-dried sample (W₂), ground to pass through 1 mm screen, directly into filter bag. Weigh one blank bag and include in the digestion to determine blank bag correction (C₁).
- Seal the bags closed within 0.5 cm from the open edge using the heat sealer.
- Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.

- A maximum of 24 bags may be placed in the bag suspender. Place three bags per tray and then stack trays on centre post with each level rotated 120 degrees. The weight is placed on top of the empty 9th tray to keep the bag suspender submerged.
- Add 2000 ml of ambient temperature acid detergent solution into ANKOM fiber analyzer vessel. Place bag suspender with samples into the solution in vessel. Turn *Agitate* and *Heat* ON and confirm that bag suspender is agitating properly. Set timer for 60 minutes and push *Start*. Close and seal lid of vessel.
- After 60 min. have elapsed turn *Agitate* and *Heat* OFF, open the drain valve and exhaust hot solution.
- After the solution has been exhausted, close valve and open the lid. Add approx. 2000 ml of hot (90-100°C) H₂O. Turn *Agitate* ON and leave *Heat* OFF and rinse for 3-5 min. Exhaust water and repeat rinsing for at least three times.
- Remove filter bags, place in beaker and cover with acetone. Allow bags to soak 3 min.
- Spread bags out and allow acetone to evaporate. Complete drying in oven at 105°C for at least 2 hours.
- Remove bags and cool and weigh bags (W₃).
- After the bags have been dried and weighed, transfer them into "Tecator" test tube and proceed with the digestion and distillation as indicated by the Kjeldahl protein method.
- ADIN is the nitrogen value as a per cent of total nitrogen in feed on a dry matter basis.

Calculation

$$\% \text{ ADF (as-is basis)} = \frac{(W_3 - (W_1 \times C_1)) \times 100}{W_2}$$

Where,

W₁ = Bag weight

W₂ = Sample weight

W₃ = Weight after extraction process

C₁ = Blank bag correction (final oven dried weight/ original blank bag weight)

Reference: ANKOM Technology, USA, -220 Manual.

8.7 Determination of cellulose by Fibertec system

To the acid detergent residues, a known quantity of acid-washed-ignited-asbestos is added. Then 72 per cent H₂SO₄ is added to the crucible and asbestos is thoroughly mixed with the residue to a fine paste and stirred with a clean rod. Refill the crucible at hourly intervals and the procedure is continued for 3 hours. After 3 hours, the acid is drained and washed free from acid with water in cold extraction unit of Fibertec system. Dry the crucible at 100°C overnight and weighed.

Calculation

$$\text{Cellulose} = \frac{W_1 - W_2}{W} \times 100$$

Where,

W₁ = Weight of crucible with ADF residue plus asbestos

W₂ = Weight of crucible with residue after 72 per cent H₂SO₄ treatment

W = Weight of the substance

Reference: Goering, H.K. and P.J. Van Soest, 1970. Forage fibre analyses. Agric. Handbook No. 379, ARS, USDA.

8.8 Determination of acid detergent lignin (ADL)

Apparatus

- Refluxing apparatus
- Tall pyrex or corning beakers (spout less) of about 500 ml capacity
- Round bottom flask as condenser
- Sintered glass crucibles with coarse porosity (Grade 1) of about 50 ml capacity
- Electronic balance
- Vacuum pump
- Hot plate
- Wash bottle
- Hot air oven and muffle furnace
- Glass tray

Reagents

- H_2SO_4 , 72 per cent by weight: Take 417 ml water in a volumetric flask and add 583 ml pure H_2SO_4 slowly with occasional swirling. The flask must be cooled in water bath (Sink) in order to add the required weight of acid.

Procedure

- 1) Prepare the acid detergent fibre.
- 2) Place the crucible in the glass tray. Have one end of the tray 2 cm higher so acid will drain away from the crucibles.
- 3) Cover the contents of crucible with cooled ($15^\circ C$) 72 per cent H_2SO_4 and stir with a glass rod to a smooth paste, breaking all lumps. Fill crucible about half way with acid and stir. Let glass rod remain in crucible, refill with 72 per cent H_2SO_4 and stir at hourly intervals as acid drain away. Crucible does not need to be kept full at all times. Three additions suffice. Keep crucible at 20 to $23^\circ C$. After 3 hours, filter off as much acid as possible with vacuum and then wash contents with hot water until free from acid. Rinse and remove stirring rod.
- 4) Dry crucible overnight at $100^\circ C$ and weigh.
- 5) Ignite crucible in a muffle furnace at 500 to $550^\circ C$ for 3 hours, and then cool and weigh.

Observations

Wt. of oven dry sample	=	----- g
Wt. of crucible and lignin	=	----- g
Wt. of crucible and ash	=	----- g

Calculation

$$ADL \% = \frac{\text{Wt. of crucible and lignin} - \text{Wt. of crucible and ash}}{\text{Wt. of sample on dry matter basis}} \times 100$$

Reference: Goering and Van Soest (1970).

8.9 Determination of acid detergent lignin (ADL) – Fibertec system

The residue left after 72 per cent H₂SO₄ acid treatment is ignited at 500-550°C for 3 hours then cooled and weighed.

Calculation

$$\text{ADL (\%)} = \frac{L}{S} \times 100$$

Where,

L = loss upon ignition after 72 per cent H₂SO₄ treatment

S = Sample weight

Reference: Goering and Van Soest (1970).

8.10 Determination of acid detergent lignin (ADL) - Ankom 220 fiber analyzer

Reagents

Sulfuric acid (72 per cent by weight)

Equipment

- ANKOM 220 fibre analyzer
- Tecator – nitrogen analyzer
- Filtration device – ANKOM –F57 filter bags
- Impulse bag sealer
- Desiccator

Procedure

- Weigh filter bag (W₁)
- Weigh 0.5 g of air-dried sample (W₂), ground to pass through 1 mm screen, directly into filter bag. Weigh one blank bag and include in the digestion to determine blank bag correction (C₁).
- Seal the bags closed within 0.5 cm from the open edge using the heat sealer.
- Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.
- Perform ADF determinations, place dried bags/ samples into 3 litre beaker and add sufficient quantity (approx. 250 ml) of 72 per cent H₂SO₄ to cover bags and agitate bags for 3 hours.
- After 3 hrs pour off H₂SO₄ and rinse with hot water (90-100°C) to remove all acid.
- Complete drying in oven at 105°C for 4 hours and cool in a desiccator (Fig. 8.1).

Calculation

$$\% \text{ ADL (as-is basis)} = \frac{(W_3 - (W_1 \times C_1)) \times 100}{W_2}$$

Where,

W₁ = Bag weight

W₂ = Sample weight

W₃ = Weight after extraction process

C₁ = Blank bag correction (final oven dried weight/ original blank bag weight)

Reference: ANKOM Technology, USA-220 Manual.



Fig. 8.1 Desiccator

9.0 ANALYSIS OF MOLASSES

9.1 Determination of density in degree brix

Apparatus

- Brix hydrometer – calibrated at 27.5°C and fitted with thermometer.
- Immersion vessel – suitable for the hydrometer used.

Procedure

Weigh 200 g of the material. Add 1400 g of cold water. Mix well till a homogeneous solution results. Determine the brix using brix hydrometer of suitable range noting simultaneously the temperature of the solution (Fig. 9.1). Apply the temperature corrections to the readings by using Table 9.1.

Calculation

Multiply the corrected brix reading by 8. This gives the density of the molasses in degree brix at 27.5°C.



Fig. 9.1 Brix hydrometer

Reference: IS:1162:1958 Indian Standards Institution, Specification for cane molasses.

Table 9.1 Temperature correction to reading of Brix Hydrometer at 27.50°C

Tem. °C	Observed Degree Brix													
	0	5	10	15	20	25	30	35	40	45	50	55	60	70
	Subtract From Reading													
10	0.75	0.82	0.89	0.96	1.02	1.08	1.14	1.20	1.24	1.28	1.31	1.33	1.35	1.37
11	0.74	0.79	0.86	0.92	0.98	1.03	1.09	1.04	1.18	1.21	1.24	1.26	1.28	1.30
12	0.72	0.76	0.82	0.88	0.93	0.98	1.04	1.08	1.12	0.14	1.17	1.19	1.20	1.22
13	0.69	0.73	0.78	0.84	0.88	0.93	0.98	1.02	1.06	1.07	1.10	1.12	1.13	1.15
14	0.66	0.70	0.74	0.79	0.83	0.88	0.92	0.96	0.99	1.00	1.03	1.05	1.06	1.07
15	0.63	0.66	0.70	0.74	0.78	0.82	0.86	0.89	0.92	0.93	0.95	0.97	0.98	0.99
16	0.60	0.62	0.66	0.69	0.73	0.76	0.80	0.83	0.86	0.86	0.88	0.90	0.91	0.92
17	0.56	0.58	0.61	0.64	0.68	0.70	0.74	0.77	0.79	0.79	0.81	0.83	0.83	0.84
18	0.52	0.54	0.56	0.59	0.62	0.64	0.68	0.70	0.72	0.72	0.74	0.75	0.75	0.76
19	0.48	0.49	0.51	0.54	0.56	0.58	0.61	0.63	0.65	0.65	0.67	0.68	0.68	0.68
20	0.43	0.44	0.46	0.48	0.50	0.52	0.54	0.56	0.58	0.58	0.59	0.60	0.60	0.60
21	0.38	0.39	0.41	0.42	0.44	0.46	0.47	0.49	0.51	0.51	0.51	0.52	0.52	0.52
22	0.33	0.34	0.35	0.36	0.38	0.40	0.40	0.42	0.43	0.43	0.43	0.44	0.44	0.44
23	0.28	0.28	0.29	0.30	0.32	0.33	0.33	0.35	0.36	0.36	0.36	0.36	0.36	0.36
24	0.22	0.22	0.23	0.24	0.25	0.26	0.26	0.27	0.28	0.28	0.28	0.28	0.28	0.28
25	0.16	0.16	0.17	0.18	0.18	0.19	0.19	0.20	0.20	0.20	0.20	0.20	0.20	0.20
26	0.10	0.10	0.11	0.11	0.11	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
27	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04

Table 9.1 Temperature correction to reading of Brix Hydrometer at 27.50°C

Tem. °C	Observed Degree Brix													
	0	5	10	15	20	25	30	35	40	45	50	55	60	70
	Add to the Reading													
28	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
29	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
30	0.18	0.18	0.18	0.18	0.18	0.19	0.19	0.20	0.20	0.20	0.20	0.20	0.20	0.20
31	0.25	0.25	0.25	0.26	0.26	0.27	0.27	0.28	0.28	0.28	0.28	0.28	0.28	0.29
32	0.32	0.33	0.34	0.34	0.35	0.35	0.35	0.36	0.36	0.36	0.36	0.36	0.36	0.37
33	0.40	0.41	0.41	0.42	0.42	0.43	0.43	0.44	0.44	0.44	0.44	0.44	0.44	0.45
34	0.48	0.49	0.49	0.50	0.50	0.51	0.52	0.52	0.52	0.53	0.53	0.53	0.53	0.53
35	0.56	0.57	0.57	0.58	0.59	0.60	0.61	0.61	0.61	0.62	0.62	0.62	0.62	0.62
36	0.64	0.65	0.65	0.67	0.68	0.69	0.70	0.70	0.70	0.71	0.70	0.70	0.70	0.70
37	0.72	0.74	0.74	0.76	0.77	0.78	0.79	0.79	0.79	0.80	0.79	0.79	0.79	0.79
38	0.81	0.83	0.83	0.85	0.86	0.87	0.88	0.88	0.88	0.89	0.88	0.88	0.88	0.88
39	0.90	0.92	0.92	0.94	0.95	0.96	0.97	0.97	0.97	0.98	0.97	0.97	0.97	0.97
40	0.99	1.01	1.01	1.03	1.04	1.05	1.06	1.06	1.06	1.07	1.06	1.05	1.05	1.05
45	1.48	1.50	1.50	1.52	1.53	1.53	1.53	1.53	1.52	1.52	1.51	1.50	1.50	1.48
50	2.03	2.04	2.04	2.05	2.06	2.05	2.04	2.03	2.01	2.00	1.99	1.97	1.96	1.92
55	2.62	2.63	2.63	2.64	2.62	2.60	2.58	2.55	2.52	2.50	2.48	2.45	2.43	2.37
60	3.26	3.28	3.28	3.25	3.22	3.18	3.13	3.09	3.04	3.02	2.98	2.94	2.90	2.83
65	3.95	3.98	3.98	3.90	3.86	3.79	3.71	3.66	3.58	3.56	3.50	3.44	3.38	3.30
70	4.70	4.74	4.70	4.60	4.54	4.44	4.32	4.24	4.14	4.12	4.03	3.96	3.87	3.78
75	5.50	5.56	5.48	5.34	5.26	5.12	4.95	4.86	4.72	4.70	4.57	4.50	4.37	4.27
80	6.35	6.41	6.32	6.12	6.02	5.84	5.62	5.51	5.32	5.30	5.15	5.04	4.88	4.77

9.2 Determination of sulphated ash

Reagent

- Concentrated sulphuric acid – sp. gr. 1.84 g/ml

Procedure

Weigh accurately about 5 g of the material into a suitable tared platinum dish or quartz crucible. Gradually add 2 ml of concentrated sulphuric acid to the material in the dish. Place the dish or crucible on hot water oven. When the charred mass does not increase in the volume any longer, continue the heating over a small flame, care being taken to avoid spurting, until the fumes of sulphur trioxide or sulphur dioxide are not evolved. Increase heat till a pinkish grey ash is obtained (If available, a muffle furnace may be used to a temperature of 550-600°C). Cool the ash and moisten it with a few drops of concentrated sulphuric acid, heat strongly on a hot plate until fumes of sulphur trioxide cease to be evolved and finally ash to constant mass.

Calculation

$$\text{Ash, sulphated, per cent by mass (calculated for } 100^{\circ}\text{C brix)} = \frac{10000 w}{WX}$$

Where,

W = mass in g of the ash

W = mass in g of the material taken for the test, and

X = density of molasses in degree brix

Reference: IS:1162:1958 Indian Standards Institution Specification for cane molasses.

9.3 Determination of total reducing matter

Reagents

Stock solution of invert sugar – Weigh accurately 9.5 g of pure sucrose on a watch glass and transfer it to a one litre volumetric flask with 100 ml water. Add 5 ml of concentrated hydrochloric acid (sp gr 1.19). Allow this to stand for 3 days at 20-25°C and then make up to volume with water (This is stable for several months).

Standard solution of invert sugar – Pipette 50 ml of the stock solution of invert sugar in a 250 ml volumetric flask. Neutralize carefully with sodium hydroxide of about one per cent (w/v) and make up to the volume.

Methylene blue indicator solution – Dissolve 1.0 g of methylene blue in water and dilute to 100 ml.

Fehling's solution (Soxhlet modification) – Prepare by mixing immediately before use, equal volumes of solution A and solution B, prepare as described under:

Solution A – Dissolve 34.639 g of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in water, and 0.5 ml of concentrated sulphuric acid of sp gr 1.84, and dilute to 500 ml in a volumetric flask. Filter the solution through prepared asbestos.

Solution B – Dissolve 173 g of rochelle salt (potassium sodium tartrate; $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) and 50 g of sodium hydroxide, analytical reagent in water, dilute to 500 ml in a volumetric flask and allow the solution to stand for two days. Filter this solution through prepared asbestos.

Standardization of Fehling's solution – Pour standard invert sugar solution into a 50 ml burette. Pipette 10 ml of Fehling's solution into a 300 ml flask and run in from the burette almost the whole of the standard invert sugar solution required to effect reduction of all the copper, so that not more than one millilitre will be required later to complete the titration. Heat the flask containing the mixture over wire gauze. Gently boil the contents of the flask for 2 minutes. At the end of 2 minutes of boiling, add, without interrupting boiling, 3 to 5 drops of methylene blue indicator solution. While the contents of the flask continue to boil, begin to add standard invert sugar solution (one or two drops at a time) from the burette till the blue colour of the indicator just disappears. (The titration should be completed within one minute, so that the contents of the flask boil altogether for 3 minutes without interruption. Note the titre (that is, the total volume in millilitres of standard invert sugar solution used for the reduction of all the copper in 10 ml of Fehling's solution). Multiply the titre (obtained by direct titration) by the number of milligrams of invert sugar in one millilitre of the standard invert sugar solution to obtain the invert sugar factor. Compare this factor with the invert sugar factor given in Table II and determine correction, if any, to be applied to the invert sugar factors derived from Table II.

TABLE II: INVERT SUGAR FACTOR FOR 10 ml of FEHLING'S SOLUTION

This table shows, for the standard method of titration, the values corresponding to integral millilitres of the sugar solution, the values corresponding to intermediate figures being obtained by interpolation.

TITRE IN MILLILITRES	INVERT SUGAR FACTOR*	MILLIGRAMS OF INVERT SUGAR PER 100 MILLILITRES SOLUTION
(1)	(2)	(3)
15	50.5	336
16	50.6	316
17	50.7	298
18	50.8	282
19	50.8	267
20	50.9	254.5
21	51.0	242.9
22	51.0	231.8
23	51.1	222.2
24	51.2	213.3
25	51.2	204.8
26	51.3	197.4
27	51.4	190.4
28	51.4	183.7
29	51.5	177.6
30	51.5	171.7
31	51.6	166.3
32	51.6	161.2
33	51.7	156.6
34	51.7	152.2

TITRE IN MILLILITRES	INVERT SUGAR FACTOR*	MILLIGRAMS OF INVERT SUGAR PER 100 MILLILITRES SOLUTION
35	51.8	147.9
36	51.8	143.9
37	51.9	140.2
38	51.9	136.6
39	52.0	133.3
40	52.0	130.1
41	52.1	127.1
42	52.1	124.2
43	52.2	121.4
44	52.2	118.7

* Milligrams of invert sugar corresponding to 10 ml of Fehling's solution.

Example

Concentration of invert sugar in standard invert sugar

solution as mg/100 ml = 200 mg

Titre obtained by direct titration = 26.2

Invert sugar factor for 26.2 ml
of standard invert sugar solution = Titre in ml x
number of
mg of invert
sugar in 1 ml
of standard

= 26.2 x 2

= 52.4

Invert sugar factor for 26.2 ml
from Table (calculated by
interpolation) = 51.32

Correction to be applied to the invert sugar
factors derived from Table II = 52.4 – 51.32

= + 1.08

Neutral lead acetate – Dissolve 100 g of lead acetate. ($\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$) in distilled water and dilute to one litre.

Sodium phosphate – Potassium oxalate solution – Dissolve 70 g of di-sodium hydrogen phosphate, dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 30 g of potassium oxalate ($\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$) in water and dilute to one litre.

Sodium hydroxide solution – Approximately 6 N, prepared by dissolving sodium hydroxide analytical reagent.

Concentrated hydrochloric acid – sp gr 1.029 at 20 deg.

Procedure

Preparation of solution – Weigh accurately about 12.5 g of molasses and transfer to a 250 ml volumetric flask. Add 25 ml of the lead acetate solution. Make up to volume, mix and filter. Reject the first few drops of the filtrate. To 100 ml of the clear filtrate in a 500 ml volumetric flask, add 10 ml of the sodium phosphate-potassium oxalate mixture. Make up to volume with water, shake and filter. Reject the first few drops of the filtrate and use the clear filtrate for preparation of invert solution.

Preparation of invert solution – To 50 ml of the filtrate, in a 100 ml volumetric flask, add 25 ml of water and 10 ml of concentrated hydrochloric acid. Heat on a water bath to 70°C and regulate heat in such a way that the temperature is maintained at 70°C. Place the flask in water bath, insert a thermometer and heat with constant agitation until the thermometer in the flask indicates 67°C. From the moment the thermometer in flask indicates 67°C, leave the flask in the water bath for exactly 5 minutes, during which time the temperature should gradually rise to about 69.5°C. Plunge the flask at once into water at 20°C. When the contents have cooled to about 35°C, remove the thermometer from the flask, rinse it and add 10 ml of 6 N sodium hydroxide solutions for neutralization of acid, leave the flask in the bath at 20°C for about 30 minutes and then make up exactly to volume with water. Mix the solution well.

Incremental method of titration – Pour the prepared solution into a 50 ml burette (see Note 5). Pipette 10 ml of Fehling's solution into a 300 ml conical flask and run in from the burette 15 ml of the prepared solution. Without further dilution, heat contents of flask over a wire gauze and boil (after the liquid has been boiling for about 15 seconds it will be possible to judge if the copper is almost all reduced by the bright red colour imparted to the boiling liquid by the suspended cuprous oxide). When it is judged that nearly all the copper is reduced, add 3 to 5 drops of methylene blue indicator solution (see Note 1). Continue boiling the contents of the flask for one to two minutes from the commencement of ebullition, and then add the prepared solution in small quantities (one millilitre or less at a time), allowing the liquid to boil for about 10 seconds between successive additions, till the blue colour of the indicator just disappears (see Note 4). In case there appears to be still much unreduced copper, after the mixture of Fehling's solution with 15 ml of the prepared solution has been boiling for a quarter of a minute, add the prepared solution from the burette in larger increments (more than one milliliter at a time, according to judgment), and allow the mixture to boil for a quarter of a minute after each addition. Repeat the addition of the prepared solution at intervals of 15 seconds until it is considered unsafe to add large increment of the prepared solution. At this stage continue the boiling for an additional one to two minutes, add 3 to 4 drops of methylene blue indicator solution and complete the titration by adding the prepared solution in small quantities (less than one millilitre at a time) (see also Note 2).

Note 1

It is advisable not to add the indicator until the neighbourhood of the end point has been reached, because the indicator retains its full colour until the end point is almost reached and thus gives no warning to the operator to go slowly.

Note 2

When the operator has had a fair amount of experience with the method, a sufficiently accurate result may often be obtained by a single estimation by the incremental method of titration, but for the utmost degree of accuracy of which the method is capable, a second titration should be carried out by the standard method of titration.

Standard method of titration

Pipette 10 ml of Fehling's solution into a 300 ml conical flask and run in from the burette almost the whole of the prepared solution required to effect reduction of all the copper so that, if possible, not more than one millilitre shall be required later to complete the titration. Gently boil the contents of the flask for two minutes. At the end of 2 minutes of boiling, add, without interrupting boiling, one millilitre of methylene blue indicator solution. While the contents of the flask continue to boil, begin to add the solution (one or two drops at a time), from the burette till the blue colour of the indicator just disappears (see Note 3). The titration should be completed within one minute, so that the contents of the flask boil altogether for 3 minutes without interruption (see Note 4).

Note 3

The indicator is so sensitive that it is possible to determine the end point within one drop of the prepared solution in many cases. The complete decolorization of the methylene blue is usually indicated by the whole reaction liquid in which the cuprous oxide is continuously churned up becoming bright red or orange in colour. In case of doubt, the flame may be removed from the wire gauze for one or two seconds and the flask held against a sheet of white paper. (A paper holder suitably fixed round the neck of the flask without risk of over balancing it). The top edge of the liquid would appear bluish if the indicator is not completely decolorized. It is inadvisable to interrupt the boiling for more than a few seconds as the indicator undergoes back oxidation rather rapidly when air is allowed free access into the flask, but there is no danger of this as long as a continuous stream of steam is issuing from the mouth of the flask.

Note 4

It should be observed that with both incremental and standard methods of titration, the flask containing the reaction mixture is left on the wire gauze over the flame throughout the titration except when it may be removed for a few seconds to ascertain if the end point is reached.

Note 5

In adding sugar solution to the reaction mixture, the burette may be held in hand over the flask. The burette may be fitted with a small outlet tube bent twice at right angles, so that the body of the burette can be kept out of the steam while adding sugar solution. Burettes with glass taps are unsuitable for this work, as the taps become heated by the steam and are liable to jam.

Calculation

Refer to table for the invert sugar factor corresponding to the titre and apply the correction previously determined.

$$\begin{array}{l} \text{Milligrams of invert sugar present} \\ \text{in 1 ml of the prepared solution} \end{array} = \frac{\text{Invert sugar factor}}{\text{Titre}}$$
$$\begin{array}{l} \text{Reducing matter, per cent by mass} \\ \text{ } \end{array} = \frac{250 C}{W}$$

Where,

C = milligrams of the invert sugar in 1 ml of the prepared solution, and

W = mass in g of the material taken for the test.

Reference: IS:1162:1958 Indian Standards Institution, Specification for cane molasses.

10.0 MINERAL ANALYSIS

10.1 Determination of moisture

Procedure

- Weigh accurately about 2-3 g of the sample in a silica dish.
- Dry the sample in vacuum oven (Fig. 10.1) at 60°C and 20" Hg pressure for a minimum of 2 hours.

Calculation

$$\text{Moisture, per cent by mass} = \frac{100 (M_1 - M_2)}{M_1 - M}$$

Where,

- M_1 = Mass in g of the dish with the material before drying
- M_2 = Mass in g of the dish with the material after drying, and
- M = Mass in g of the empty dish

Reference: IS 1664: 2002.



Fig. 10.1 Vacuum oven

10.2 Determination of total ash

Procedure

- Weigh accurately about 2-3 g of moisture free DCP / mineral mixture (As described under moisture estimation) in silica dish.
- Complete the ignition by keeping in a muffle furnace (Fig. 10.2) at 550°C for 2 hours.
- Cool in desiccator and weigh.
- Total ash in the moisture free sample can be calculated.



Fig. 10.2 Muffle furnace

Calculation

$$\text{Total ash, per cent by mass} = \frac{100 (M_2 - M)}{M_1 - M}$$

Where,

- M_2 = Mass in g of the dish with the material after ashing in muffle furnace at 550°C for 2 hours.
- M_1 = Mass in g of the dish with the material after drying in vacuum oven at 60°C.
- M = Mass in g of the empty dish.

Reference: IS 1664: 2002.

10.3 Determination of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc – using Atomic Absorption Spectrometry

Reagents and materials

Use only reagents of recognized analytical grade.

- Water, complying with at least grade 3 in accordance with ISO 3696.
- Concentrated hydrochloric acid. $c(\text{HCl}) = 12 \text{ mol/l}$ ($\rho = 1.19 \text{ g/ml}$).
- Hydrochloric acid, $c(\text{HCl}) = 6 \text{ mol/l}$.
- Dilute hydrochloric acid. $c(\text{HCl}) = 0.6 \text{ mol/l}$.



Fig. 10.3 Atomic Absorption Spectrophotometer (AAS)

- Lanthanum nitrate solution - Dissolve 133 g of $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ in 1 litre of water.
- Caesium chloride solution - Dissolve 100 g of CsCl in 1 litre of water.

Stock solution of Cu, Fe, Mn and Zn

Mix 100 ml of water and 125 ml of concentrated hydrochloric acid in a 1 litre volumetric flask.

Weigh out the following:

- 392.9 mg of copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)
- 702.2 mg of ammonium iron (II) sulfate hexahydrate [$(\text{NH}_4)_2\text{SO}_4 \cdot \text{FeSO}_4 \cdot 6\text{H}_2\text{O}$]
- 307.7 mg of manganese sulfate monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$)
- 439.8 mg of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)

Transfer the weighed salts to the volumetric flask and dissolve them in water. Dilute to the mark with water.

The contents of Cu, Fe, Mn and Zn of this stock solution each are $100 \mu\text{g/ml}$.

Note : Ready-prepared commercially available solutions may be used.

Standard solution of Cu, Fe, Mn and Zn

Dilute 20.0 ml of the stock solution with water to 100 ml in a volumetric flask. The contents of Cu, Fe, Mn and Zn of this solution are each $20 \mu\text{g/ml}$. Prepare the solution fresh on the day of use.

Stock solution of Ca, K, Mg and Na

Weigh out the following:

- 1.907 g of potassium chloride (KCl)
- 2.028 g of magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
- 2.542 g of sodium chloride (NaCl).

Transfer the weighed salts to a 1 litre volumetric flask.

Add 50 ml of hydrochloric acid (6 mol/l) to a beaker. Weigh into the beaker 2.497 g of calcium carbonate (CaCO_3).

Standard solution of Ca, K, Mg and Na

Dilute 25.0 ml of stock solution with dilute hydrochloric acid (0.6 mol/l) to 250 ml in a volumetric flask.

The contents of Ca, K and Na of this solution are 100 $\mu\text{g}/\text{ml}$ each; the content of Mg of the solution is 20 $\mu\text{g}/\text{ml}$.

Prepare the solution fresh in the week of use and store it in a polyethylene bottle.

Lanthanum / caesium blank solution

Add 5 ml of lanthanum nitrate solution, 5 ml of caesium chloride solution and 5 ml of hydrochloric acid (6 mol/l) to a 100 ml volumetric flask. Dilute to the mark with water.

Apparatus

- Analytical balance, capable of weighing to the nearest 0.1 mg.
- Incineration dishes of platinum, quartz or porcelain, free from potassium and sodium with a smooth un-detached inner surface, upper internal diameter 4 to 6 cm, lower internal diameter 2 to 2.5 cm, and a height of about 5 cm. Before use, boil with hydrochloric acid.
- Glassware of hard borosilicate glass.
- Electric hot plate or gas burner.
- Boiling water bath.
- Electric muffle furnace, capable of being maintained at $(550 \pm 15^\circ\text{C})$
- Atomic absorption spectrometer (Fig. 10.3). Suitable for measuring at the wavelengths specified and provided with an air-acetylene flame and a facility for correction, or measurement of background absorption.
- Hollow cathode lamps or electrode-less discharge lamps for the determination of Ca, Cu, Fe, K, Mg, Mn, Na or Zn.
- Filter paper which does not release minerals.

Procedure

Detection of presence of organic matter

Heat a spatula with some test sample in a flame.

If the test sample melts without smoke, little organic matter is present.

If the test sample changes in color and melting does not occur, the test sample contains organic matter.

Test portion

Depending on the expected content, weigh 1 to 5 g of the prepared test sample to the nearest 1 mg into an incineration dish.

If the test sample contains organic matter, proceed for dry ashing.

If the test sample contains little or no organic matter, proceed for decomposition.

Dry ashing

Heat the incineration dish on a hot plate or over a gas burner until the test portion has been completely carbonized in fume hood chamber (Fig. 10.4). Avoid burning the test portion.

Transfer the dish to the muffle furnace, which has already been at a temperature of 550°C for 15 min. Ash the sample for 3 h at this temperature.

Allow the sample to cool down then moisten the contents of the dish with 2 ml of water. If many carbon particles are present, dry the dish over the water bath.

Ash for another 2 h in the muffle furnace set at 550°C.

Allow to cool down then add 2 ml of water.

Decomposition

While swirling, add 10 ml of hydrochloric acid (6 mol/l), first drop wise until effervescence (possible development of carbon dioxide) has ceased, then faster. Swirl and heat the contents of the dish until almost dry. While drying, take care to avoid loss by splattering.

Dissolve the residue by heating with 5 ml of hydrochloric acid (6 mol/l) and transfer the solution quantitatively with some 5 ml portions of water to a 50 ml volumetric flask.

Allow to cool, then dilute to the mark with water and mix. Allow the particles to settle and filter the solution if it is not clear after 4 h.

Blank solution

Prepare for each measuring series a blank solution by carrying out the procedure according to test portion, dry ashing and decomposition without the test sample.



Fig. 10.4 Fume hood chamber

Determination of copper, iron, manganese and zinc

Measuring conditions

Adjust the atomic absorption spectrometer in accordance with the manufacturer's instructions. Optimize the response of the instrument for measurement with the air-acetylene flame. For the determination of Cu, Fe, Mn and Zn set the following wavelengths:

Cu: 324.8 nm;

Fe: 248.3 nm;

Cu: 279.5 nm;

Cu: 213.8 nm

Preparation of calibration curve

Prepare a series of appropriate calibration solutions by diluting the standard solution with dilute hydrochloric acid (0.6 mol/l).

Measure the absorbance of the hydrochloric acid (0.6 mol/l). Measure the absorbance of the calibration solutions and subtract the absorbance measured for hydrochloric acid (0.6 mol/l).

Draw a calibration curve by plotting the corrected absorbance against the respective contents of Cu, Fe, Mn and Zn.

Measurement of test solution

Measure parallel to the calibration solutions, under identical circumstances, the absorbance of the test solution and the blank solution. Subtract the latter absorbance from the first absorbance.

If necessary, dilute a quantity of the test solution and blank solution with dilute hydrochloric acid (0.6 mol/l) to obtain an absorbance in the linear part of the calibration curve.

Proceed in accordance with expression of results

Determination of calcium, magnesium, potassium and sodium

Measuring conditions

Adjust the atomic absorption spectrometer in accordance with the manufacturer's instructions. Optimize the response of the instrument for measurement with the air-acetylene flame. For the determination of Ca, K, Mg and Na set the following wavelengths:

Ca : 422.6 nm;

K : 766.5 nm;

Mg : 285.2 nm;

Na : 589.6 nm

Preparation of calibration curve

Dilute the standard solution with water. Add per 100 ml of diluted standard solution 5 ml of lanthanum nitrate solution, 5 ml of caesium chloride solution and 5 ml of hydrochloric acid (6 mol/l). Choose the dilutions so that appropriate calibration solutions are obtained.

Measure the absorbance of the lanthanum / caesium blank solution.

Measure the absorbance of the calibration solutions and subtract the absorbance measured for the lanthanum / caesium blank solution.

Draw a calibration curve by plotting the corrected absorbance against the respective contents of Ca, K, Mg and Na.

Measurement of test solution

Dilute a quantity of the test solution and blank solution with water. Add per 100 ml of diluted solution 5 ml of lanthanum nitrate solution, 5 ml of caesium chloride solution and 5 ml of hydrochloric acid (6 mol/l).

Measure parallel to the calibration solutions, under identical circumstances, the absorbance of the diluted test solution and the diluted blank solution. Subtract the latter absorbance from the first absorbance.

If necessary, dilute a quantity of the test solution and blank solution with lanthanum / caesium blank solution to obtain an absorbance in the linear part of the calibration curve.

Expression of results

Calculate the content of each of the elements calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc starting from the calibration curve and taking into account the mass of the test portion and the dilutions applied.

Express the result in milligrams per kilogram or grams per kilogram.

Reference: IS: 15121: 2002, ISO 6869: 2000.

10.4 Determination of calcium – Precipitation method

Reagents

- Hydrochloric acid – 25 ml of concentrated hydrochloric acid diluted to 100 ml.
- Methyl red indicator – Dissolve 0.15 g of methyl red in 500 ml of water.
- Ammonium hydroxide solution – 50 per cent (v/v).
- Dilute ammonium hydroxide solution – 2 per cent (v/v).
- Ammonium oxalate solution – Saturated.
- Concentrated sulphuric acid
- Standard potassium permanganate solution – 0.1 N.

Procedure

1. Ashing and extraction – Accurately weigh about 3 g of the material into a silica dish. Char carefully and continue the ashing in a muffle furnace at a temperature not above 450°C until the ash is white or almost so. Cool the ash, moisten with a few millilitres of distilled water and add 3 to 5 ml of concentrated hydrochloric acid drop by drop. Evaporate to dryness on a water-bath and continue heating on the water bath for one hour to render silica insoluble. Moisten the residue with 20 ml distilled water and add about 2 to 3 ml of concentrated hydrochloric acid. Heat on a water bath for a few minutes and filter through medium filter paper into a 250 ml volumetric flask. Wash the filter paper thoroughly with hot water, cool the filtrate and make it up to volume, shake thoroughly.
2. Transfer a 25 ml aliquot of the solution prepared as in (1) to a 400 ml beaker, dilute to about 100 ml with water and add two drops of methyl red indicator solution. Add ammonium hydroxide solution drop wise till a brownish – orange color is obtained (pH 5.6). Add two drops of hydrochloric acid so that the color of solution is pink (pH 2.5 to 3.0). Dilute to about 150 ml, bring to the boil and add slowly, with constant stirring, 10 ml of hot ammonium oxalate solution. If the red color of the solution changes to orange or yellow, add hydrochloric acid drop wise until the color again changes to pink. Leave overnight to allow the precipitate to settle. Filter the supernatant liquid through ash-less filter paper and wash the precipitate thoroughly with dilute ammonium hydroxide solution. Place the paper with the precipitate in the beaker which precipitation was carried out and add a mixture of 125 ml of water and 5 ml of concentrated sulphuric acid, heat to 70 to 90°C and titrate with the standard potassium permanganate solution until the first slight pink colour is obtained.

Calculation

$$\text{Calcium (as Ca) (on moisture-free basis), per cent by mass} = \frac{2000 AN}{m (100-M)}$$

Where,

A = volume in ml of the standard potassium permanganate solution required in the titration

N = normality of the standard potassium permanganate solution

m = mass in g of the material taken for the test and

M = per cent moisture content

Reference: IS:7874 (part-II) – 1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.5 Determination of phosphorus – Precipitation method

Reagents

- Concentrated nitric acid
- Nitric acid (1:1) – A mixture of equal volumes of concentrated nitric acid and water.
- Ammonium molybdate stock solution – Take 200 g of powdered ammonium molybdate in a stoppered graduated cylinder of 1000 ml capacity, add to it 800 ml of water and shake well for 25 minutes to dissolve the ammonium molybdate. Add gradually 25 per cent ammonium hydroxide solution till the solution is clear (about 100 to 140 ml of ammonium hydroxide may be required). Avoid adding excess of ammonia. Make up the volume to one litre. If necessary, filter the solution through a fluted filter paper and stock this solution.
- Nitric acid solution – 2 per cent (m/v).
- Potassium nitrate solution – 3 per cent (m/v)
- Standard sodium hydroxide solution – 0.1 N.
- Standard nitric acid solution – 0.1 N.
- Phenolphthalein indicator solution – Dissolve 0.1 g of phenolphthalein in 100 ml of 60 per cent (m/v) rectified spirit.

Procedure

Precipitation

Take 10 ml aliquot of the prepared solution (As in ashing and extraction of calcium) in a 150 ml beaker. In a dry beaker, prepare ammonium molybdate solution by pouring into it, quickly and simultaneously 10 ml of the ammonium molybdate stock solution and 10 ml of concentrated nitric acid; or take 10 ml of concentrated nitric acid first in the beaker and into this pour quickly 10 ml of the ammonium molybdate stock solution, whirling the beaker during addition. Pour this freshly prepared clear liquid quickly into the beaker containing the aliquot and stir.

Filtration and washing

Allow the precipitate to stand overnight and then filter through a disc of Whatman filter paper No. 42 in a gooch crucible by suction or through a 9 cm Whatman filter paper No. 42 over an ordinary funnel. As far as possible only the supernatant liquid is passed through the filter paper, retaining the precipitate in the beaker. When the supernatant liquid is decanted off, the precipitate is washed twice with dilute nitric acid and then with potassium nitrate solution until the washings is free from acid. If ordinary funnel and filter paper are used, freedom from acidity may be tested by collecting sufficient filtrate in test tube to which a few drops of phenolphthalein indicator solution and one drop of the standard sodium hydroxide solution are added. If the pink color appears with one drop of the standard alkali, the precipitate is free from acid.

Titration

Transfer the precipitate with the filter paper back to the beaker in which precipitation was carried out. When gooch crucible is used for filtration, transfer the whole crucible along with the filter paper to the beaker in which precipitation was carried out. Add sufficient quantity of the standard sodium hydroxide solution from a burette just sufficient to dissolve the precipitate and then add 5 ml in excess. See that no yellow precipitate sticks to the filter paper. Note the total volume of the standard sodium hydroxide solution added. Add about 10 drops of phenolphthalein indicator solution and titrate the excess of alkali with the standard nitric acid.

Calculation

$$\text{Phosphorus (on moisture-free basis) per cent by mass} = \frac{336.75 (AN_1 - BN_2)}{m (100 - M)}$$

Where,

- A = volume in ml of the standard sodium hydroxide solution used,
- N₁ = normality of the standard sodium hydroxide solution,
- B = volume in ml of the standard nitric acid used in to neutralize the excess alkali
- N₂ = normality of the standard nitric acid,
- m = mass in g of the material taken for the test and,
- M = per cent moisture content

Reference: IS:7874 (part – II) – 1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.6 Determination of phosphorus – Photometric method

Apparatus

- UV-VIS spectrophotometer

Reagents

- Molybdovanadate reagent – Dissolve 40 g ammonium molybdate 4H₂O in 400 ml hot H₂O and cool. Dissolve 2 g ammonium metavanadate in 250 ml hot H₂O and cool; add 250 ml 70% HClO₄. Gradually add molybdate solution to vanadate solution with stirring, and dilute to 2 litre.
- Phosphorus standard solutions – (i) Stock solution -2 mg/ml. Dissolve 8.788 g KH₂PO₄ in H₂O and dilute to 1 L (ii). Working solution – 0.1 mg/ml. Dilute 50 ml stock solution to 1 litre.
- Preparation of standard curve
- Transfer aliquots of working standard solution containing 0.5, 0.8, 1.0 and 1.5 mg P to 100 ml volumetric flasks. Treat as mentioned in determination (d). Prepare standard curve by plotting mg P against per cent T on semi log paper.
- Determination using UV-VIS spectrophotometer (Fig. 10.5).

Ash 2 g sample in 150 ml beaker about 4 h at 600°C. Cool, add 40 ml HCl (1+3) and several drops HNO₃ and bring to boiling point. Cool, transfer to 200 ml volumetric flask and dilute to volume with H₂O. Filter and place aliquot containing 0.5-1.5 mg P in 100 ml volumetric flask. Add 20 ml molybdovanadate reagent, dilute to volume with H₂O and mix well. Let stand 10 min; then read per cent T at 400 nm against 0.5 mg standard set at 100% T (Use 15 mm diameter cells). Determine mg P from standard curve.



Fig. 10.5 UV-VIS spectrophotometer

Calculation: P (%) = mg P in aliquot / (g sample in aliquot x 10)

Reference: AOAC Official Method 965.17.

10.7 Determination of available phosphorus

Reagents

- Aminonaphtholsulphonic acid: Place 195 ml of 15 per cent sodium bisulphate solution in a glass-stoppered cylinder. Add to it 0.5 g of 1, 2, 4- aminonaphtholsulphonic acid and 5 ml of 20 per cent sodium sulphite solution. Stopper the cylinder and shake well to dissolve the powder. If the powder is not dissolved completely add more sodium sulphite solution, 1 ml at a time, with shaking but avoid excess. Transfer the solution to a brown-glass bottle and store in the cold. The solution, if stored as described, may be used for about four weeks.
- Sodium bisulphite solution – 15 per cent: Weigh accurately 30 g of sodium bisulphate in a beaker and add 200 ml of water. Stir to dissolve, and if the solution is turbid allow to stand well-stoppered for several days and then filter. Keep the solution well stoppered.
- Sodium sulphite solution: Dissolve 20 g of anhydrous sodium sulphite in water and dilute to 100 ml, if necessary, filter the solution. Keep the solution well-stoppered.
- Calcium chloride solution: 10 per cent, saturated with calcium hydroxide at pH 8.8.
- Calcium chloride solution: 20 per cent
- Hydrochloric acid: Dilute
- Molybdate I: Dissolve 25 g of reagent grade ammonium molybdate in about 200 ml of water. Place in one-litre graduated flask, 500 ml of 10 N sulphuric acid and add to it the molybdate solution. Dilute to one litre with water. Mix well. The solution keeps stable indefinitely.
- Molybdate II: Dissolve 25 g of reagent grade ammonium molybdate in about 200 ml of water. Place in one-litre graduated flask, 300 ml of 10 N sulphuric acid and add to it the molybdate solution. Dilute to one litre with water. Mix well. The solution keeps stable indefinitely.
- Phenolphthalein indicator solution: Dissolve 0.1 g of phenolphthalein in 100 ml of 95 per cent (m/v) ethyl alcohol.
- Sodium hydroxide solution - Saturated.
- Standard phosphate solution: Weigh exactly 0.351 g of pure dry mono-potassium phosphate and dissolve in water. Transfer quantitatively to a one-litre graduated flask. Add to it 10 ml of 10 N sulphuric acid and make up the volume to the mark. Shake thoroughly. This solution contains 0.4 mg of phosphorus in every 5 ml of the solution.
- Sulphuric acid – 10 N: Add carefully 450 ml of concentrated sulphuric acid to 1300 ml of water. To check, dilute 10 ml of this solution to 100 ml in graduated flask, mix and titrate a 10 ml portion of this solution with standard 1 N sodium hydroxide solution. From the titration results, adjust, if necessary, the normality of the original solution to make it exactly 10 N.
- Trichloroacetic acid – 5 per cent: Dissolve 5 g of the reagent grade trichloroacetic acid in water and dilute to 100 ml.

Apparatus

- Colorimeter

Procedure

1. Weigh accurately about 20 g of the ground material and transfer it to a 250 ml beaker. Add 100 ml of trichloroacetic acid (maintained at about 5°C) and stir occasionally for 15 minutes. Allow it to stand for 2 hours.

2. Transfer the contents to a 250 ml graduated flask and make up the volume to the mark with trichloroacetic acid (maintain at about 5°C). Stir the content of the flask thoroughly and allow to stand for 30 minutes. Filter about 120 ml of the supernatant liquid and transfer 100 ml of the filtrate to a 250 ml beaker. Neutralize with the sodium hydroxide solution using phenolphthalein as the indicator. Add to it 2 ml of calcium chloride solution and allow it to stand at room temperature for 10 minutes. Centrifuge the precipitate and wash with a small volume of water containing the calcium chloride solution. Filter and wash. Place the funnel containing the filter paper and the precipitate on an empty 100 ml graduated flask. Dissolve the precipitate with dilute hydrochloric acid, wash the filter paper, and then make up the volume of the filtrate to the mark.
3. Transfer 5 ml of the filtrate to a 10 ml graduated cylinder and add to it 1 ml of the molybdate II reagent. Shake thoroughly and add 0.4 ml of the aminonaphtholsulphonic acid reagent and mix again. Make up the volume to the 10 ml mark with water, mix, and allow the contents to stand for 5 minutes.
4. For taking the colorimetric measurement, compare in the colorimeter against a standard prepared at the same time as given below:
5. Transfer 5 ml of the standard phosphate solution containing 0.4 mg phosphate solution containing 0.4 mg phosphorus, to a 100 ml graduated flask and add 50 ml of water. Add 10 ml of molybdate I reagent. Mix thoroughly and add 4 ml of aminonaphtholsulphonic acid reagent. Dilute with water to the 100 ml mark, mix well and allow the contents of the flask to stand for 5 minutes. Compare the standard against itself in the colorimeter before taking a reading of the unknown solution. If the colour of the unknown is particularly strong, repeat the reading the unknown a few minutes later, to make sure that the maximum colour development has taken place.
6. Calculate the percentage of the available phosphorus in the material from the reading of the colorimeter.

Reference: IS 1374: 1992.

10.8 Determination of iron – Colorimetric method

Apparatus

- Heat resistant glass tube – of 50 ml capacity, marked at 30 ml.
- Centrifuge – suitable for clarifying the isoamyl alcohol phase.
- Photoelectric colorimeter – capable of measuring optical density at 495 nm. (Fig. 10.6)

Reagents

- Distilled water – redistilled.
- Concentrated sulphuric acid – r.d. 1.84
- Perchloric acid – 60 per cent (m/m) solution.
- Concentrated nitric acid – 60 per cent (m/m).
- Ammonium hydroxide solution – 25 per cent (m/m).
- Concentrated hydrochloric acid – 35 per cent (m/m)
- Hydrogen peroxide solution – 0.1 per cent (m/m) solution in water stored in a brown bottle in a refrigerator.
- Isoamyl alcohol – of boiling point 129 to 132°C.



Fig. 10.6 Photoelectric colorimeter

- Potassium thiocyanate solution – Dissolve 50 g of potassium thiocyanate (KSCN) in 100 ml of water.
- Standard iron solution – Dissolve 0.7022 g of ferrous ammonium sulphate ($\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$) in 100 ml of water, add 5 ml of concentrated sulphuric acid, warm slightly and add potassium permanganate solution (approximately 0.1 N) drop by drop until the solution shows a slight pink coloration. Make up the volume to one litre in a graduated flask. Pipette 10 ml of this solution into a one litre graduated flask, add 10 ml of hydrogen peroxide solution and make up the volume with water. This solution contains 1 microgram of iron per millilitre.

Procedure

Preparation of the test solution

1. Weigh accurately about 2.0 g of the material and transfer to a 200 ml Erlenmeyer flask. Add 2 ml of concentrated sulphuric acid, 3 ml of perchloric acid and 5 ml of concentrated nitric acid. Digest until a clear solution is obtained and while fumes of sulphuric acid are evolved. Dilute with 10 ml of water and make up the volume to 200 ml with water in a graduated flask. Preserve this solution for the determination of copper and cobalt.
2. Take a suitable aliquot of the test solution containing about 10 microgram of iron and transfer to the heat resistant glass tube. Add ammonium hydroxide solution until the solution is just alkaline to phenolphthalein. Add 1 ml of concentrated hydrochloric acid and 1 ml of hydrogen peroxide solution and make up the volume in the tube to 30 ml with distilled water. Add 10 ml of isoamyl alcohol, accurately measured, and 2 ml of potassium thiocyanate solution, stopper the tube and shake for 20 seconds. Transfer enough of the isoamyl alcohol phase meant for colour measurement to the centrifuge tubes, and centrifuge for 5 minutes at about 3000 rev/min. Measure the absorption of the solution in a suitable photo-electric colorimeter at 495 nm setting the reading of the blank at zero absorption. The blank is prepared simultaneously by using the same quantities of acid employed in the digestion, making up the volume and developing the colour in the same size aliquot and in the same manner as in the case of the test solution.
3. Prepare a series of standards by treating aliquots of the standard iron solution in the same manner as the test solution. From the absorption of the standard solutions, prepare a standard curve plotting absorption values against concentrations. From this curve, obtain the mass of iron present in the test solution and calculate the quantity of iron present in 100 g of the material on moisture free basis.

Reference: IS:7874 (part-II) – 1975. Methods for animal feeds and feeding stuffs. Part II. Minerals and trace elements.

10.9 Determination of copper – Colorimetric method

Apparatus

1. Heat resistant glass tube – of 50 ml capacity and marked at 30 ml.
2. Centrifuge – capable of clarifying the isoamyl alcohol phase.
3. Photoelectric colorimeter – capable of measuring the optical density at 430 nm.

Reagents

- Sodium citrate solution – saturated
- Ammonium hydroxide solution – 20 per cent (m/v).
- Isoamyl alcohol – boiling point 129 to 132°C
- Sodium diethyldithiocarbonate solution – 0.1 per cent (m/v) aqueous.

- Standard copper solution- Dissolve 0.393 g cupric sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) of analytical grade in distilled water, add few drops of concentrated sulphuric acid and make up the volume to one litre in a graduated flask. Shake well. Pipette out of 10 ml of this solution into a one litre graduated flask and make up the volume. This solution contains 1 microgram of copper per ml.

Procedure

1. Pipette a suitable aliquot of the test solution as prepared in iron determination containing about 10 microgram of copper, in the glass tube marked at 30 ml. Add 3 ml of sodium citrate solution and ammonium hydroxide solution until just alkaline to phenolphthalein, followed by a 3 ml of ammonium hydroxide solution. Make up the volume to 30 ml with distilled water. Add 10 ml of isoamyl alcohol accurately measured and 1 ml of sodium diethyldithiocarbamate



Fig. 10.7 Centrifuge

2. Stopper the tube and shake vigorously for 20 seconds. Transfer enough of the isoamyl alcohol phase meant for color measurement to centrifuge tubes and centrifuge for 2 minutes at about 3000 rpm in centrifuge machine (Fig. 10.7). Measure the absorption of the solution in a suitable photo-electric colorimeter at 430 nm, setting the reading of blank at zero absorption. The blank is prepared simultaneously by using the same quantities of acid employed in the digestion, making up the volume and developing the color in the same size aliquot and in the same manner as in the case of the test solution.
2. Prepare a series of standards by rating aliquots of the standard copper solution in the same manner as the test solution. From the absorption of the standard solutions prepare a standard curve plotting absorption values against concentrations. From this curve, obtain the mass of copper present in the test solution and calculate the quantity of copper present in 100 g of the material of moisture-free basis.

Reference: IS:7874 (part-II)-1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.10 Determination of manganese – Colorimetric method

Apparatus

- Photoelectric colorimeter – capable of measuring optical density of 520 nm.

Reagents

- Concentrated sulphuric acid – r.d 1.84.
- Sulphurous acid – saturated solution stored in an amber bottle in a cool place.
- Solution A – Mix 42 ml of water, 2 ml of sulphuric acid, 5 ml of sulphurous acid solution and 1 ml of phosphoric acid. This solution should be freshly prepared before use.
- Potassium periodate
- Sodium metabisulphite solution – 10 per cent (m/v), aqueous.
- Phosphoric acid – r.d 1.70
- Concentrated nitric Acid – r.d 1.42

- Standard manganese solution – Dissolve 0.5756 g of dry potassium permanganate in about 50 ml of water in a beaker of suitable size. Add 40 ml of concentrated sulphuric acid and reduce the permanganate by careful addition of sodium metabisulphite solution until the manganese solution just becomes colourless. Oxidize the excess sulphurous acid in the hot solution by the addition of a little nitric acid. Cool and transfer the solution quantitatively to a 2 litre graduated flask. Make up the volume and store the solution in a glass stoppered reagent bottle. This solution contains 0.1 mg of manganese per millilitre.

Procedure

1. Weigh accurately about 5 g of the material into a silica dish, char carefully and ask it in a muffle furnace at 600 to 700°C. Cool, extract the ash with 10 ml of solution A for 2 minutes and transfer to a 150 ml beaker. Rinse the dish first with 40 ml of solution A and then with distilled water, collecting the rinsing until the volume is 100 ml. Heat to the boil on a hot plate and evaporate the solution, using a boiling tube until the volume is reduced to 20 ml. Care should be taken not to allow the solution to bump. Allow the solution to stand overnight. Filter through a small disc of ash less filter paper under slight suction into a 150 ml beaker. Wash the filter paper and dilute and filtrate with water to about 100 ml. Add 2 ml of phosphoric acid and 0.3 g of potassium periodate. Boil to oxidize the manganese and continue boiling for about 15 minutes after the colour has been apparently fully developed. The final volume should not be less than 50 ml (If necessary, boiling water may be added to the solution while boiling). Cool and dilute to 100 ml. Measure the absorption of the solution at 52 nm by means of a suitable photo electric colorimeter.
2. Simultaneously carry out a control determination under the same conditions as above step 1, adding 5 ml of the standard manganese solution, 2 ml of sulphuric acid and 2 ml of phosphoric acid to 100 ml of water and oxidizing with potassium periodate as described in step 1.
3. Measure the absorption at 520 nm of a series of aliquots of the standard manganese solution treated in the same manner as the test solution. Plot a curve of these absorption values against concentration. From this curve, obtain the mass of manganese in the test solution and calculate the quantity of manganese present in 100 g of the material on moisture-free basis.

Reference: IS:7874 (part-II) – 1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.11 Determination of cobalt – Colorimetric method

Apparatus

- Spectrophotometer or photoelectric colorimeter – of a suitable type.

Reagents

- Citric acid – 0.2 M. Prepare by dissolving 42 g of citric acid in 100 ml of water and standardize against standard sodium hydroxide solution using phenolphthalein as indicator.
- Bromophenol blue indicator solution – Dissolve 40 mg of bromophenol blue in 100 ml of water containing 5.7 ml sodium hydroxide (0.01 N).
- Methyl red indicator solution – Dissolve 25 mg of methyl red in 100 ml of ethyl alcohol (60 per cent v/v).
- Dithizone solution in chloroform – 0.2 per cent (m/v). Keep in a dark bottle in a refrigerator.

- Dithizone solution in carbon tetrachloride – 0.05 per cent (m/v). Keep in a dark bottle in a refrigerator.
- Phenolphthalein indicator solution – Dissolve 50 mg of phenolphthalein in 100 ml ethyl alcohol (50 per cent m/v).
- Buffer solution – Dissolve 6.184 g of boric acid and 25.62 g of Sorensen's salt ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) in 500 ml of standard sodium hydroxide (1.0 N) and make up the volume to one litre with distilled water.
- Concentrated nitric acid – 60 per cent (m/v).
- Perchloric acid – 60 per cent.
- Concentrated sulphuric acid – r.d. 1.84.
- Cresol red indicator solution – Dissolve 40 mg of cresol red in 100 ml of water containing 10.5 ml of sodium hydroxide (0.01N).
- Nitroso-R salt – 0.2 per cent (m/v) aqueous solution stored in dark.
- Standard cobalt solution – Dissolve 4.7694 g of cobalt sulphate ($\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$) in distilled water, add one millilitre of concentrated sulphuric acid and make up the volume to one litre. Take one millilitre of this solution and make up volume to one litre with water in a graduated flask. This solution contains 1 microgram of cobalt per millilitre.

Procedure

1. Take an aliquot of 5 ml of the test solution as prepared in iron determination. Evaporate the water cautiously until all but a trace of sulphuric acid is removed. Add 7.5 ml of nitric acid to the residue and wash the solution into a 100 ml separating funnel. Dilute to about 30 ml with water.
2. Extraction with dithizone – Add 5 drops of bromophenol blue to the solution. Run in sodium hydroxide solution (1.0 N) until a distinct greenish blue colour appears through the yellowish tint due to the ferric citrate. The solution should still be acid to methylred. Dilute the solution to 50 ml. Extract the solution with successive 20 ml portions of dithizone solution in chloroform. Shake vigorously and run off the chloroform layer. When the chloroform layer retains the original green colour of the dithizone solution, the test solution is washed once with pure chloroform.
3. Adjust the pH of the aqueous phase to approximately 8.3 by adding a few drops of phenolphthalein and cautiously titrating with the buffer solution until the first sign of a purplish pink colour appears. Extract the cobalt with successive 10 ml portions of dithizone solution in carbon tetrachloride until the carbon tetrachloride phase retains the green colour of original dithizone solution. Boil off the carbon tetrachloride from a heat resistant boiling tube. Add to the residue 1 ml of nitric acid, 0.5 ml of perchloric acid and 0.2 ml of sulphuric acid and heat till it becomes colourless. Heat the boiling tube for a few minutes in a muffle furnace at a temperature not above 350°C to ensure complete removal of sulphuric acid.
4. Production of the cobalt – Nitroso-R salt complex – Dissolve the residue in 1 ml of citric acid and dilute with a little water so that the total volume is not more than 5 ml. Add accurately 1.2 ml of the buffer solution to adjust the pH. The pH is checked with cresol red by withdrawing a small drop of the solution. Develop the cobalt-nitroso-R salt complex by introducing 0.5 ml of the cobalt-nitroso-R salt solution while shaking. Boil for one minute. Cool and dilute to 10 ml. Measure the absorption of the solution in a suitable spectrophotometer or photoelectric colorimeter at 510 nm setting the reading of the blank at zero absorption. The blank is prepared simultaneously by using the same quantities of the reagents employed in the digestion and in the subsequent procedure. Make up the volume to 100 ml and develop the color in the same size aliquot and in the same manner as in the case of the test solution.

5. Prepare a series of standards by treating aliquots of the standard cobalt solution in the same manner as the test solution. From the absorption of the standard solutions, prepare a standard curve plotting absorption values against concentrations. From this curve, obtain the mass of cobalt present in the test solution and calculate the quantity of cobalt present in the test solution and calculate the quantity of cobalt present in 100 g of the material on moisture-free basis.

Reference: IS:7874 (part-II) – 1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.12 Determination of zinc – Colorimetric method

Apparatus

- Colorimeter – A photo electric colorimeter or suitable spectrophotometer capable of measuring optical density at a wavelength of 540 nm.

Reagents

1. Copper sulphate solution – Prepared by dissolving 8.0 g copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in distilled water and diluted to one litre.
2. Ammonium citrate solution – Dissolve 225 g ammonium citrate ($(\text{NH}_4)_3\text{C}_2\text{H}_5\text{O}_7$) in distilled water, make alkaline to phenol red ($\text{pH} \pm 7.4$) with concentrated ammonium hydroxide (25 per cent) and add a further 75 ml. Dilute to 2 litre. Before use, purify by adding a slight excess of dithizone solution and extract with successive portions of carbon tetrachloride until the solvent layer has a clear bright green colour. Remove the dithizone remaining in the solution by means of successive extractions with chloroform followed by a final extraction with carbon tetrachloride (The dithizone shall be entirely removed to prevent loss of zinc during the removal of cobalt).
3. α -Nitroso- β -Naphthol solution – Dissolve 0.25 g α -Nitroso- β -Naphthol in chloroform and make the volume up to 500 ml with chloroform.
4. Chloroform – redistilled. Store in an amber bottle.
5. Alizarin indicator solution – Dissolve 0.02 g of sodium alizarin sulphonate in water and make up the volume to 100 ml.
6. Dilute hydrochloric acid – 0.05 N.
7. Buffer solution – Dissolve 0.1 g of hydroxylamine.
8. Standard zinc solution – Dissolve 0.500 g of pure granulated zinc in a slight excess of hydrochloric acid. Dilute to 1 litre with double distilled water. One millilitre of this solution contains 0.5 microgram of zinc.
9. Standard zinc working solution – Dilute 10 ml of standard zinc solution with 0.04 N hydrochloric acid to make 1 litre. One millilitre of this solution contains 5 mg of zinc.
10. Ammonium hydroxide solution – An aqueous solution of ammonia containing approximately 5 per cent ammonia (m/m).
11. Hydrochloric acid solution – Concentrated hydrochloric acid diluted with distilled water in the proportion 1:6.
12. Bromine water – A saturated solution of bromine in water.
13. Hydrogen sulphite
14. Methyl red indicator solution – Dissolve 25 mg methyl red in 100 ml of 60 per cent ethyl alcohol.
15. Phenol red indicator solution – Dissolve 100 mg phenol red sodium salt in 100 ml distilled water.

Procedure

1. Dilute 10 ml of the test solution to about 40 ml. Add 2 drops of methyl red indicator and 1 ml copper sulphate solution and neutralize with ammonia. Add sufficient hydrochloric acid solution to bring the concentration of this acid to 0.15 N. The pH value of the solution should now be between 1.9 and 2.1. Pass a stream of hydrogen sulphite through the solution until precipitation is complete. Filter through a fine filter paper (previously washed with hydrochloric acid solution and with water) into a 250 ml beaker. Wash the precipitate and filter paper with three or four small portions of water, adding the washings to the filtrate. Boil the solution until all trace of hydrogen sulphite has been removed, add 5 ml of bromine water and continue boiling until free from bromine. Cool, neutralize to phenol red with ammonium hydroxide solution and add 0.6 ml hydrochloric acid solution. Make up to a suitable volume and take an aliquot containing 4 to 20 microgram of zinc for the determination. Adjust the volume of the aliquot to about 20 ml by the addition of distilled water and transfer to a 125 ml separating funnel. Add 5 ml ammonium citrate solution and 10 ml α -nitroso- β -naphthol solution. Shake for 2 minutes, allow the phases to separate and discard the solvent layer.
2. Wash the aqueous layer with small portions of chloroform to remove residual α -nitroso- β -naphthol. If necessary, adjust the pH value of the aqueous solution to 8.0 to 8.2 by the addition of ammonium hydroxide or hydrochloric acid solutions and add 2 ml dithizone solution and 10 ml carbon tetrachloride. Shake for 2 minutes, allow the phases to separate and, using a pipette, withdraw the aqueous phase as completely as possible and discard it. Wash down the sides of the separating funnel with 25 ml distilled water and again withdraw the aqueous phase and discard it. Add 25 ml of 0.04 N hydrochloric acid to the content of the separating funnel, shake for 1 minute to transfer zinc to the aqueous phase, allow the phases to separate and discard the solvent layer. To the aqueous solution remaining in the separating funnel add 5 ml ammonium citrate solution and adjust the pH value, if necessary, to 8.8 to 9.0. Add 10.0 ml carbon tetrachloride accurately measured. Determine the quantity of dithizone solution to be added.
3. To a separating funnel containing 4.0 ml of the standard zinc working solution (20 microgram zinc) made up to 25 ml with 0.04 N hydrochloric acid add 5.0 ml ammonium citrate solution and 10 ml carbon tetrachloride; then add dithizone solution from a burette in 0.1 ml increments, shaking after each addition, until a faint yellow color in the aqueous phase indicates a slight excess of reagent. Multiply the volume of dithizone solution used by 1.5 and add this quantity to the test solution. Shake for 2 minutes and allow the phases to separate. Pipette 5 ml of the solvent phase into a test tube, dilute with 10 ml carbon tetrachloride and use for the determination of the absorption. Prepare a series of standards containing 5, 10, 15 and 20 microgram zinc diluted in each case to 25 ml with 0.04 N hydrochloric acid solutions and treated in the same manner as the test solution. Prepare a blank simultaneously by using the same quantities of reagents as were used in the digestion of the test sample and in the subsequent procedure, making up to 200 ml and developing the color in the same size aliquot and in the same manner as in the actual determination. Measure the absorptions of the standard and test solutions at 540 nm in the colorimeter. From the absorptions of the standard solutions prepare a graph by plotting absorptions against concentrations and from it determine the concentration of zinc in the test solution. Express the result as percentage of zinc in the test sample.

Reference: IS:7874 (part-II) – 1975. Methods of tests for animal feeds and feeding stuffs. Part – II, Minerals and Trace elements.

10.13 Determination of fluorine - Distillation method

Apparatus

- Distillation flasks
- Nessler tubes – of 50 ml capacity
- Micro-burette

Reagents

1. Lime water free from fluorine – Dissolve lime in an excess of perchloric acid. Boil for 15 minutes. Dilute, cool and neutralize with fluorine-free sodium hydroxide. Filter through a Buchner funnel and wash. Make a saturated solution in distilled water using the lime thus free from fluorine.
2. Perchloric acid solution – 60 to 70 per cent (m/v). Heat some quantity for an hour or longer at 140 to 150°C.
3. Silver perchloric solution – 1 per cent (m/v). Prepare by adding sufficient sodium hydroxide solution to a solution of silver nitrate to cause precipitation. Filter and wash the precipitate with water. Dissolve the precipitate in perchloric acid and dilute.
4. Sodium hydroxide solution – 0.5 N.
5. Alizarin Indicator solution – Dissolve 0.02 g of sodium alizarin sulphonate in water and make up the volume to 100 ml.
6. Dilute hydrochloric acid – 0.05 N.
7. Buffer solution – Dissolve 0.1 g of hydroxylamine hydrochloride in water and make up the volume to 100 ml.
8. Thorium nitrate solution – Dissolve 0.5 g of hydrated thorium nitrate ($\text{Th}(\text{NO}_2)_4 \cdot 12\text{H}_2\text{O}$) in distilled water and make up the volume to one litre.
9. Standard fluorine solution – Dissolve 2.211 g of sodium fluoride in water and make up the volume to one litre in a graduated flask. Pipette out 10 ml of this solution into a one litre graduated flask and make up the volume. This solution contains 0.01 mg of fluorine per millilitre.

Procedure

1. Weigh accurately about 5 g of the material. Moisten with lime water. Dry on a water bath and ignite in a muffle furnace at about $550 \pm 20^\circ\text{C}$. When the ashing is complete, cool and transfer to distillation flask, washing it with water. Dissolve the residual ash in 10 to 15 ml of perchloric acid and transfer to the same distillation flask. Add sufficient silver perchlorate to bring about complete precipitation. Steam-distil at $132 \pm 3^\circ\text{C}$ into another flask containing 2 ml of the sodium hydroxide solution. Collect about 150 ml of the distillate. Transfer the distillate to a 200 ml graduated flask and make up the volume to the mark with water.
2. Transfer a suitable aliquot of the test solution containing 10 to 30 microgram of fluorine to a Nessler tube. Add 1 ml of alizarin indicator solution. Take the same quantity of the indicator solution in another Nessler tube. If necessary, the alkali in the test solution is neutralized with a drop or two of hydrochloric acid. To each tube, add 1 ml of the buffer solution 2.0 ml of hydrochloric acid and dilute to about 45 ml. The colour of the solutions should be straw-yellow. Add the thorium nitrate solution from a microburette to the test solution until a permanent slight pink colour appears (0.5 to 2.5 ml of thorium nitrate solution would be required). Add an equal volume of the thorium nitrate to the other. Nessler tube. Adjust the colour of the solution in this Nessler tube by adding the standard fluorine solution from a micro-burette to the same intensity as the colour of the test solution. When the colour in both the tubes

matches, the amount of fluorine in the standard fluorine solution added is equal to the amount of fluorine present in the aliquot of the test solution. From this, calculate the amount of fluorine present in 100 g of the material on moisture-free basis.

Reference: IS:7874 (part-II) – 1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.14 Determination of fluorine - Ion selective electrode (ISE) method

Apparatus

- Ion analyzer (Fig. 10.8)
- Single junction reference electrode
- Solid state fluoride electrode
- Magnetic stirrer & stir bars
- Plastic lab ware

Required solutions

- Distilled or deionised water
- 100 ppm fluoride standard solution
- Reference electrode filling solution
- Total Ionic Strength Adjuster Buffer (TISAB-III). To provide constant background ionic strength, decomplex fluoride and adjust solution pH.



Fig. 10.8 Ion analyzer

Preparation of sample

Weigh accurately about 0.3 g sample into 100 ml plastic beaker. Add 5 ml of 5 M hydrochloric acid and mix it well to dissolve all the material. Transfer the solution into 100 ml volumetric flask and make the volume 100 ml with deionised water. Take 25 ml aliquot in a plastic beaker and add 2.5 ml TISAB-III and measure the concentration on Ion analyzer.

Preparation of standard

Stock solution (500 ppm): Accurately weigh 1.105 g NaF (reagent grade, dried 4 hours at 100°C) into 1 litre volumetric flask. Dissolve and dilute to volume with H₂O and mix thoroughly. Store in plastic bottle at room temperature.

Preparation of working standard from readily available 100 ppm fluoride solution:

- 1) 1 ppm fluoride standard solution: Take 1 ml stock solution (100 ppm) into 100 ml volumetric flask and dilute to volume with H₂O and mix. It gives 1 ppm fluoride concentration.
- 2) 10 ppm fluoride standard solution: Take 10 ml stock solution (100 ppm) into 100 ml volumetric flask and dilute to volume with H₂O and mix. It gives 10 ppm fluoride concentration.

Take 25 ml aliquot and add 2.5 ml TISAB-III and use for meter calibration.

Note: Use plastic lab ware for fluorine estimation.

Determination of fluoride concentration

Connect fluoride and single junction reference electrodes to ISE meter, place electrodes in standards for calibration of ISE meter. After calibration of meter with two or three standards, place electrodes in sample and stir the solution at constant rate, read concentration of standard and unknown solution directly from the meter.

Calculation

$$\% \text{ Fluorine} = \frac{\text{Meter reading (ppm)} \times 100 \times 10^{-4}}{\text{Weight of sample (g)}}$$

Reference: AOAC (1995) Official Methods of Analysis. 975.08.

10.15 Determination of iodine (as KI) – Titration method

Reagents

1. Methyl orange indicator – Dissolve 0.50 g of methyl orange in water and dilute to one litre.
2. Dilute sulphuric acid – approximately 2 N.
3. Bromine water – Saturated aqueous solution. Determine the approximate concentration (mg/ml) by adding (from a burette) a measured volume to a flask containing 5 ml of 10 per cent potassium iodide solution, adding 5 ml of dilute sulphuric acid and titrating the liberated iodine with 0.1 N sodium thiosulphate solution.
4. Sodium sulphite solution – approximately one per cent (m/v).
5. Phenol solution – approximately 5 per cent (m/v).
6. Potassium iodide solution – approximately 10 per cent (m/v).
7. Standard sodium thiosulphate solution – 0.005 N (freshly standardized).
8. Starch solution – 1 per cent (freshly prepared) (m/v).
9. Sodium chloride solution – Dissolve 10 g of sodium chloride in water and make up the volume to 100 ml.
10. Potassium iodide control solution – Dissolve 0.3280 g of potassium iodide in water and then make up the volume to 250 ml. Dilute 50 ml of this solution to 250 ml, and use 5 ml control (that is, 1.0 mg iodine or 0.308 mg potassium iodide).
11. Concentrated sulphuric acid – r.d. 1.84.

Preparation of sample solution

Weigh accurately about 50 g of the material and suspend in 100 ml of water. Add 2 g of takadiastase and allow to stand at 37°C for 2 hours. Filter the solution and wash the residue with water. Collect the filtrate and washings and make up the volume to 250 ml in a graduated flask.

Procedure

Pipette 50 ml of the prepared sample solution into a 200 ml Erlenmeyer flask. Neutralize to methyl orange indicator with dilute sulphuric acid. Add bromine water drop wise from burette in a quantity equivalent to 20 mg of bromine. After a few minutes, destroy most of the remaining free bromine by adding sodium sulphite solution drop wise with stirring. Wash down the neck and sides of the flask with water and completely remove free bromine by addition of a drop or two ml of potassium iodide solution and titrate the liberated iodine with standard sodium thiosulphate solution adding 1 ml of the starch indicator near the end of the titration. Carry out a blank determination of reagents and make one or more control determinations, using 50 ml of sodium chloride solution to which have been added appropriate quantities of the potassium iodide control solution.

Calculation

$$\text{Iodine (as KI), on moisture free basis, per cent by mass} = \frac{1384 (V_1 - V_2) N}{m (100 - M)}$$

Where,

V_1 = Volume in ml of the standard sodium thiosulphate solution required for the test with the prepared sample solution,

V_2 = Volume in ml of the standard sodium thiosulphate solution required for the blank determination

N = Normality of the standard sodium thiosulphate solution.

M = Mass in g of the material taken, and

M = Per cent moisture content

Reference: IS:7874 (part-II) – 1975. Methods for animal feeds and feeding stuffs. Part-II. Minerals and trace elements.

10.16 Determination of iodine - Ion selective electrode method

Required equipment

- Ion Selective Electrode meter
- Electrodes
- Magnetic stirrer, stir bars – for stirring of samples and standards.

Required solutions

- a) Distilled or de-ionize water – to prepare all solutions and standards.
- b) Reference electrode filling solution.
 - i) Inner chamber filling solution.
 - ii) Outer chamber filling solution
- c) Standard solutions
 - i) Ionic strength adjustor (ISA) 5M NaNO_3 (to adjust to a constant back ground ionic strength)
 - ii) Iodide standard solution (0.1 M NaI)
- d) Preparation of 1000 ppm iodide stock standard from 0.1 M NaI:
Dilute the 0.1 M iodide standard, by pipetting 78.7 ml of standard into 1 litre volumetric flask. Add distilled water to make up the volume.
Store stock standards in plastic bottles and prepare fresh weekly. Lower concentration working standards used for calibration should be prepared daily.

Preparation of sample

Prepare the sample same as in fluoride determination. Take 50 ml aliquot and add 1 ml Ionic Strength Adjuster (ISA) in each sample and measure the concentration of iodine on ISE meter after calibration.

Preparation of working standard from the 1000 ppm iodide stock solution

1. 1 ppm iodide standard solution: Pipette 0.1 ml stock solution into 100 ml volumetric flask, dilute to volume with distilled water.
2. 10 ppm iodide standard solution: Pipette 1.0 ml stock solution into 100 ml volumetric flask, dilute to volume with distilled water.
3. 100 ppm iodide standard solution: Pipette 10.0 ml stock solution into 100 ml volumetric flask, dilute to volume with distilled water.

Take 50 ml aliquot and add 1 ml Ionic Strength Adjuster (ISA) in each standard and use for meter calibration.

Determination of iodide concentration

Connect iodide and double junction reference electrodes to ISE meter, place electrodes in standards for calibration of meter. After calibration of meter with two or three standards, place electrodes in sample and stir the solution at constant rate, read concentration of standard and unknown solutions directly from the meter.

Calculation

$$\% \text{ Iodide} = \frac{\text{Meter reading (ppm)} \times 100 \times 10^{-4}}{\text{Weight of sample (g)}}$$

Reference: Orion, Iodide electrodes instruction manual.

10.17 Determination of sulphur

Apparatus

- Magnetic stirrer
- Spectrophotometer
- Measuring spoon (0.2 to 0.3 ml)
- 250 ml flasks and stirring bars

Reagents

1. Conditioning solution: Mix 50 ml glycerol with a solution containing 30 ml concentrated HCl, 300 ml distilled water. 100 ml 95 per cent ethyl alcohol and 75 g sodium chloride.
2. Barium chloride, A.R. grade crystals.
3. Standard sulphur solution: Dissolve 5.438 g reagent grade potassium sulphate in 1 litre water to make standard stock solution. Dilute 10 ml of the stock solution to 100 ml. This solution contains 0.01 mg sulphur / ml.

Procedure

Take 5 to 10 ml aliquot of HCl extract in a 250 ml flask and add water to bring the volume to 95 ml. Add 5 ml of conditioning solution. Place the flask on the magnetic stirrer and add a magnetic stirrer bar. The stirring speed should be constant for each run. While stirring, add a spoonful of barium chloride crystals and stir for exactly 1 minute. Just after stirring measure the absorbance at 350 nm. A blank in which no barium chloride is added is run parallel. Prepare a suitable standard curve extends from 0 to 1.2 mg of sulphur per 100 ml solution.

Calculation

$$\text{Sulphur, per cent by mass} = \frac{T \times V \times 100}{A \times W \times 1000}$$

Where,

T = mg of sulphur in test sample

V = volume of extract made

A = Aliquot taken; W = Wt. of sample in g

Reference: IS: 1664 – 2002. Specification of mineral mixtures for supplementing cattle feeds (Fourth revision).

11.0 ANALYSIS OF BYPASS PROTEIN AND FAT SUPPLEMENTS

11.1 *In vitro* determination of degree of protein protection in bypass protein supplement

Equipment

- Stopped ground glass 30 ml test tubes – Quick fit catalogue number MF 24/2/6 socket size 19/26 plus stoppers – catalogue number SB19.
- Test tube racks to fit both sizes of test tubes
- Suba seals
- Wide rubber bands
- Vortex mixer
- Acid dispensers and precision pipettes
- Tecator Kjeltac Auto 1030 nitrogen analyzer with digestion system 20 and scrubbing unit together with the necessary digestion tubes and racks.



Fig. 11.1 Shaking incubator

Notes:

- Weigh all samples the day before and cover with foil.
- Turn on incubator the night before and set temperature to 39°C.
- Keep strained rumen fluid at 39°C flushed with N₂ and commence ruminal incubation within an hour of collection.
- Keep test tubes with samples warm
- Keep rumen fluid warm and flush continuously with N₂ whilst dispensing

Procedure

1. Weigh 100 mg of protected protein, unprotected protein, known standards, pure casein and blanks into ground glass stoppered test tubes in triplicate.
2. Pipette 10 ml of strained rumen fluid into test tube.
3. Flush the sample triplicate set with nitrogen using a pasteur pipette attached to a gas cylinder via rubber tubing.
4. Cap with suba seals and tightly seal with rubber bands to maintain anaerobic conditions.
5. Incubate samples in a shaking incubator (Fig. 11.1) at 39°C for 20-24 hours.
6. To stop incubation add 2 ml of 1 N sulphuric acid.
7. Filter the content through cotton plug in other test tubes.
8. Take 2 ml of filtrate into Tecator tubes.
9. Proceed to titrate using a Tecator distillation set up.

Calculation for the degradation of protein

$$\% \text{ Protein Degradation (RDP)} = \frac{(\text{mls titrant} - \text{blank titrant}) * 0.1(\text{N}) * 14.01 * 6.25 * 6.25 * 100}{1000 * \text{sample wt.} * \text{protein} / 100 * \text{dry matter} / 100}$$

$$\% \text{ Protection (RUP)} = 100 * (1 - \text{protein degradation} / \text{casein protein degradation})$$

References: Gulati S.K. (1976). Protected triacylglycerol and sterol supplements for ruminants. MSc thesis, Macquarie University, North Ryde, NSW, Australia.; Ashes *et al.* (1979) J. Amer. Oil Chem Soc. 56:522; Gulati *et al.* (1999). 90th AOCS, Florida, USA. S41-S42.

11.2 *In sacco* digestibility determination

Apparatus

- Nylon bag
- Plastic tube
- Rubber band/ nylon thread
- Hot air oven
- Washing machine
- Rumen cannulated animal

Procedure

Preparation of Sample

- Weigh 3.0 g sample for dry feeds (hays, straw etc) or 5.0 g sample for protein supplements and transfer it to nylon bag (35-50 μ pore size).
- Record the weight of bag plus sample.
- Attach the nylon bags to the respective plastic tubes for incubation and place them with bags in the rumen and tie the bags with cannula with a piece of nylon thread.
- Incubate the bags for 4, 8, 16, 24, 48, 72 and 96 h for roughage and for protein sample 2, 4, 8, 16, 24 and 48 h.
- Withdraw the bags and immediately place in a bucket of cold water to prevent further fermentation and to wash off the feed particles adhering to the outside of the bags.
- Transfer the bags in washing machine for 20 min for cold water washing. The bags could also be washed under running cold water in laboratory until the washing is clear.
- Detach the nylon bags from the tubes by cutting the rubber bands.
- Dry the bags at 60-65°C for 48h and weigh the bags immediately after drying.

Calculation

Empty bag weight	(W)
Bag + feed sample before incubation	(W ₁)
Bag + residue after incubation	(W ₂)
Per cent dry matter in the feed sample	(DM %)
Sample dry matter weight	(W ₁ -W) x DM % = (W ₃)
Residue dry matter weight	(W ₂ -W) = (W ₄)

$$\text{DM disappearance (\%)} = \frac{W_3 - W_4}{W_3} \times 100$$

Degradation kinetics

The degradation kinetics of the incubated feedstuffs may be calculated by curvilinear regression of DM and other degradable components of the feed determined by nylon bag technique.

$$\text{Potential degradability (P)} = a + b (1 - e^{-ct})$$

Where,

a = y-axis intercept at time 0 that represents soluble and completely degradable substrate washed out of the bags (0h disappearance)

b = The difference between the intercept (a) and the asymptote that represents the insoluble but potentially degradable substrate

t = incubation time

c = The rate of disappearance of component 'b' per hour (rate constant)

a+b = The asymptote of the disappearance curve

1 - (a+b) = The un-degradable portion of a sample

$$\text{Effective degradability (E)} = a + bc / (c + k)$$

Where,

k = rumen small particle out flow rate.

The above equations assume that component 'b' disappears at a constant fractional 'c' per hour.

Reference: Orskov and McDonald, I. (1979). The estimation of protein degradability in the rumen from incubating measurements weighed according to rate of passage. *J. Agric. Sci. (Camb)* 92: 499.

11.3 Determination of protein-bound formaldehyde

The methods described below are applicable to any sample of formalin treated protein of feed. The estimated formaldehyde includes both CH₂O which is loosely bound and that which is tightly bound to the protein and which in the literature are often referred to as the "free" and "irreversibly bound" forms. The samples must be ground through a 1 mm screen.

Reagents

- Distilled water
- Anhydrous sodium sulphate
- Phosphoric acid, concentrated
- Chromotropic acid
- Sulphuric acid, 36 N.
- Formaldehyde solution – dilute 0.25 ml of formalin to a litre.

Equipment

- 500 ml Kjeldahl flasks
- Quickfit macro Kjeldahl distillation unit
- Electrical heating unit
- 50 ml volumetric flask
- Spectrophotometer – set at 570 m μ .

Procedure

Distillation: (this two-step distillation procedure takes approximately 1 hour)

1. Accurately weigh 1 g of feed sample (or 5 ml of milk) into a 500 ml Kjeldahl flask.
2. Add 50 ml of distilled water, 2.0 g of anhydrous sodium sulphate and 3 ml of concentrated phosphoric acid and anti-bumping granules.
3. Place the flask on an electrical heating unit and connect it to a condenser through a trap, claisen head and plug funnel.
4. Place a 100 ml stoppered measuring cylinder with a wide neck under the condenser, containing 5 ml of distilled water, and begin the distillation until 40 ml have been collected.
5. Allow the Kjeldahl unit to cool, and then add another 50 ml of distilled water through the plug funnel and continue the distillation until 90 ml have been collected.
6. Make up to 100 ml with distilled water and mix.

Color development

1. Weigh 100 ± 10 mg of chromotopic acid into a 30 ml beaker.
2. Add 1.0 ml aliquot of the distillate.
3. Evaporate the solution to dryness by placing the beaker in an air oven at 100°C for 30 minutes.
4. When the residue has cooled down to room temperature, 5 ml of 36 N sulphuric acid is added and the resulting solution is heated for 30 minutes in an air oven at 100°C .
5. After cooling, the solution is diluted to 50 ml with distilled water in a volumetric flask.
6. The absorbance of this solution is measured against a reagent blank at 570 nm using a 1 cm light path cell in a spectrophotometer.

Calibration curve

1. To a series of solutions containing from 0 to $100 \mu\text{g}$ of CH_2O carefully add into 30 ml beakers 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml aliquots of a standard formaldehyde solution prepared by diluting 0.25 ml of formalin to a litre (The concentration is checked by titration). Make up to 1 ml with distilled water.
2. Proceed for the color development as for the samples.
3. Prepare a calibration curve by plotting absorbance versus micrograms formaldehyde.
4. By linear regression analysis, determine the slope of the best line fit.

Calculation

$$W = \frac{As}{S}$$

Where,

- W = micro grams formaldehyde found
As = absorbance units for sample
S = slope of calibration curve

Reference: Van-Dooren (1975), *J. Sci Fd. Agric.* 26: 1265-1271.

11.4 Amino acid analysis by hydrolysis method (Non Oxidised)

This method is used for the quantitative determination of amino acids in materials such as grain, feedstuff, milk, etc., which do not just consist of pure protein.

Reagents

- 6 N Hydrochloric acid
- Lithium citrate buffer: pH 2.20

Equipment

- Serial heating mantle set up for 250 ml round bottom flasks with water-cooled condensers.
- Rotary evaporator
- Amino acid analyser (Fig. 11.2)

Procedure

1. Accurately weigh 200 mg of sample into a 250 ml round bottom flask. Add 2 glass beads.
2. Add 160 ml of 6 N HCl. Reflux at 110°C for 23 hours.
3. Filter solution into a 250 ml measuring cylinder, make it up 200 ml with DI water.



Fig. 11.2 Amino acid analyzer

4. Take a 50ml aliquot, place it into a 250 ml round bottom flask, and remove the acid using a rotary evaporator connected to a vacuum pump (water bath temperature 60°C). Rinse the residue twice with DI water and evaporate to dryness. Store 50 ml in a specimen jar in case you need to repeat the procedure discard once you are happy with the results.
 5. Pipette 5 ml of diluting buffer into the residue, and transfer to a plastic scintillation vial.
 6. Filter about 0.5 ml through a 2 um filter, into a vial, so that it is ready to be used, with the appropriate buffer dilution. The samples are run under the "Hydrolysis program" of the Biochrom 20.
- The dilution is worked out according to the Nitrogen% of the sample, i.e. the higher the Nitrogen content the higher the dilution.

Hydrolysis method (Oxidised)

This method is used for the quantitative determination of cystine and methionine, (both acids must be converted into their stable oxidized forms using performic acid) to convert them to cysteic acid and methionine sulphone. During the oxidation tyrosine and histidine will be almost completely destroyed.

Reagents

- Performic acid/hydrogen peroxide oxidation mixture:

To make	10 ml	20 ml	30 ml
Hydrogen peroxide 30%	1 ml	2 ml	3 ml
Performic acid 88%	9 ml	18 ml	27 ml
Liquid phenol	50 mg	100 mg	150 mg

Weigh the liquid phenol into a 50 or 100 ml conical flask, then add the other two reagents and mix, incubate for 1 hour at 30°C., using a water bath.

- Sodium disulphite.
- 6 N Hydrochloric acid + phenol: (50 ml 6 N HCl, containing 50 mg phenol).
- Lithium citrate buffer: pH 2.20.

Equipment

- Serial heating mantle set up for 250 ml round bottom flasks with water-cooled condensers.
- Rotary evaporator and amino acid analyser

Procedure

1. Accurately weigh 100 mg of sample into a 100 ml round bottom flask, and place them with enough ice to cover a third of the flask.
2. Prepare the oxidation mixture, cool it together with the samples in ice.
3. Add 5 ml of solution to each sample, swirl the flask to mix in the solution.
4. Cover each flask with parafilm, and leave in the fridge for 16 hours. This is where the oxidation occurs.
5. To stop the reaction, add 840 mg of Sodium bisulphate.
6. The hydrolysis is performed with 60 ml of 6 N HCl containing 60 mg phenol, add boiling chips, and reflux at @ 110°C for 23 hours.
7. Filter solution into a 100 ml measuring cylinder, make it up to 100 ml with DI water.
8. Take a 50 ml aliquot, place it into a 250 ml round bottom flask, and remove the acid using a rotary evaporator connected to a vacuum pump (water bath temperature 60°C). Rinse the residue twice with DI water and evaporate. Store 50 ml into a specimen jar in case you need to repeat the procedure, discard once you are happy with the results.
9. Pipette 5 ml of diluting buffer into the residue, and transfer to a plastic scintillation vial.
10. Filter about 0.5 ml through a 2 um filter, into a vial, so that it is ready to be used, with the appropriate buffer dilution.*

*The dilution is worked out according to the Nitrogen % of the sample, i.e. the higher the Nitrogen content the higher the dilution.

References: Biotronic amino acid analyser LC 5001 Manual.

11.5 Total fat extractions using acidic chloroform: methanol (2:1 v/v) method

This procedure is to be used for samples containing protected lipid supplement either pure or as part of a ration.

Equipment

- Soxhlet extraction apparatus with individually controlled serial heating mantle.
- Extraction thimbles to fit soxhlet apparatus
- Rotary film evaporator
- Drying oven and boiling chips

Reagents

- Chloroform / Methanol 2:1 v/v
- 1 N HCL

Procedure

1. A 5-10 g sample is weighed into a 30 x 80 mm soxhlet thimble.
2. The sample is extracted by refluxing for 5-6 h in a soxhlet apparatus using approximately 150 ml of chloroform – methanol (2:1, v/v) plus 1 ml of 1 N HCl.
3. The solvent extract is allowed to cool and filtered and the volume measured in a 250 ml stoppered measuring cylinder.
4. The filtrate is washed with 1/5 volume of water.
5. The mixture is allowed to separate into two clear phases; the upper aqueous phase is removed by aspiration and discarded.
6. The lower organic phase is evaporated to dryness in a tarred 250 ml round bottom flask using a rotary film evaporator and the remaining lipid is estimate gravimetrically after drying at 100°C for 1h.

Calculation

$$\% \text{ Fat} = \frac{(\text{wt flask} + \text{fat}) - \text{wt flask} * 100}{\text{wt of sample}}$$

References: Gulati S.K (1976). Protected triacylglycerol and sterol supplements for ruminants. MSc thesis, Macquarie University, North Ryde, NSW, Australia.

11.6 Fatty acid analysis of feed

Reagents

- Ethanol (absolute)
- 5 N sodium hydroxide (NaOH) (200 g of NaOH pellets / 1 litre of distilled water).
- 5 N hydrochloric acid (HCL) (500 ml of concentrated acid added to 500 ml of distilled water)
- Petroleum ether (PE) (Boiling range 40-60°C)
- 1% sulphuric acid in methanol.
- 5% Salt solution (NaCl)

Procedures

Saponify

- Mix thoroughly and take a representative sample.
- Grind sample to a fine consistency.
- Add 2-5 ml of ethanol
- Add 2-5 ml of 5 N sodium hydroxide (NaOH)
- Shake well and cover with foil
- Place in oven @ 80°C for 1.5-2 h and then allow to cool.

Acidify

- Add 5 N hydrochloric acid approx 3 ml and invert test tube with care.
- pH must be checked for each sample using pH paper till acid (must be pink)
- When sample has cooled sufficiently
- Extract fatty acids with 4 ml of petroleum ether, shake well, pipette the supernatant into a labelled 15 ml test tube.
- Repeat above step – pooling the extracts
- Evaporate pooled PE extracts to dryness in a warm water bath under a stream of nitrogen.

Methylate

- To the dried sample add 3 ml of 1% sulphuric acid in methanol
- Reflux on a heating block at 50-60°C for 1.5 h
- Add 3 ml of 5% NaCl (salt solution)
- Add 3 ml of PE
- Cool, shake well and centrifuge and decant supernatant into GLC vial

Reference: Christie, W.C. 1993, Gas chromatography and lipids. The Oily Press Ltd., Dundie, Scotland, UK.; Gulati S.K. (1976). Protected triacylglycerol and sterol supplements for ruminants. MSc thesis.

11.7 Fatty acid analysis of milk

Reagents

- Ethanol (absolute)
- 5 N sodium hydroxide (NaOH) (200 g of NaOH pellets / 1 litre of distilled water).
- 5 N hydrochloric acid (HCL) (500 ml of concentrated acid added to 500 ml of distilled water)
- Petroleum ether (PE) (Boiling range 40-60°C)
- 1% sulphuric acid in Methanol.
- 5% Salt solution (NaCl)

Procedures

Mix milk samples thoroughly by shaking.

Saponify

- Pipette 2 ml of a milk sample into a 25 ml stoppered test tube
- Add 1 ml of ethanol
- Add 1 ml of 5 N sodium hydroxide (NaOH)
- Shake well and cover with foil
- Place in oven @ 80°C for 1.5-2 h.
- Remove from oven
- Allow to cool.

Acidify

- Add 5 N hydrochloric acid (HCL) approx 2 ml.
- Invert test tube with care.
- pH must be checked for each sample using pH paper till acid (must be pink)
- When sample has cooled sufficiently
- Extract fatty acids – add 4 ml of petroleum ether, shake well, pipette the supernatant into a labelled 15 ml test tube.
- Repeat above step – pooling the extracts
- Evaporate pooled PE extracts to dryness in a warm water bath under a stream of nitrogen.

Methylate

- To the dried sample add 3 ml of 1% sulphuric acid in methanol
- Reflux on a heating block at 50-60°C for 1.5 h
- Add 3 ml of 5% NaCl (salt solution)

- Add 3 ml of PE
- Cool, shake well
- Centrifuge
- Decant supernatant into GLC vial and cap
- Make sure samples are clearly labelled with number and date
- Analyze the fatty acid composition using gas chromatograph (Fig. 11.3).



Fig. 11.3 Gas chromatograph

Reference: Christie, W.C. 1993, Gas chromatography and lipids. The Oily Press Ltd., Dundie, Scotland, UK.; Gulati S.K. (1976). Protected triacylglycerol and sterol supplements for ruminants. MSc thesis, Macquarie University, North Ryde, NSW, Australia.

11.8 *In vitro* determination of degree of protection in bypass fat supplement

Equipment

1. Stoppered ground glass 15 & 30 ml test tubes
2. Suba seals
3. Wide rubber bands
4. Vortex mixer
5. Solvent, acid and caustic dispensers and precision pipettes
6. Nitrogen evaporator
7. Nitrogen gas cylinder
8. Thermostatically controlled orbital shaking incubator plus black cloth to use as a cover
9. Thermostatically controlled heating block to fit 15 ml tubes
10. Laboratory oven
11. Pasteur pipettes

Procedures

1. Weigh 130 mg of protected lipid, untreated lipid, known standards and blanks. This should be done a day in advance, and tubes stored in the refrigerator.
2. Pipette 10 ml of strained rumen fluid into test tube.
3. One tube from each sample remains un-incubated (zero hour). Proceed to the saponification step with these.
4. Flush the remaining duplicate sample sets with nitrogen using a pasteur pipette attached to a gas cylinder via a rubber tubing.
5. Cap with suba seals and seal tightly with rubber bands to maintain anaerobic conditions.
6. Incubate samples in a shaking incubator at 39°C for 24 hours.
7. Remove at the end of incubation, allow to cool, and carefully remove Suba seals, Proceed to the saponification step.

Saponification

1. Add 2 ml of ethanol
2. Add 2 ml of 5 N sodium hydroxide (NaOH).
3. Shake well and cover with foil.
4. Place into oven @ 80°C for 1.5-2 h.
5. Remove from oven
6. Allow to cool.

Acidify

1. Add 5 N hydrochloric acid (HCL) approx 2 ml.
2. Invert test tube with care.
3. pH must be checked for each sample using pH paper till acid (must be pink)
4. When sample has cooled sufficiently
5. Extract fatty acids – add 4 ml of petroleum ether, shake well, pipette the supernatant into a labelled 15 ml test tube.
6. Repeat above step – pooling the extracts
7. Evaporate pooled PE extracts to dryness in a warm water bath under a stream of nitrogen.

Methylate

1. To the dried sample add 3 ml of 1% sulphuric acid in methanol (freshly made).
2. Reflux on a heating block at 50-60°C for 1.5 h.
3. Add 3 ml of 5% NaCl (salt solution).
4. Add 2 ml of PE.
5. Cool, shake well.
6. Centrifuge at 2000 rpm for 3 minutes or allow to stand until phases clear.
7. Decant supernatant into GLC vial and cap.
8. Make sure sample vials are clearly labelled with number, date and name of operator.
9. Run on GC.

Calculation

$$\text{Protection (\%)} = \frac{\% C_{18:2} \text{ after incubation}}{\% C_{18:2} \text{ before incubation}} \times 100$$

Reference: Gulati S.K. (1976). Protected triacylglycerol and sterol supplements for ruminants. MSc thesis, Macquarie University, North Ryde, NSW, Australia.; Gulati, S.K., Ashes, J.R. and Scott, T.W. (1977). Assessing the degradation of fat supplements in ruminants. Anim. Feed Sci. & Tech. 64:127.

11.9 Determination of acid value

Acid value gives the measure of proportion of free fatty acids (FFA). It can be defined as mg of potassium hydroxide (KOH) required to neutralize free fatty acids present in 1 g of sample. A known quantity of (5.1000 ± 0.1000 g) sunflower acid oil was dissolved in 50 ml of neutral solvent (ether: 95 per cent alcohol: phenolphthalein = 25: 25: 1 ml and neutralized with N/10 NaOH). The contents were titrated against 0.1 N KOH in the presence of phenolphthalein as indicator. End point was the appearance of a faint pink color.

The acid value was then calculated by using the formula:

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of sample}}$$

Reference: AOAC method 969.17, 16th edition 1995.

11.10 Determination of saponification number

Saponification number is the measure of chain length of fatty acids. It may be defined as mg of KOH required to saponify one gram of oil. The saponification number was estimated according to the method of AOAC (1995). A known quantity (5.1000 ± 0.1000 g) of filtered sunflower acid oil was mixed with 50 ml of 4 per cent alcoholic KOH by draining it from the burette for definite time. The contents were refluxed for one hour and then cooled. The cooled solution was titrated against 0.5 N HCl using phenolphthalein indicator. Simultaneously a blank was run adapting similar procedures. The saponification number was calculated by the formula:

$$\text{Saponification number (mg KOH/g)} = \frac{28.05 (B - S)}{\text{Sample (g)}}$$

Where,

B = ml 0.5 N HCl required by blank

S = ml 0.5 N HCl required by sample

Reference: AOAC method 920.160, 16th edition 1995.

11.11 Determination of free fatty acids

The free fatty acids (FFA) content of sunflower acid oil was determined as per the method of AOAC (1995). Accurately 7.05 g of well mixed oil was weighed into a 250 ml flask, to this was added 50 ml of alcohol which was neutralized previously by adding 2 ml phenolphthalein solution and enough 0.1 N NaOH to produce faint permanent pink. The contents were titrated with 0.25 N NaOH with vigorous shaking until permanent pink color appears and persists for more than one minute. Titre value itself corresponds to the per cent FFA expressed as per cent oleic acid.

Reference: AOAC method 940.28, 16th edition.

12.0 ESTIMATION OF GROSS ENERGY IN BIOLOGICAL SAMPLES USING BOMB CALORIMETER

Gross energy (GE) is the amount of heat liberated when a feed or feed ingredient completely burnt to carbon dioxide and water. It is also known as heat of combustion and is usually determined by a bomb calorimeter (Fig. 12.1).

Type of bomb calorimeter

1. Adiabatic bomb calorimeter
2. Ballistic bomb calorimeter

Adiabatic bomb calorimeter

Determination of bomb equivalent: The water equivalent or bomb equivalent of the bomb calorimeter is determined with calorimetric grade benzoic acid having $GE=6324$ cal/g or 26460 j/g.

Sample preparation

1. Take 0.35 g of benzoic acid and make into a pellet with the help of the pelleting machine.
2. Place the pellet in a pre-weighed metallic crucible; weigh the pellet + crucible accurately.



Fig. 12.1 Bomb calorimeter

Bombing procedure

1. Clean the electrodes with emery paper. Wet rubber ring in bomb top with distilled water. Put the bomb top on the stand. Thread fuse wire through the electrodes so as to give a taut piece of wire across the electrode when the slides are pushed down.
2. Tie a piece of cotton thread (10 cm) to the centre of fuse wire. Place the crucible containing the sample in the ring support. Using tweezers put the cotton thread under the sample. Tighten the nut holding the ring support.

3. Pipette 1 ml of distilled water into the bomb body.
4. Place the electrode assembly into the bomb body ensuring that it fits correctly.
5. Fill the bomb to 25 atmospheres pressure with oxygen.
6. Adjust the weight of the can and water to exactly 3 kg on the balance using 10 ml pipettes.
7. Put the can into the calorimeter console ensuring that the peg on the can locates in the socket of the support.
8. Place the bomb into the calorimeter ensuring that it locates correctly in the base of the can.
9. Gently slide the top of the calorimeter console into the bomb. Switch the main on and press down the centre contact to the bomb (Then *ready to fire light* gets on).
10. Turn on the cooling water to fast flow until the temperature of the water jacket thermometer is 0.5°C below that of the main thermometer. The cooling water flow rate is adjusted to about 300 ml/minutes.
11. Allow 2 minutes for the temperature to stabilize. Press the thermometer vibrator button, then read the initial temperature on the thermometer (to 0.001°C). Check again about 30 seconds later.
12. Note the initial temperature when stable. Press fire switch.
13. After 8 minutes read temperature on main thermometer. Note the final temperature when it stabilizes.
14. Switch off main switch. Slide up console top. Remove bomb and can.
15. Allow the bomb to settle for 10 minutes. The pressure is released using the pressure release cap. Undo the top of the bomb and wash the electrodes, inside of the top and inside of the bomb with distilled water from a wash bottle. These washings are retained in a beaker for corrections for N and S content (S correction is not required with Benzoic acid).
16. The bomb equivalent is thus calculated using the following equation.

$$\text{Bomb equivalent (Cal/}^\circ\text{C)} = \frac{(6234 \times A) + C}{B}$$

Where,

A = Weight of benzoic acid (g); B = Rise in temperature (°C); C = Correction factor for wire, thread, N and sulphur (Heat of combustion of thread and wire maybe taken as 3962 cal/g and 1400 cal/g (or 2.3 cal/cm), respectively).

12.1 Gross energy of feed and faeces

Preparation of sample

- 1) Weight 0.5 to 1 g of the finely ground samples and make a pellet with the help of a pellet press.
- 2) Weigh the empty bomb crucible. Put the pellet into the crucible and weigh again.
- 3) Weigh out in duplicate some of the dried material into the moisture cup at the same time as pellets are prepared (for moisture determination). Dry for overnight at 100°C.

Bombing procedure

Follow the steps as described, in case of bomb equivalent.

Calculation

The gross energy may thus be calculated by using the following equation.

$$\text{GE (cal/g)} = \frac{(\text{Bomb equivalent} \times T) \times A}{\text{Dry weight of sample (g)}}$$

Where,

T = Rise in temperature (°C)

A = Correction factors for wire, thread, N and sulphur

12.2 Gross energy of urine

Sample preparation

- 1) Cut out 12 cm diameter discs from 50 gauge polyethylene sheet and weigh the disc.
- 2) Take 20 ml of urine sample in a glass beaker. Adjust its pH to about 6.0 with dilute sulphuric acid.
- 3) Then take 15 ml of urine from the above sample into a previously weighed polyethylene sheet of known energy value in a evaporating basin. Dry the urine on the polyethylene sheet in the basis at 40°C in a vacuum drying oven.
- 4) After drying, carefully fold up the dry urine and polyethylene.

Bombing procedure

The steps (1) to (15) as described in case of bomb equivalent determination may be repeated.

Calculation

The gross energy of the urine sample may thus be calculated using the following equation:

$$\text{GE (cal/ ml)} = \frac{(\text{Bomb equivalent} \times T) \times B \times A}{\text{Amount of the urine (ml)}}$$

Where,

T = Rise in temperature (°C)

B = Gross energy of polyethylene used for the sample

A = Correction factor for wire, thread, N and sulphur

12.3 Gross energy of milk

Sample preparation

- 1) Take 5 ml of milk sample in a bomb crucible.
- 2) Keep it for drying at 40°C as in case of urine in the vacuum drying oven.
- 3) 59. After drying, scratch with a needle and burn the sample in the bomb calorimeter as given above.

Calculation

The gross energy of milk sample may be calculated using the following formula:

$$\text{GE (cal/ml)} = \frac{(\text{Bomb equivalent} \times T) \times A}{\text{Volume of milk (ml)}}$$

Correction for nitrogen and sulphur

- 1) After the sample is burnt, open the bomb, wash all interior surfaces of bomb with distilled water (Fig. 12.2).
- 2) Collect the washings in 250 ml beaker for estimation of H_2SO_4 and HNO_3 formed during combustion from sulphur and nitrogen.
- 3) Boil the washings collected in the beaker for about 5 minutes.
- 4) Cool and titrate against N/10 $Ba(OH)_2$ solution using phenolphthalein indicator.
- 5) Add 20 ml N/10 Na_2CO_3 solution and boil again.
- 6) Cool the contents, filter through Whatman No.1 filter paper and wash with hot distilled water (2-3 washings).
- 7) Titrate the washings against N/10 HCl using methyl orange indicator.
- 8) Heat liberated by H_2SO_4 and HNO_3 can be calculated by using the following factors:
1 ml of N/10 $Ba(OH)_2$ solution = 3.60 cal
1ml of N/10 Na_2CO_3 solution = 1.43 cal

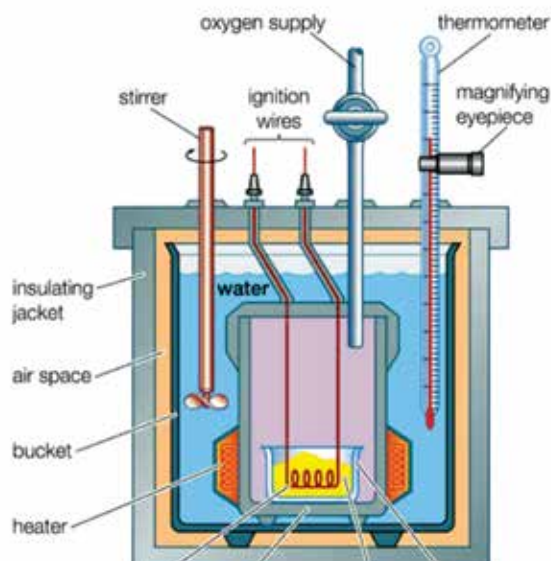


Fig. 12.2 Schematic diagram

Calculations

- a) Amount of N/10 $Ba(OH)_2$ solution used = A ml
 - b) Amount of N/10 Na_2CO_3 solution added = B ml
 - c) Amount of N/10 HCl used = C ml
- Therefore, Nitric acid correction = 1.43 (B-C) cal
Sulphuric acid correction = 3.60 [A-(B-C)] cal

Ballistic oxygen bomb calorimeter

In this calorimeter, a known weight of sample is ignited electrically and burnt in an excess of oxygen in the bomb and the maximum temperature rise of the bomb is measured with the thermocouple and galvanometer system. By comparing this rise, with that obtained when a standard sample of known calorific value is burnt, the calorific value of the sample material can be determined. In this case, calibration constant is calculated by combusting benzoic acid in bomb calorimeter.

GE determination of feed and faeces

- 1) Take 1 g of ground, dried sample. Make the pellet of this using pelleting machine.
- 2) Weigh the empty steel crucible and then with sample.
- 3) Disconnect the thermocouple and remove the safety cover and bomb body.
- 4) Place the crucible on the support pillar in the base of the bomb.
- 5) Take one strand of 50 mm length of cotton, insert one end of the cotton between the coils of the firing wire and dip the other end into centre of the sample in the crucible.
- 6) Replace the safety cover and plug the thermo couple into hole in the top of the bomb body.
- 7) Fill the bomb with oxygen, keeping the pressure maximum up to 25 atmospheres.

- 8) By mean of "Galvo zero" known on the control box and the galvanometer mechanical zero arm, bring the light spot index of the galvanometer to zero, and leave for about 30 seconds to check that the temperature is stable.
- 9) Stand back from the bomb, press and release the firing button.
- 10) Note the maximum deflection of the galvanometer (which occurs about 40 seconds after firing).

For urine and milk

Sample preparation for urine and milk is same as in case of adiabatic bomb calorimeter described earlier. After the sample preparation, bombing of sample is done as described above from steps (2) to (10).

Standardizing the apparatus

1. **Correction for constant heat gain:** During every test a small, constant amount of heat is released in the bomb by firing cotton. To measure this, a test is carried out without any sample in the crucible.
2. **Calibration with standard sample:** This calibration is done to establish the relationship between the galvanometer deflection and the amount of heat released by the combustion of the sample. Thermo-chemical grade benzoic acid is recommended standard material but any other pure material such as salicylic acid or sugar of known calorific value can be used. With benzoic acid, calorific value 6.32 kcal per g, about 0.7 g of benzoic acid is required.

Calculation of results

- | | | | |
|----|---|---|--------------------------------|
| 1) | Mass of benzoic acid | = | W g |
| 2) | Calorific value of benzoic acid | = | 6.32 kcal/g |
| 3) | Heat released from benzoic acid | = | 6.32 W kcal |
| 4) | Galvanometer deflection without sample | = | O ₁ divs |
| 5) | Galvanometer deflection with benzoic acid | = | O ₂ divs |
| 6) | Deflection due to Benzoic acid and calibration constant | = | $\frac{6.32 W}{O_2 - O_1} = y$ |

Gross energy of sample

- | | | | |
|----|--|---|--|
| 1) | Mass of sample | = | Z gms |
| 2) | Galvanometer deflection with sample | = | O ₃ divs |
| 3) | Galvanometer deflection without sample | = | O ₁ divs |
| 4) | Therefore, galvanometer deflection due to sample | = | O ₃ -O ₁ divs |
| 5) | Heat release from sample | = | (O ₃ -O ₁) Y kcal |
| 6) | Calorific value of sample (kcal/g) | = | $\frac{(O_3 - O_1) Y}{Z}$ |

Reference: NUKAMP, H.J., 1965, "Some remarks about the determination of the heat of combustion and the carbon content of urine" fide EAAP, publication No.11, Editor, Blaxter, K.L., Academic Press, London, New York, pp.147-157.

13.0 ANALYSIS OF STRAINED RUMEN LIQUOR

13.1 *In vitro* gas production technique

A system for feed evaluation based on *in vitro* gas production was developed in Germany by Menke *et al.* (1979) and Menke and Steingass (1988). This system is basically a Tilley and Terry (1963) system but in this method, gas production rather than dry matter loss measured.

The amount of gas (CO₂ and CH₄) released when feeds are incubated *in vitro* with rumen liquor is closely related to digestibility and to the energetic feed value of diets for ruminants. The feeds of different digestibility produce different volume of gases with in a stipulated time. A sample is introduced into a calibrated glass syringe with buffer rumen liquor medium and incubated at 39°C. At the end of incubation the gas produced is recorded from glass syringe. Unlike Tilley and Terry where disappearance of substrate is measured in this method fermentation production i.e. gas is measured.

Equipment

- Incubator
- Glass syringes 100 ml (graduated 1/1, with capillary attachment)
- Silicone tube (50 mm per glass syringe)
- Analytical balance
- Suction pump
- Bottle with stopper
- CO₂ cylinder
- Filling equipment for rumen fluid



Fig. 13.1 Incubation of samples by *in vitro* gas production technique

Reagents

1. Main element solution

Na ₂ HPO ₄	-	2.70 g
KH ₂ PO ₄	-	6.2 g
MgSO ₄ ·7H ₂ O	-	0.6 g

Make up to 1 L with distilled water

2. Trace element solution

CaCl ₂ ·2H ₂ O	-	13.2 g
MnCl ₂ ·4H ₂ O	-	10.0 g
CoCl ₂ ·6H ₂ O	-	1.0 g
FeCl ₂ ·6H ₂ O	-	0.8 g

Make up 100 ml with distilled water

3. Buffer solution

NaHCO ₃	-	35 g
NH ₄ CO ₃	-	4 g

Make up 1 L with distilled water

4. Resazurine solution

Resazurine	-	100 mg
Distilled water	-	100 ml

5. Reduction solution

1 N NaOH	-	2 ml
Na ₂ S·7H ₂ O	-	0.285 g
Distilled water	-	47.5 ml

It should be prepared fresh at the time of use.

Procedure

Day 1

- Prepare all the solutions except reducing solution
- Feed sample should be prepared by milling using 1 mm screen and weigh 200 mg dry feed.
- Prepare syringes in the following way:
Weigh 200 mg sample in each numbered syringes
After weighing, grease the plungers with Vaseline and place in incubator at 39°C
Keep three syringes without feed for incubation as blank.

Day 2

- Prepare reduction solution as mentioned in reagents.
- Prepare medium by mixing the ingredients sequentially as given below:

Water	-	400 ml
Micro mineral solution	-	1.0 ml
Buffer solution	-	200 ml
Macro mineral solution	-	200 ml
Resazurine solution	-	1.0 ml
Reduction solution	-	2.40 ml

- a. Maintain the medium at 39°C then add reducing solution
- b. Put magnet in the flask and gently bubble CO₂ through the solution until the blue color turns to pink and then clear.
- c. After bubbling raise the CO₂ tube above the level of the contents of the flask and stream CO₂ throughout the dispensing procedure.
 - Collect rumen fluid from animal.
 - Strain through cheese cloth/gauze in a warm (39°C) beaker.
 - Prepare the rumen fluid and medium mixture:
 - a) Add rumen fluid to the medium when it becomes colorless at the ratio of 1:2 (v/v).
 - b) Bubble CO₂ for 15 min and then with raised tube during filling.
 - Transfer 30 ml rumen fluid-medium mixture with semiautomatic pipette in preheated (39°C) syringe containing 200 mg feed.
 - Fix one end of the rubber tube properly to the attachment of the syringe and another end to the pipette to join the syringe and pipette.
 - Remove the air bubbles by gentle shaking and moving the piston upward.
 - Shut the clamp on the tube and record the volume of the mixture in the syringe.
 - Incubate the syringe at 39°C (Fig. 13.1).
 - Record the gas volume after 24 h.
 - Mix gently each syringe 2-3 times during first day, as well as at the time of reading.
 - Reading time can be selected to suit the type of substrate tested. For forages 3, 6, 12, 24, 48 and 96 h are suitable but for concentrate more readings in first 24 h are required.
 - The sample should be done in triplicate.
 - Three syringes containing only rumen fluid buffer solution with no feed sample are processed as blank and three syringes with standard hay sample are also incubated to monitor the activity of rumen fluid.

Calculation

$$\text{Gas production (ml/200 mg DM)} = \frac{(V - V_o - G_o)}{\text{Feed (mg)}} \times 200$$

- V = Reading after 24 h of incubation
 V_o = Reading just before incubation
 G_o = Gas produced in blank syringes

Note: The prediction equations have been developed by which the organic matter (OM) digestibility and metabolisable energy (ME) can be estimated.

Digestible organic matter

$$\text{DOM (\%)} = 14.88 + 0.889 * \text{gas (ml/200 mg DM)} + 0.45 * \text{CP} + 0.65 * \text{Ash.}$$

Metabolisable energy content of feed

For concentrates

$$\text{ME (KJ/kg DM)} = 1.06 + 0.157 * \text{gas (ml/200 mg DM)} + 0.0084 * \text{CP} + 0.022 * \text{EE} - 0.0081 * \text{Ash}$$

For roughages

$$\text{ME (KJ)} = 2.20 + 0.136 * \text{gas (ml/200 mg DM)} + 0.0057 * \text{CP} + 0.0029 * \text{EE}^2$$

A common equation useful both for roughage and concentrate

$$\text{ME (KJ)} = 1.24 + 0.146 * \text{gas (ml/200 mg DM)} + 0.007 * \text{CP} + 0.0224 * \text{EE}$$

* CP (crude protein), EE (ether extract) and Ash in g/kg DM

Precaution

Sample of rumen liquor should be drawn after 2-3 h of feeding.

Anaerobic condition must be maintained throughout the experiment.

References: Menke, K.H. and Steingass, H. (1988). Estimation of the energetic feed value obtained by chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Dev.*, 28:7.; Menke, K.H., Raab. L., Salewski, A. Steingass, H., Fritz. D and Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedstuff from the gas production when they are incubated with rumen liquor.

13.2 Estimation of volatile fatty acids

Principle

In the presence of oxalate buffer (pH 3.5) volatile fatty acids volatilize when heated with steam which is collected in the form of distillate through condenser and is equivalent to standard alkali (NaOH) used for neutralization.

Reagents

- Standard sodium hydroxide solution (N/50)
- Oxalic acid solution 5 % (w/v) with H₂O
- Potassium oxalate solution 10 % (w/v) with H₂O
- Scaris-brickbuffer. Equal volume of reagent 2 and 3 are mixed just before use.
- Phenolphthalein indicator

Apparatus

- Markham's distillation apparatus

Procedure

1. Rumen liquor thus collected is preserved with saturated mercuric chloride (2-3 drops in 10 ml) or 25% H₂SO₄ (1-2 drops).
2. 2 ml rumen liquor is distilled in presence of 2 ml oxalates buffer put at the same time in the boiling chamber of the Markham's distillation apparatus (Fig. 13.2).
3. Putting the knob the content is steam distilled and the distillate is collected under ice-cold condition.
4. About 2/3rd is collected in a 100 ml conical flask and immediately titrated against N/50 NaOH using phenolphthalein as indicator.



Fig. 13.2 Markham's distillation apparatus

Calculation

$$\text{TVFA (m.eq/l)} = \frac{\text{Titre} \times 0.02}{\text{Vol. of sample taken}} \times 1000$$

Note: 1 N VFA = 1 N NaOH, so 1 ml N/50 NaOH = 0.02 m.eq. VFA.

Reference: Applied Animal Nutrition Research Techniques by Raman Malik and SK Sirohi, NDRI, Karnal – 132 001, Haryana.

13.3 Estimation of total volatile fatty acids by gas chromatography

Chromatography is a physical method for separating components in a mixture. The basis of separation lies within the separating column, which is small-diameter tubing packed with a medium of large surface area. A mobile phase percolates through the stationary medium. The name gas chromatography denotes that the mobile phase is a gas.

The gas liquid chromatography (GLC) has a stationary phase, a liquid spread over the surface of a solid support. In GLC, a stream of carrier gas flows through the column. A sample is injected into the carrier gas near the head of packed chromatographic column. Separation of various components of the sample occurs as a result of multiple forces by which the column material tends to retain these components. In the gas phase the components move towards the outlet, but they are selectively retarded by the stationary phase. Consequently, all components pass through the column at varying speeds and emerge in the inverse order of their retention by the column material. Upon emerging from column the gaseous phase immediately enters a detector attached to the column. Here the individual components register a series of signals which appear as peaks on the chromatogram.

The chromatographic peaks are used for qualitative and quantitative detection of the components. A typical chromatogram consists of a baseline and the peaks. The distance between peaks is influenced by the rate of flow of gas, the column conditions and column temperature. However, peak area is related to the concentration of component. Therefore, under constant conditions, a comparison between a standard reference with that of sample will tell about the concentration of a component.

Preparation of sample extract

Draw rumen liquor from a cannulated animal and filter it through 4 layers of muslin cloth.

Reagents

- VFA (C₂-C₅ standard solution (25 mg/ml) – Transfer 2.5 g of individual volatile fatty acid (acetic, propionic, butyric, valeric and iso-valeric), in separate 100 ml volumetric flask and make up the volume with distilled water.
- Lactic acid standard solution (100 mg/ml) – Transfer 2.5 g lactic acid in a 25 ml volumetric flask and make up the volume.
- Pivalic acid internal standard (2.5 mg/ml) – Dissolve 0.5 g pivalic acid in distilled water and dilute to 200 ml.
- Oxalic acid (0.12 M) – Dissolve 3.782 g oxalic acid dehydrate in water and dilute to 250 ml.
- Composite working standard – Pipette 1 ml of C₂-C₅ standard solution (reagent 1), 2.5 ml lactic acid standard solution (reagent 2), 10 ml pivalic acid (reagent 3) and 25 ml oxalic acid solution (reagent 4) into a 100 ml volumetric flask and dilute to volume.

Procedure

1. Set the GLC according to manufacturers' instructions to the following operating conditions:

Gas flows	Nitrogen	24 ml/min
	Hydrogen	50 ml/min
	Air	500 ml/min

Temperature

Injection	220°C
Detector	240°C
Oven	175°C

2. Make repeat 1.0 μ l injections of the composite working standard until a stable response is obtained. The total chromatographic run time is approximately 30 minutes.

Examination of the sample extract

Pipette 5 ml of strained rumen liquor into a 10 ml volumetric flask, add 1 ml of the pivalic acid solution (reagent 3) and 2.5 ml of oxalic acid solution (reagent 4), then dilute to volume. Centrifuge the resulting extract at 2600 g for 5 minutes. Inject 1.0 μ l of the supernatant and integrate the peaks eluted.

Calculation

Calculate mg/ml of each acid in the strained rumen liquor from the expression:

$$\text{Conc. of unknown} = (\text{RF}_1/\text{RF}_2 \times \text{standard conc.} \times 2)$$

Where,

$$\text{RF}_1 = \frac{\text{Area of sample component peak}}{\text{Area of internal standard}}$$

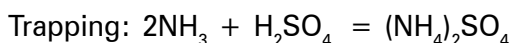
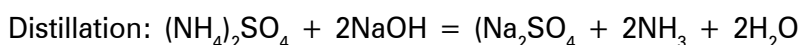
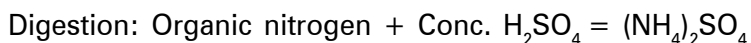
$$\text{RF}_2 = \frac{\text{Area of known peak}}{\text{Area of internal standard}}$$

Reference: Applied Animal Nutrition Research Techniques by Raman Malik and SK Sirohi, NDRI, Karnal – 132 001, Haryana.

13.4 Determination of total nitrogen in SRL

Principle

When sample containing nitrogen is digested with H_2SO_4 , the total nitrogen converts into ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$. In the presence of alkali, ammonia is released from the ammonium sulphate and released ammonia is distilled and trapped in a known volume of standard acid, which is then back titrated with the standard alkali. The whole process is completed in three steps. The reactions during estimation are as follows:



Apparatus

- Balance
- Micro kjeldahl distillation assembly
- Digestion bench
- Kjeldahl flasks
- Pipette
- Conical flask
- Beaker
- Burette
- Volumetric flask

Reagents

- Digestion mixture ($\text{K}_2\text{SO}_4 + \text{CuSO}_4$ in the ratio of 9:1): 90 g potassium sulphate and 10 g copper sulphate mixed together.
- Concentrated H_2SO_4
- 40% NaOH solution

- 0.01 N NaOH
- 0.01 N H₂SO₄
- Methyl red indicator: Dissolve 0.1 g methyl red indicator in 60 ml ethanol and add distilled water to make the volume 100 ml.

Procedure

Digestion

- Take 5 ml strained rumen liquor in a Kjeldahl flask.
- Add 10 ml concentrated H₂SO₄.
- Add 2-3 g digestion mixture.
- Keep the flask on digestion bench and allow gentle boiling. Bumping should be avoided.
- When the solution becomes clear blue, then remove the flask from the digestion bench and cool it.
- Add 5 to 10 ml distilled water to the Kjeldahl flask. Transfer the whole material in 100 ml volumetric flask with repeated washings of distilled water. Make the volume to 100 ml.

Distillation

- Set the Kjeldahl distillation assembly.
- Take 10 ml 0.01 N H₂SO₄ in a conical flask and add 2-3 drops of indicator
- Keep the flask under the condenser in such a way that the tip of the condenser should be dipped in acid, to avoid ammonia loss during distillation.
- Take 10 ml aliquot of digested sample and transfer it into the kjeldahl assembly.
- Add 15-20 ml 40% NaOH to make the aliquot contents alkaline and put the stopper immediately.
- Allow distillation for 15 min.

Titration

- Remove the flask after washing tip of the condenser with distilled water.
- Titrate the contents of the flask with standard 0.01 N NaOH till the pink color develops (end point).
- Record the volume of alkali used for titration on the burette.
- Run a blank using all reagents but no sample and following the whole procedure to estimate the nitrogen contents of the reagents, if any.

Calculation

1 ml 0.01 N H₂SO₄ = 0.00014 g nitrogen

$$\text{Nitrogen/100 ml rumen liquor} = \frac{V \times 0.00014 \times D \times 100}{v \times A}$$

Where,

$$V = A - B$$

D = Dilution (Volume made in volumetric flask)

v = Initial volume of rumen liquor taken for the digestion

A = Aliquot taken (10 ml)

Total nitrogen of sample = Total nitrogen of sample – Total nitrogen of blank

Reference: Laboratory manual of animal nutrition. IVRI, Izatnagar, U.P. – 243 122.

13.5 Determination of TCA precipitable nitrogen

TCA precipitable nitrogen is the nitrogen coming from the true protein because by adding trichloroacetic acid, only proteins are precipitated. These precipitated proteins are analyzed further for nitrogen contents by Micro Kjeldahl method.

Procedure

1. Take 5.0 ml rumen liquor in a centrifuge tube.
2. Add 5.0 ml of 20% TCA
3. Leave the tubes overnight.
4. Centrifuge the tubes at 2,000 rpm for 10 min.
5. Transfer the whole precipitate with repeated washings of distilled water in Kjeldahl flask.
6. Proceed for digestion, distillation and titration as described above.

Soluble nitrogen

TCA precipitable nitrogen subtracted from total nitrogen gives soluble nitrogen contents of rumen liquor.

Ammonia nitrogen

In alkaline medium, ammonia is released from the rumen liquor and the released ammonia is analyzed for nitrogen contents by Micro-Kjeldahl method.

Procedure

1. Take 5.0 ml strained rumen liquor in the distillation assembly and 5 ml of 40% NaOH.
2. Proceed for distillation and titration as described above.

Reference: Laboratory manual of animal nutrition. IVRI, Izatnagar, U.P. – 243 122.

13.6 Determination of lactic acid in SRL

Lactic acid, when heated with concentrated H_2SO_4 , converts into acetaldehyde, which reacts with p-hydroxydiphenyl to give purple color in the presence of copper ions.

Reagents

1. Copper sulphate 20%: Dissolve 200 g $CuSO_4 \cdot 5H_2O$ in 500 ml distilled water and make the volume to one litre. The solution is stable indefinitely.
2. Copper sulphate 4%: Take 200 ml of reagent 1, and make the volume to one litre.
3. Calcium hydroxide ($Ca(OH)_2$).
4. Concentrated H_2SO_4
5. NaOH 5% - 5 g NaOH, dissolve in 100 ml distilled water.
6. p-hydroxydiphenyl reagent : Take 1.5 g p-dydroxydiphenyl in a 100 ml volumetric flask. Add 100 ml of 5% NaOH and 100 ml distilled water. Warm it with constant stirring to dissolve. Make the volume to 100 ml. Store it in amber color bottle.
7. Stock standard lactic acid: Take 0.1065 g lithium lactate in 100 ml volumetric flask and dissolve in about 50 ml distilled water. Add 0.1 ml concentrated H_2SO_4 and make up the volume to 100 ml with distilled water. The solution contains 1 mg lactic acid per ml and the solution is stable for a long period in refrigerator.
8. Working standard lactic acid solution: Dilute 1 ml of stock lactic acid solution to 100 ml with distilled water. It contains 0.01 mg lactic acid per ml. Prepare fresh working solution at the time of analysis.

Procedure

- Take 1 ml strained rumen liquor in a centrifuge tube. Add 1 ml of 20% CuSO_4 and make the volume to 10 ml.
- Add 1 g Ca(OH)_2 , shake vigorously to make the mixture homogenous.
- Leave the tubes for 90 minutes with periodic shaking.
- Centrifuge at 3000 rpm for 10 min.
- Take 1 ml supernatant in a test tube in duplicate.
- Add 0.05 ml of 4% CuSO_4 .
- Add 6ml concentrated H_2SO_4 drop by drop with continuous shaking.
- Keep the tubes in boiling water bath for 5 minutes.
- Cool the tubes at room temperature
- Add 0.1 ml p-hydroxydiphenyl reagent drop by drop. The pipette tip should not touch the wall of the tube. Mix the contents immediately and vigorously.
- Incubate the tubes at 30°C for 30 min with periodic shaking.
- Keep the tubes in boiling water bath for 90 sec. Remove the tubes and cool to room temperature.
- To plot the standard curve prepare the standard tubes in duplicate as follows:
- Proceed for color development (step 5 to 12).
- Read absorbance (optical density) of all the tubes at 560 nm.
- Find out the concentration of sample on standard curve and multiply by 10 (dilution) to give μg lactic acid/ml rumen liquor.

Tube No.	1	2	3	4	5	6
Distilled water (ml)	1.0	0.9	0.8	0.6	0.4	0.2
Standard lactic acid solution (ml)	0.0	0.1	0.2	0.4	0.6	0.8
Lactic acid concentration (μg)	0.0	1.0	2.0	4.0	6.0	8.0

Reference: Laboratory manual of animal nutrition. IVRI, Izatnagar, U.P.-243 122.

14.0 URINE ANALYSIS

14.1 Determination of allantoin - Colorimetric method

Chemistry

The manual method adapted to measure allantoin is based on the calorimetric method described by Young and Conway (1942). In this procedure, allantoin is hydrolyzed firstly under a weak alkaline condition at 100°C, to allantoic acid which is further degraded to urea and glyoxylic acid in weak acid solution. The glyoxylic acid then reacts with phenyl hydrazine hydrochloride to produce a phenyl hydrazone of the acid. The product can then form an unstable chromophore with potassium ferricyanide. The color is read at 522 nm.

Apparatus

- Spectrophotometer
- Boiling water bath. If you have a temperature controlled water bath, you may use polyethylene glycol (PEG MW 400) solution instead of water in the bath and set the temperature at 100°C. The temperature can be better controlled since PEG has a boiling point greater than 100°C.
- Ultrasonic bath (Fig. 14.1)



Fig. 14.1 Ultrasonic bath

Chemicals

- Sodium hydroxide (NaOH) 0.5 M
- NaOH 0.01 M
- Hydrochloric acid (HCl) 0.5 M
- Phenyl hydrazine hydrochloride 0.023 M freshly prepared before use.
- Potassium ferricyanide 0.05 M freshly prepared before use.
- Concentrated hydrochloric acid (11.4 N) cooled at -20°C at least 20 min before use.
- Alcohol bath, 40% (v/v) alcohol, kept at -20°C. You may use 40% NaCl solution instead of alcohol solution.
- Allantoin

Standards

Prepare a stock allantoin solution 100 mg/L. Dilute it to give working concentrations of 10, 20, 30, 40, 50 and 60 mg/L.

- 1) Weigh 50 mg of allantoin and transfer it to a 500 ml volumetric flask. Dissolve in about 100 ml 0.01 M NaOH, and top up to volume with distilled water. The addition of NaOH is only to help to dissolve allantoin.
- 2) To prepare 50 ml of the working standards 10, 20, 30, 40, 50 and 60 mg/L respectively pipette 5, 10, 15, 20, 25 and 30 ml of stock solution into 50 ml volumetric flasks and make up to volume with distilled water. If you do this analysis routinely, it is better to prepare the standard solution in larger volumes.
- 3) Store each working standards as small aliquots in the freezer. Only the necessary quantities are thawed and any leftover discarded. This ensures that fresh standards are used for each analysis run.

Preparation

Before the analysis:

- 1) Put the alcohol bath into the freezer over night.
- 2) Put the concentrated hydrochloric acid into the freezer just before the beginning of the analysis.
- 3) Switch on the water bath.
- 4) Check the samples. If any precipitate is visible, place the samples in an ultrasonic bath for 20 min. to break up the particles.
- 5) Prepare the fresh solutions of Phenyl hydrazine hydrochloride and Potassium ferricyanide.

Phenyl hydrazine hydrochloride and potassium ferricyanide solution:

Prepare solutions of phenyl hydrazine hydrochloride and potassium ferricyanide on the day of analysis (keep these solutions in fridge before use). Fifty ml is enough for 10 samples in duplicate. Weigh 0.1663 g of phenyl hydrazine hydrochloride, dissolve in a small beaker and transfer to a 50 ml volumetric flask. Top up to volume with distilled water. Weigh 0.835 g potassium ferricyanide and transfer to a 50 ml volumetric flask, dissolve and make up to volume with distilled water.

Procedure

This procedure requires critical timing of the reactions. The reading of standards and samples OD must be done within a shortest possible time-span, since OD decrease with time. Therefore, no more than 10 samples in duplicate should be processed in each run. A set of standards and a blank (using distilled water) in duplicate are processed.

- Pipette 1 ml of sample, standard or distilled water (blank) into 15 ml tubes.
- Add 5 ml of distilled water.
- Add 1 ml of 0.5 M NaOH.
- Mix the contents of the tubes by vortexing.
- Put the tubes in the boiling water bath for 7 min.
- Remove from the boiling water and cool the tubes in cold water.
- Add to each tube 1 ml of HCl (0.5 M). The pH after adding the HCl must be in the range 2-3.
- Add 1 ml of the phenyl hydrazine solution. Mix and transfer the tubes again to the boiling water for exactly 7 min.
- Remove from the boiling water and dump it immediately into the icy alcohol bath for several min.
- Pipette 3 ml of concentrated HCl (operate in a fume cupboard) and 1 ml of Potassium ferricyanide. Perform this for all samples within a shortest possible span. Start the timer.
- Mix thoroughly and transfer some to 4.5 ml cuvettes at room temperature.
- Read the absorbance at 522 nm after exactly 20 min. on the timer. Once started, do it as quickly as possible (because the color will disappear). It is important that OD for samples and standards be read at a shortest possible time span.

Calculation

Standard curve:

The standard curve should be linear. Therefore, we can fit a linear regression between the known allantoin concentrations (standards) (X) and the corresponding OD (Y). The slope of the line is usually 0.16-0.18. Calculate the concentration of the unknown based on this equation.

Reference: Young, E.G and Conway, C.F., 1942. On the estimation of allantoin by the Rimini-Schryver reaction. J. Biol. Chem., 114: 243-248.

14.2 Determination of uric acid by uricase method

Principle

Uric acid absorbs UV at 293 nm, although other compounds may also absorb at this wavelength. When samples are treated with uricase, uric acid is converted to allantoin and other compounds that do not absorb UV at 293 nm. Therefore, the reduction in OD reading after treatment with uricase is correlated with the concentration of uric acid in the sample. After treatment, the OD of the standards should be zero if the conversion is complete.

Reagents

- KH_2PO_4 buffer, 0.67 M, pH 9.4. Adjust the pH with KOH.
- Uricase from porcine liver. Prepare an enzyme solution of 0.12 unit/ml buffer.
- Uric acid.

Equipment

Spectrophotometer

Water bath

Ultrasonic bath (optional)

Standards

Prepare uric acid standard working concentrations of 5, 10, 20, 30 and 40 mg/L.

Procedure

- Pipette 1 ml of urine or standard or blank (distilled water) into 10 ml tubes. Mix with 2.5 ml phosphate buffer. Prepare two sets of tubes.
- To one set, add 150 μl buffer and to the other add 150 μl of uricase solution. Mix well.
- Incubate in the water bath at 37°C for 90 min (Fig. 14.2).
- Remove from water bath, mix and transfer the solutions to cuvettes and read the OD at 293 nm.
- If the enzymatic conversion is complete, the OD of the standards with uricase added should be zero. If not, incubate in water bath for an additional 30 min and read again.

Standard curve and calculation

- Standard curve is curvilinear. When both X and Y are transformed to Ln functions, Ln (Y) is linearly correlated to Ln (X).
- Use the OD reading of the set without addition of uricase for the construction of standard curve.
- Calculate the net reduction in OD (AOD) for the samples due to uricase treatment $\text{OD} = \text{OD without enzyme} - \text{OD with enzyme}$.
- Calculate the uric acid concentration from OD based on the established standard equation.

Reference: I.A.E.A. 1997. Estimation of rumen microbial protein production from purine derivatives in urine. A laboratory manual, IAEA – TECDOC – 945. IAEA, Vienna.



Fig. 14.2 Water bath

14.3 Estimation of creatine & creatinine

Estimation of creatinine

Principle

An alkaline picrate solution is added to a filtrate of urine and the creatinine contained therein reacts to form a complex orange-red coloured compound (Jaffe Reaction). The colour may be due to formation of picramic acid or to an enolized form of the picric acid itself.

Reagents

1. NaOH solution (10% w/v): Dissolve 10 g of NaOH in water and dilute to 100 ml.
2. Picric acid (saturated): Dissolve 13 g picric acid to 800 ml hot distilled water, cool and makeup the volume to 1 litre.
3. Alkaline picrate solution: Mix 5 volumes of saturated picric acid solution to one volume of NaOH.
4. Standard solution:

Stock solution : Creatinine solution is prepared by dissolving 1.0 g of pure dry creatinine in 0.1 N HCl and diluted to 1 litre with acid.

Working solutions : Dilute 3 ml of stock solution to 500 ml with water using 50 ml of 0.1 N HCl (0.006 mg/ml).

Procedure

1. Dilute urine with water in a ratio of 1:20 and filter. Add 1 ml alkaline picrate solution to 2 ml filtrate, mix well and allow the colour to develop for 20 min at room temperature.
2. Include a blank tube containing 2 ml water & 1.0 ml of alkaline picrate solution.
3. Standard course is prepared in the same way by taking 0.5, 1.0, 1.5 and 2.0 ml working standard and adding water to make volume 2.0 ml.
4. Read the O.D. of unknown and standards against blank at 520 nm. The colour is stable for at least 30 min.

Calculation

$$\text{Urine creatinine (mg/100 ml)} = \frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \text{Dilution factor} \times 100$$

Estimation of creatine

Principle

The creatine in the presence of mineral acid is transformed into creatinine by autoclaving. By determining the creatinine content before and after treatment with acid, the amount of creatine present may be obtained by difference.

Reagent

1 N HCl

Procedure

- Add 2 ml diluted urine and 1.0 ml of 1 N HCl in stoppered test tube and autoclave the tube at 14 lb pressure at 120°C for 20-30 min.
- Determine the creatine as described above.

Calculation

Urine creatine = Total creatinine – Preformed creatinine

Reference: Wootton, I.D.P. 1964. Micro- analysis in Medical Biochemistry, 4th edn., J. and A. Churchill Ltd., London, pp. 174 – 175.

15.0 ESTIMATION OF AFLATOXIN B₁ IN FEED

15.1 Estimation of Aflatoxin B₁ – HPLC method

Reagents

- Acetonitrile AR grade
- Distilled water
- Acetonitrile HPLC grade
- Water HPLC grade
- Nitrogen gas
- Aflatoxin B₁ standard from Sigma.

Apparatus

- Amber colored beaker
- Amber colored conical flask
- HPLC



Fig. 15.1 High performance liquid chromatography

Procedure

Extraction

- Weigh 25 g of ground sample into an Erlenmeyer flask or small blending jar.
- Add 100 ml of 80/20 acetonitrile/ water to the sample.
- Secure a lid tightly and shake for 1 hour or blend on high for minutes.
- Filter the extract into a sample jar and cover with a lid.

Purification process

- Place the purification column in a 15 x 125 mm test tube.
- Pipette 2 ml of extract and spiking solution into the top of the purification column.
- Push approximately 500 µl of solutions through the column.

HPLC conditions

- Mobile Phase : Combine 350 ml of water with 650 ml of Acetonitrile.
- Flow Rate : 0.5ml/min; Approximate Retention Time : 2.9
- Fluorescence Detector: Emission = 430 nm; Excitation = 369 nm

Preparation of standards

Take required quantity of standards (0, 0.25, 0.5, 1, 5, 10, 20, 50, 75, 100 and 200 ng) in clean vials and dry it under stream of nitrogen gas. Then add 150 ml of trifluoro acetic acid for derivatisation and after an interval of 10 minutes, add 850 ml of mobile phase to make it 1ml. Inject 20 µl of this solution in HPLC (Fig. 15.1) for standard peaks with areas.

HPLC injections

Take 100 ml extract from purified extraction and dry it under stream of nitrogen. Add 150 ml of trifluoro acetic acid and 850 ml of mobile phase to make 1 ml. Now it is ready for HPLC injection. Sample peak and area will be compared with standards.

Calculations

$$\text{Aflatoxin B}_1 (\mu\text{g/Kg}) = \frac{\text{Baf} \times \text{Vext}}{\text{Vb} \times \text{W} \times \text{Vf}/100}$$

Baf (ng)- Concentration of aflatoxin B₁ from calibration curved solution

Vex - Volume in which dried extraction dissolved

Vb - Volume injected in HPLC.

W - Weight of the sample.

Vf - Volume of filtrate taken in column.

100 - 100 ml of extraction solution.

Reference: AOAC (1997) 16th edition

15.2 Estimation of Aflatoxin B₁ - Elisa kit method

Intended use

The HELICA Low Matrix Aflatoxin Assay is a competitive enzyme-linked immunoassay for the quantitative detection of aflatoxins B₁, B₂, G₁ and G₂ in grains, nuts, cotton seed, cereals and all commodities which are difficult to measure due to high matrix effect such as silage and most spices.

Principle

The HELICA Low Matrix Total Aflatoxin Assay is a solid phase direct competitive enzyme immunoassay. An aflatoxin specific antibody optimized to cross react with all four subtypes of aflatoxin is coated to a polystyrene micro well. Toxins are extracted from a ground sample with either 80% methanol or 80% acetonitrile and after dilution, added to the appropriate well. If aflatoxin is present, it will bind to the coated antibody. Subsequently, aflatoxin bound to horse-radish peroxidase (HRP) is added and binds to the

antibody not already occupied by aflatoxin present in the sample or standard. After this incubation period, the contents of the wells are decanted, washed and an HRP substrate is added which develops a blue color in the presence of enzyme. The intensity of the color is directly proportional to the amount of bound conjugate and inversely proportional to the amount of aflatoxin in the standard or sample. Therefore, as the concentration of aflatoxin in the sample or standard increases, the intensity of the blue color will decrease. An acidic solution is added which changes the chromogen color from blue to yellow. The micro wells are measured optically by micro plate reader with an absorbance filter of 450 nm (Fig. 15.2). The optical densities of the samples are compared to the OD's of the kit standards and a result is determined by interpolation from the standard curve.



Fig. 15.2 Elisa reader

Reagents Provided

- | | |
|--------------------------------------|--|
| 1 pouch: Antibody coated micro wells | - 96 wells (12 eight well strips) in a micro well holder coated with a mouse anti-aflatoxin monoclonal antibody |
| 1 plate: Mixing wells (red) | - 96 non-coated wells (12 eight well strips) in a micro well holder. |
| 6 vials: Aflatoxin standards | - 1.5 ml/vial of aflatoxin at the following concentrations: 0.0, 0.02, 0.05, 0.1, 0.2, and 0.4 ng/ml in aqueous solution |
| 1 bottle: Aflatoxin HRP-conjugate | - 12 ml of aflatoxin B ₁ , conjugated to peroxidase in buffer with preservative |
| 2 bottles: Assay Diluent | - 2 x 12 ml propriety assay diluent |
| 1 bottle: Substrate Solution | - 12 ml stabilized tetramethylbenzidine (TMB) |
| 1 bottle: Stop Solution | - 12 ml acidic solution |
| 1 pouch: Washing Buffer | - PBS with 0.05% Tween20, bring to 1 litre with distilled water and store refrigerated |

Materials required

Extraction procedure

- Grinder: Sufficient to render sample to particle size of fine instant coffee.
- Collection container: Minimum 125 ml capacity
- Balance: 20 g measuring capability
- Graduated cylinder: 100 ml.
- Methanol or acetonitrile: 80 ml reagent grade per sample.
- Distilled or de-ionized water : 20 ml per sample
- Filter paper: Whatman no. 1 or equivalent filter funnel.

Assay procedure

- Pipettor with tips : 100 μ l and 200 μ l
- Timer
- Wash bottle
- Dilution tubes
- Absorbent paper towels
- Micro plate reader with 450 nm filter

Precautions

1. Bring all reagents to room temperature (19-27°C) before use.
2. Store reagents at 2 to 8°C and do not use beyond expiration date(s). Never freeze kit components.
3. Do not return unused reagents back into their original bottles. The assay procedure details volumes required.
4. Adhere to all time and temperature conditions stated in the procedure.
5. Never pipette reagents or samples by mouth.
6. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin. Wear protective gloves and safety glasses when using this kit.
7. Dispose of all materials, containers and devices in the appropriate receptacle after use.

Extraction procedure

Note: The sample must be collected according to established sampling techniques.

Note: The ratio of sample to extraction solvent is 1:5 (w/v).

1. Grind a representative sample to the particle size of the fine instant coffee (50% passes through a 20 mesh screen).
2. Prepare extraction solvent (80% methanol of 80% acetonitrile) by adding 20 ml of distilled or de-ionized water to 80 ml methanol or acetonitrile for each sample to be tested.
3. Weigh out a 20 g ground portion of the sample and add 100 ml of the extraction solvent.
4. Mix by shaking in a sealed container or in a blender for a minimum of 2 minutes.
5. Allow the particulate matter to settle, then filter 5-10 ml of the extract through a Whatman no. 1 filter paper (or equivalent) and collect the filtrate to be tested. Alternatively, the sample may be centrifuged to clarify.
6. Dilute an aliquot of the extract 1 in 10 with reconstituted wash buffer.
7. The sample is now ready. The standards required no pre-dilution before use.

Assay procedure

1. Bring all the reagents to room temperature before use. Reconstitute the PBS-Tween packet by washing out the contents with a gentle stream of distilled water into a 1 litre container. Make to 1 litre with distilled water and store refrigerated when not in use.
2. Place one mixing well in a micro well holder for each standard and sample to be tested. Place an equal number of antibody coated micro titre wells in another micro well holder.
3. Dispense 200 μ l of the assay diluent into each mixing well.

4. Using a new pipette tip for each add, 100 μ l of each standard and prepared sample to appropriate mixing well containing diluent. Mix by priming pipettor at least 3 times.
5. Using a new pipette tip for each, transfer 100 μ l of contents from each mixing well to a corresponding. Antibody coated micro titre well. Incubate at room temperature for 30 minutes. The mixing wells contain enough solution to run each standard and/or sample in duplicate if so desired.
6. Decant the contents from micro wells into a discard basin. Wash the micro wells by filling each with PBS-Tween wash butter, then decanting the wash into a discard basin. Repeat wash for a total of 3 washes.
7. Tap the micro wells (face down) on a layer of absorbent towels to remove residual water.
8. Add 100 μ l of conjugate to each antibody coated well and incubate at ambient temperature for 30 minutes.
9. Repeat steps 6 and 7.
10. Measure the required volume of substrate reagent (1 ml/strip or 120 μ l well) and place in a separate container. Add μ l to each micro well; incubate at room temperature for 10 minutes.
11. Measure the required volume of stop solution (1ml/strip or 120 μ l /well) and place in a separate container. Add 100 μ l in the same sequence and at the same place as the substrate was added.
12. Read the optical density (OD) of each micro well with a micro titre plate reader using a 450 nm filter. Record the optical density (OD) of each micro well.

Note: This assay may be used for detecting aflatoxin in potable water. In this case 100 μ l of the sample should be used without pre-dilution with wash buffer.

Interpretation of results

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0.0) standard against the aflatoxin content of the standard. Unknowns are measured by interpolation from the standard curve.

The information contained on the label of a standard vial refers to the contents of that vial. However, the sample has been diluted at a 5:1 ratio by extraction solvent and also 10:1 in wash buffer and so the level of aflatoxin shown by the standard must be multiplied by 50 in order to indicate the ng per gram (ppb) of the commodity as follows:

Standard (ng/ml)	Commodity (ppb)
0.00	0.0
0.02	1.0
0.05	2.5
0.10	5.0
0.20	10.0
0.40	20.0

The sample dilution results in a standard curve from 1 to 20 ppb. If a sample contains aflatoxin at greater than the highest standard, it should be diluted appropriately in 80% extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

In the case of potable water there is no pre-dilution so it is measured with a sensitivity equal to the lowest standard which is twenty parts per trillion.

Assay characteristics

Data from ten consecutive standard curves gave the following results:

ppb	B/B ₀	% CV
0	100	-
1	81.0	1.3
2.5	59.0	2.0
5	34.7	5.5
10	17.5	4.9
20	12.6	7.6

As an example of a high matrix effect commodity, thirteen silage samples, 5 corn, 2 wheat, 3 hay and 3 snap, which had measured less than 1 ppb each for B₁, B₂, G₁ and G₂ by HPLC were extracted with either 80% methanol or 80% acetonitrile.

Following extraction with 80% methanol, 12/13 measured less than 1 ppb, with a single wheat silage sample measuring 1.2 ppb. After extraction with 80% acetonitrile 8/12 measured less than 1 ppb with 5 samples average 1.5 ppb. No sample measured more than 2 ppb.

Recoveries of a 5 ng/gm spike into four of the silage samples were as follows:

Acetonitrile extract		
	ppb	% Recovery
Spike	4.8	100
Corn	4.1	85
Wheat	4.8	100
Hay	4.6	96
Snap	4.6	96

Methanol extract	
ppb	% Recovery
5.1	100
2.5	49
2.7	53
2.7	53
2.9	57

In a similar experiment, extraction of paprika, pistachio and peanut by either methanol or acetonitrile was less than 1 ppb before extraction and after a 5 ppb spike recoveries were 96, 93 and 67% respectively for acetonitrile and 67, 69 and 58% for methanol.

Acetonitrile is the preferred extraction solvent but methanol may be used if its extraction efficiency is taken into account.

Cross-reactions

The assays will cross-react with aflatoxin analogues as follows:

B₁ 100%, B₂ 77%, G₁ 64% and G₂ 25%.

Reference: Patey, A.L., Sharman. M and Gilbert. J., 1992. Determination of total aflatoxin levels in peanut butter by enzyme linked immunosorbent assay: collaborative study. *J of AOAC Intern.*, 75: 693-697.

15.3 Estimation of aflatoxin B₁ - TLC method

Principle

Methods of aflatoxin analysis are mainly based on their solubility characteristics in different solvents as well as on their characteristic fluorescent properties. The solubility of aflatoxins in organic solvents like chloroform, methanol, ethanol, acetone, acetonitrile, benzene etc., helps in their quantitative extraction from the various samples. Their insolubility in diethyl ether, petroleum ether and hexane helps in separating them from certain interfering pigments and fats. Their characteristic fluorescence and absorption in the long wavelength range have visible and UV light aids their detection and estimation.

Thin layer chromatography

Thin layer chromatography (TLC), also known as flat bed chromatography or planar chromatography is one of the most widely used separation techniques in aflatoxin analysis. Since, 1990 it has been considered the AOAC official method and the method of choice to identify and quantitative aflatoxins at levels as low as 1 ng/g. The TLC method is also used to verify findings by newer, more rapid techniques.

Reagents

- Petroleum ether (40-60°C)
- Chloroform
- Anhydrous sodium sulphate
- Silica gel
- Diethyl ether
- Hexane
- Methanol
- Benzene
- Acetone
- Nitrogen gas
- Aflatoxin B₁ standard

Apparatus

- Amber colored beaker
- Amber colored conical flask
- Rotary vacuum evaporator
- Mechanical shaker
- Chromatography column (300 x 22)

Procedures

Extraction

Weigh accurately 50 g of the well mixed sample in a 500 ml glass stoppered conical flask. Add 150 ml of petroleum ether, mix and keep overnight. Filter out the ether using an ordinary filter paper. To defatted sample in the flask, add 250 ml of chloroform mix and place in the mechanical shaker for 30 min. Filter the contents through an ordinary filter paper followed through a bed of anhydrous sodium sulphate.

Column chromatography

Prepare a chromatographic silica gel column (300 mm x 22 mm i.d.) using glass wool, anhydrous sodium sulphate (5 g), slurry of activated (at 110 ± 1 °C for 1 h) silica gel (10 g in almost an equal amount of chloroform) and anhydrous sodium sulphate (7.5 g), in the



Fig. 15.3 Rotary vacuum evaporator

order mentioned. Pour 50 ml of the prepared extract on the column and elute it. Then pour 150 ml of diethyl ether: hexane mixture (3:1, v/v). Elute the aflatoxin from the column using 150 ml of chloroform: methanol mixture (97:3, v/v) and collect the elute in a 250 ml round bottom flask.

Concentrate the extract in a rotary vacuum evaporator (Fig. 15.3) and then dry the concentrated extract on a water bath under a gentle stream of inert (nitrogen) gas.

Preparation of activated TLC plate

Prepare TLC plates of silica gel G (8-10 g with about 20 ml of water on a plate of 20 x 20 cm size) using an applicator (0.40 mm thickness). Air-dry the plates overnight. Activate the plates in oven at $110 \pm 1^\circ\text{C}$ for 1 h. Dilute the dried extract to a fixed volume (e.g. 200 μl) with benzene: acetonitrile mixture (98:2, v/v). Apply 5 or 10 μl of the diluted extract on the marked TLC plates (3 cm above the bottom edge of the plate) along with standard aflatoxin B₁ solution representing 2.5, 5.0, 7.5 and 10 μg concentrations. Run the plates to about 15 cm height in a solvent system (chloroform: acetone, 90:10 v/v). Remove the plates and air dry. Observe the plates under UV light at 360 nm in a UV chromatography view cabinet. Compare the intensities of the fluorescent of the spots produced by the sample with those produced by the standard for quantifying the aflatoxin content in the sample.

$$\text{Aflatoxin B}_1 \text{ content } (\mu\text{g/kg}) = \frac{C \times V_1 \times V_3}{M \times V_2}$$

Where,

- C - Concentration of aflatoxin B₁ from calibration curved solution ($\mu\text{g/ml}$)
- V₁ - Final volume of extract
- V₂ - Volume of extract applied on the TLC plate.
- V₃ - Volume of standard B₁ solution applied on the TLC plate whose fluorescence intensity is similar to that of sample (μg)
- M - Weight of the sample.

Reference: IS 1992b.

15.4 Estimation of Aflatoxin B₁ - Modified Romer's method

Outline of the method

The aflatoxin is extracted with acetone, treated with cupric carbonate and ferric gel to eliminate fluorescent materials other than aflatoxin, washed with acid and alkali and extracted with chloroform, dried, re-diluted with chloroform and spotted in an activated TLC plate with standards and ascertained the concentration by visual comparison method in a UV viewing cabinet.

Reagents required

- 0.2 M NaOH: 8 g of NaOH in 1000 ml of solution
- 0.41 M FeCl₃: Dissolve 70 g FeCl₃ to make one litre of solution with distilled water
- 0.03% H₂SO₄: 0.3 ml of Conc. H₂SO₄ in one litre of distilled water
- 4. 0.02 M KOH & 1% KCl : Dissolve 1.222 g of KOH and 10 g of KCl in one litre standard flask with distilled water
- Silica gel

Apparatus

- Amber colored beaker and conical flask
- Hot air oven
- Mixer

Preparation of activated TLC plate

To prepare 2 plates (10 cm x 10 cm or 10 cm x 5cm) of 0.2 mm thickness, dissolve 16 g of silica gel in 35 ml of distilled water, apply on the plates using applicators and allow it to natural dry. Then keep the plates at 105°C for 1 hour and cool. Draw lines with 1 cm space such that standards are at the middle of the plate and four sample spots can be applied on either side of the standard.

Procedure

1. Take 10 g of the sample. Add 40 ml of distilled water.
2. Add 60 ml of acetone and again beat it for two minutes. Contents may slightly be heated up. High temperature should be avoided.
3. Filter the contents. Take 30 ml of the filtrate and add approximately 0.6 g of cupric carbonate in beaker (A)
4. In another beaker (B), take 34 ml of 0.2 M NaOH + 6 ml of FeCl₃ (0.41 M) and swirl the contents.
5. Add the contents in the beaker (B) to beaker (A) and again mix it slowly by swirling movements.
6. Filter the contents through Whatman No.1 filter paper.
7. Take 40 ml of the filtrate in a 250 ml separating funnel.
8. Add 40 ml of (0.03 %) H₂SO₄ and 10 ml of chloroform. Mix it slowly.
9. Collect the chloroform layer in a 100 ml beaker, add again 10 ml of chloroform, mix thoroughly, allow to settle and collect the chloroform in the same 100 ml beaker.
10. In a second separating funnel, take 40 ml of 0.02 M KOH and 1 % KCl mixture.
11. To this, add the collected 20 ml chloroform extract. Mix it slowly and collect the layer through anhydrous sodium sulphate bed drop by drop to remove any traces of moisture.
12. Keep the chloroform extract in an oven at 50°C till it becomes dry.
13. The dry aflatoxin film is re-diluted with 0.2 ml chloroform and spot on the TLC plate taking exactly 5, 10, 20 and 40 µl besides the standard spots of 5 µl and 10 µl.

Preparation of aflatoxin standard

Carefully prepare the given aflatoxin in a suitable standard flask with benzene: acetonitrile (98+2) mixture to give a concentration of 10 µg/ml. Standardize the concentration of the stock solution using spectrophotometer. From this stock solution, prepare aflatoxin solution in benzene:acetonitrile containing 4 µg/ml in a suitable standard flask which is the working standard. After spotting the standards and sample, develop the spots in an unsaturated developing tank containing chloroform: acetone: water in the ratio 88:12:1. After developing three fourth of the plate, the plate is carefully removed from the tank, dried well and viewed in a UV cabinet viewer using long wavelength (364 nm)

Calculation

$$\text{Aflatoxin content in ppb} = \frac{S \times C \times d}{T \times 1.714} \times 1000$$

Where,

S = Standard which compares with the sample in fluorescent intensity

C = Concentration of the standard

d = Dilution factor

T = Sample which compares with standard in fluorescent Intensity

1.714= Effective weight

Reference: Modified AOAC Official Method 975.36, Romer mini column method.

16.0 VITAMIN ANALYSIS

16.1 Determination of vitamin A – Using HPLC

Principle

The sample is saponified with ethanolic potassium hydroxide solution and the vitamin A is extracted into light petroleum. The light petroleum is removed by evaporation and the residue is dissolved in 2-propanol. The vitamin A concentration in the 2-propanol extract is determined by reverse-phase liquid chromatography using conditions that give a single peak for all retinol isomers.

Reagents

- Water, HPLC grade
- Potassium hydroxide solution (Dissolve 500 g of KOH in water and dilute to 1 litre)
- Ethanol- 95%
- 2-Propanol
- Light petroleum (40-60°C)
- Vitamin A standard
- All-trans-retinyl acetate, vitamin A acetate ($C_{22}H_{32}O_2$), 328.5 g/mol, with a purity of at least 90%.
- All-trans-retinol, vitamin A alcohol ($C_{20}H_{30}O$), 286.5 g/mol, with a purity of at least 90%
- Methanol, HPLC grade
- Mobile phase for liquid chromatography: Mix together methanol and water (770 + 30 v/v)
- Sodium sulfate (Na_2SO_4) anhydrous
- Sodium ascorbate solution, $p = 100$ g/l.

Apparatus

- High-performance liquid chromatograph (Column: length 250 mm, ID 4.6 mm, packed with a stationary phase consisting of octadeca (C_{18}) groups bonded to silica).
- UV-Visible spectrometer
- Boiling water bath
- Rotary vacuum evaporator
- Extraction apparatus
- Membrane filter-0.45 μm pore size

Procedure

Saponification

- Weigh about 0.1 g sample in 1 litre conical flask.
- Add 200 ml of ethanol. Swirl the flask.
- Add 2 ml of sodium ascorbate and 50 ml of potassium hydroxide solution and mix by swirling.
- Fit a reflux condenser to the flask and immerse the flask in the boiling water bath.
- Allow the contents of the flask to reflux for 60 min.
- Remove and cool the flask to room temperature as rapidly as possible under a stream of cold water.

Extraction of vitamin A (retinol)

- Transfer the contents of the flask to extraction cylinder.
- Rinse the flask with two 25 ml portions of ethanol and transfer the rinsing to cylinder.
- Repeat the rinsing of the flask with two 125 ml of petroleum and one 250 ml of water, each time transferring the rinsing to the cylinder.
- Stopper the cylinder and shake well for 1 min.
- Cool under a stream of cold water while waiting for the two liquid phases to separate, before removing the stopper.

When the layers have separated, remove the stopper, wash the sides of the stopper with a few ml of light petroleum and insert the adjustable tube, positioning the lower open end so that it is just above the level of the interface. By application of a slight pressure of inert gas to the side-arm tube, transfer the upper, light petroleum layer to a 1 litre separating funnel.

- Add 125 ml petroleum to cylinder and shake for 1 min.
- Allow the layers to separate and transfer the upper layer to the separating funnel using the adjustable tube. Repeat above step again.
- Wash the combined light petroleum extracts with four 100 ml portions of water using at first only gentle inversion then only gentle shaking in order to keep emulsion formation to a minimum.
- Transfer the washed extract through a medium/fast filter paper containing 60 g of anhydrous sodium sulfate, into a flask suitable for vacuum evaporation.
- Rinse the separating funnel with two 20 ml of light petroleum and add rinsing, through the filter to the evaporation flask.
- Wash the filter further with two 25 ml of light petroleum and collect the washings in the evaporation flask.
- Evaporate the petroleum extract to dryness under vacuum at a temperature not exceeding 40°C.
- Restore atmospheric pressure by admitting inert gas.

High performance liquid chromatography

- Dissolve the residue in the minimum volume of 2 propanol and transfer quantitatively to a 20 ml volumetric flask.
- Rinse the evaporation flask with three small portions of 2-propanol, transferring the rinsing to the volumetric flask. Dilute to volume with 2 propanol and mix and inject 10 μ l of the sample extract into HPLC.
- Calculate the peak area.

Note: 1 IU of vitamin A is equal to 0.300 μ g of all-trans-retinol.

1 IU of vitamin A is equal to 0.344 μ g of all-trans-retinyl acetate.

Hydrolysis of all-trans-retinyl acetate for calibration

- Prepare a solution of all-trans-retinyl acetate in ethanol, so that 1 ml contains approximately 15000 IU of vitamin A.
- Transfer 2.5 ml of this solution to a 150 ml flask.
- Add 20 ml of ethanol, 1 ml of potassium hydroxide and 5 ml of sodium ascorbate solution.
- Fit a condenser to the flask. Immerse the flask in the boiling water bath and allow to reflux for 60 min.
- Cool the flask to room temperature under a stream of cold water and transfer the contents to a separating funnel.
- Rinse the flask with 50 ml of water, followed by 25 ml of ethanol, adding the rinsing to the separating funnel.

- Extract the aqueous phase with one 80 ml of petroleum and then with two 50 ml portions of light petroleum.
- Combine petroleum extracts, and then wash with two 50 ml portions of water. Add 2 g of anhydrous sodium sulfate.
- Transfer petroleum extract to 250 ml volumetric flask and dilute to volume. The retinol concentration of this solution (solution 1) is approximately 150 IU/ml.

Standardization of retinol solution for calibration

- Pipette 5 ml solution 1 into a 50 ml volumetric flask and remove the solvent, at ambient temperature, with a stream of inert gas.
- Dissolve the residue in 2-propanol, and then dilute to volume with 2-propanol.
- Measure the absorbance (A) of the solution, using 2-propanol as reference, at wavelengths of 310 nm, 325 nm and 334 nm. The absorbance values will be approximately 0.7 to 0.8. If necessary, an intermediate dilution may be used.

Using the following equation, calculate the corrected absorbance at 325 nm:

$$A_{325,corr} = 6.815 \times A_{325} - 2.555 \times A_{310} - 4.26 \times A_{334}$$

If $A_{325,corr} / A_{325}$ is less than 0.97, use the value of $A_{325,corr}$ for the standardization; otherwise use A_{325}

The retinol concentration of solution 1 is given by :

$$\begin{aligned} \text{Concentration (IU/ml)} &= A_{325} \times 183 \text{ IU/ml, or} \\ \text{Concentration (IU/ml)} &= A_{325,corr} \times 183 \text{ IU/ml.} \end{aligned}$$

Preparation of a retinol standard for chromatography

- Prepare a retinol solution in 2-propanol. For each 1000 IU of vitamin A/kg of sample, a retinol concentration of 2.5 IU/ml is expected in the extract.
- Evaporate an aliquot volume of solution 1 to dryness at ambient temperature with a stream of inert gas. Dissolve the residue in the appropriate volume of 2-propanol to provide the required retinol concentration and mix it.
- If necessary, filter the standard solution through a membrane filter.
- For calibration purposes, a standard solution of vitamin A (retinol) in 2-propanol prepared by diluting a stock standard solution of all-trans-retinol made by dissolving an appropriate quantity of all-trans-retinol standard substance directly in 2-propanol (5.4) may be used.
- In this case, check the vitamin A standard by measuring the absorbance of the standard solution in quartz cells at wavelengths of 300 nm, 325 nm, 350 nm and 370 nm against 2-propanol as reference. Determine the A/A325 ratio at each of the wavelengths for all-trans-retinol. If the ratio does not exceed 0.602, 0.432 and 0.093 at 300 nm, 350 nm and 370 nm respectively, the standard substance is suitable for use.

Calculation

Calculate the numerical value of the vitamin A content of the test sample by the equation.

$$wA = 20,000 \times \frac{c}{m}$$

where,

wA is the numerical value of the vitamin A content of the test sample, in IU/kg.

c is the numerical value of the retinol concentration of the extract, in IU/ml.

m is the numerical value of the mass of the test sample, in grams.

Reference: Indian Standards, IS 15120 : 2002.

16.2 Simultaneous estimation of vitamins A & E – HPLC method

Reagents

- Absolute alcohol
- Potassium hydroxide (AR grade)
- Distilled water
- Petroleum ether (40-60°C)
- Acetonitrile HPLC grade
- Tetrahydrofuran HPLC grade
- Water HPLC grade
- Nitrogen gas
- Vitamin A and E standards

Apparatus

- Amber colored conical flask
- Amber colored vial
- Separating funnel
- Water bath shaker

Procedure

Extraction

- Saponify about 1 g feed sample with 10 ml of 95% ethanol and 2 ml of 60% KOH in 50 ml conical flask.
- Keep the content on the boiling water bath till one or two bubbles appears.
- Thereafter, cool by ice bath for 5 minutes and add 10 ml of petroleum ether.
- Shake the contents for 15 minutes in water bath at 39°C and collect the extract in the vial.
- Repeat the same for three times.
- Collect pooled ether extract and treat with 10 ml of 0.5 N KOH in a separating funnel (Fig. 16.1).
- Wash the solution with distilled water for three times to remove the excess alkali.
- Finally collect ether and filter through sodium sulfate. Dry the ether extract under nitrogen gas.
- Residue is ready for injection to HPLC.

Operating conditions

Mobile phase	- Acetonitrile: Tetrahydrofuran: Water (47:42:11)
Flow rate	- 1.5 ml/min
Column	- C 18 ODS (150 x 40 mm)
Oven temperature	- 17°C
Detector	- UV/PDA
Wave length	- 325 nm (vit A)
Wave length	- 290 nm (vit E)
Total run time	- 6 minutes.

Calculations

$$\text{Vitamin A/E } (\mu\text{g/g}) = \frac{V_e \times S_A \times S_dC}{V_i \times S_dA} \times \text{purity of vitamin}$$



Fig. 16.1 Separating funnel

Where,

- Ve - Volume in which the dried was dissolved
- SA - Sample area from peak
- SdC - Standard concentration (Vit A/E)
- Vi - Volume injected
- SdA - Standard area from the peak

Reference: Rajiv Chawla and Harjit Kaur 2001. Isocratic HPLC method for simultaneous determination of beta carotene, retinol and alfa tocopherol in feeds and blood plasma. *Ind. J.Dai.Sci.* 54: 84-90.

16.3 Estimation of vitamin D₃ – HPLC method

Reagents

- Diethyl ether
- Acetone
- Dichloromethane
- Methanol
- Di-sodium hydrogen orthophosphate
- Acetonitrile HPLC grade
- Nitrogen gas
- Vitamin D₃ standard

Apparatus

- Beaker
- Amber colored vial
- Freezer

Procedure

Extraction

- Add about one gram feed sample with 5 ml diethyl ether and keep it in freezer with acetone containing beaker till the lower portion freezes.
- Collect the supernatant in another vial.
- Repeat the same for three times.
- Add 4 ml mixture of dichloromethane and methanol (3:1) in the collected supernatant of each vial.
- Collect again supernatant from above mixture and treat with 0.1 M of phosphate buffer.
- The supernatant of ether extract is collected and dried under nitrogen gas.
- Residue is ready for HPLC injection.

Operating conditions

- | | |
|------------------|-------------------------|
| Mobile phase | - Acetonitrile (100%) |
| Flow rate | - 2 ml/min |
| Column | - C 18 ODS (150 x 40mm) |
| Oven temperature | - 25°C |
| Detector | - UV/PDA |
| Wave length | - 265 nm |
| Total run time | - 10 minutes. |

Calculation

$$\text{Vitamin A/E } (\mu\text{g/g}) = \frac{V_e \times SA \times SdC}{V_i \times SdA} \times \text{purity of vitamin}$$

Where,

V_e - Volume in which the dried was dissolved

SA - Sample area from peak

SdC - Standard concentration (Vit A/E)

V_i - Volume injected

SdA - Standard area from the peak

Reference: Vitamin Analysis for the Health and Food Sciences. By Ronald R Eirenmiller, W.O.Landen 1998.

17.0 ESTIMATION OF ORGANOCHLORINE PESTICIDE RESIDUES – GC METHOD

Reagents

- Acetonitrile
- Distilled water
- Petroleum ether (40-60°C)
- Sodium chloride (AR grade)
- All standards from Sigma.

Apparatus

- Conical flask
- Separating funnel
- Graduated cylinder
- Florisil column
- Blender

Procedure

Extraction

- Blend about 50 g sample with 350 ml acetonitrile: water mixture (65:35) for 5 minutes at high speed, (the addition of water to acetonitrile increases the extractability of organochlorine pesticide residues in dry fodders and straws) and filter with the help of filter paper (Whatman No.1) into 500 ml conical flask.
- Transfer the filtrate to 250 ml graduated cylinder and record the volume.
- Transfer the filtrate into 1000 ml separating funnel.
- Measure 100 ml petroleum ether and pour into the separating funnel.
- Shake vigorously (1-2 mts) and add about 10 ml saturated sodium chloride solution and 600 ml of water mixed gently and thoroughly.
- Allow to separate.
- Discard aqueous layer and washed the solvent layer twice with 100 ml portions of water. Discard washings.
- Transfer the solvents to 100 ml stoppered graduated cylinder and recorded the volume.
- This solvent is concentrated to 5-10 ml before it is passed through the column.
- Extract the concentrated mixture and feed ingredients for OCPRs along with fat by the soxlet extraction. About 0.5 g extracted fat was dissolved in 5 ml portion of petroleum ether and passed through a column containing florisil for separation.

Clean up of the residue

The extract is ready for column chromatography, on partially deactivated florisil cartridges (Bond elut @ Varian).

Gas chromatographic analysis

The cleaned up residues were measured using a gas chromatograph equipped with ECD (Fig. 17.1).

Column - Equity TM -5, 30 m x 0.25 mm ID x 0.25 micro m



Fig. 17.1 Pesticide residues estimation by GC

Operating conditions

Injection port temperature	- 225°C
Column oven temperature	-100°C (2 min), 15-160°C/min, 5-300°C/min (10min)
Detector temperature	- 310°C
Carrier gas	- Helium, 30 cm/sec @ 100°C
Total run time	- 35 min.

$$\text{Pesticide residues } (\mu\text{g/l}) = \frac{A \times B \times C \times D}{E \times F \times G}$$

Where,

- A - ng of standard pesticide
- B - Peak area of sample
- C - Extracted volume (μl)
- D - Dilution factor
- E - Peak area of standard
- F - Volume of extract injected (μl)
- G - Volume of sample extracted (ml)

Reference: AOAC (1997) 16th edition.

18.0 ESTIMATION OF METHANE EMISSION IN RUMINANTS BY SULFUR HEXAFLUORIDE TRACER TECHNIQUE

The sulfur hexafluoride (SF_6) tracer technique was developed at Washington State University, USA by Johnson *et al.* (1994). In this technique, a small permeation tube containing sulfur hexafluoride is placed into the rumen. SF_6 release rate of permeation tube is determined prior to the placement in the animal. A halter fitted with a capillary tube is placed on the animals head and connected to an evacuated canister. As the vacuum in the sampling canister slowly dissipates a steady breath sample is taken. After collection of the sample (Fig. 18.1), canister is pressurized with nitrogen gas and analyzed for methane and SF_6 concentration using gas chromatograph.

Methane emission rate is calculated as the product of the permeation tube emission rate and the ratio of methane to SF_6 in the sample.

Equipments & parts required

- Gas chromatograph equipped with flame ionization detector (FID) and electron capture detector (ECD)
- Methane standards
- Sulfur hexafluoride standards
- Molecular sieve 5A column
- Porapak N column
- Pure sulfur hexafluoride gas
- Nitrogen gas
- Water bath
- Hot air oven
- Vacuum pump
- Pressure gauge
- Liquid nitrogen



Fig. 18.1 Collection of breath samples

Preparation of permeation tube & determination of SF_6 release rate:

Parts required

- Brass tube body
- Teflon window
- 9/16" wrench + locking pliers
- Stainless steel frit (2μ)
- Glass receptacle in 39°C water bath
- 1/4 " Swagelok TM nut
- Pure SF_6 gas
- Liquid nitrogen
- Nitrogen gas

Procedure

- Drill a 3/16" hole in a 1.25" x 7/16" O.D. brass rod to a depth of about 1" in one end of the rod to make SF_6 cavity.
- On the open end, thread the outside of the rod to allow attachment of a 1/4" swage lock nut along with a thin teflon window and a stainless steel frit and put an identification at the bottom of the rod.
- Weigh the assembled empty permeation tube.

Note:

- The thickness and type of teflon dictate the permeation rate.
- Teflon of 12 mm thickness and a 2 μ frit will normally provide SF₆ permeation rates in the range of 1000-2000 ng/min at 39°C.

Filling of SF₆ in permeation tube

- Weigh the assembled empty permeation tube (including swage lock nut, teflon window and stainless steel frit).
- Remove the nut, teflon window and frit.
- Immerse the tube body in liquid nitrogen and allow it to reach cryogen temperature.
- Remove the tube from liquid nitrogen and quickly fill with SF₆ and cap it with teflon window, frit and swage lock nut (before filling any liquid inside the tube is poured out of the cavity).
- Weigh the permeation tube after filling (Fig. 18.2).



Fig. 18.2 Permeation tube

Determination of SF₆ release rate of permeation tube

- Place the SF₆ filled permeation tube in a glass receptacle in a water bath (at 39°C) and maintain a flow of clean N₂ gas to purge the glass receptacle of SF₆ emissions.
- Weigh the tubes at weekly intervals to determine the release rate of SF₆.
- After six weeks, calculate the SF₆ release rate.

Construction of breath sampling apparatus

- Breath sampling apparatus consists of collection canister, a modified halter and capillary tubing.

Halter construction

- Halter size depends on the size of the animal. The size of the halter is very important, as the location of the inlet over the nostril is critical to the success of sampling (Fig. 18.3).
- Punch new holes in the halter straps for smaller animals to ensure a snug fit to the nose band.
- Rivet or sew a leather flap to the halter nose band to provide support for the capillary tube inlet.



Fig. 18.3 Halter

Capillary tubing

- The length of the capillary tubing regulates the sampling rate.
- Stainless steel tubing with an inside diameter of 0.005" and an outside diameter of 1/16" serves as the flow restrictor and transfer line.
- To determine the sampling rate of a piece of capillary tubing connect it to an evacuated canister and allow it to fill for several hours while periodically checking the pressure. Calculate the fill rate and compare it to the desired length of time.
- The canister should fill to approximately 1/2 atmospheric pressure after the desired collection time has completed. Filling to 1/2 atmospheric pressure ensures the fill rate is a linear and constant one.

- Capillary tubing has been found to be very different both within and among different lots. It is necessary to check all fill rates prior to sampling.
- After the appropriate length of capillary tubing has been selected and tested, attach a 50 micron filter to the upstream end of the capillary tube. This will also require a 1/8" to 1/16" reducing union and appropriate swage lock.
- The purpose of the filter is to protect the capillary tubing from filling with dust and debris. Attach the filter with leather noseband so that the filter and tubing on the end will be located on the above nostril of the animal.
- To the downstream end of the tubing install another 1/8" to 1/16" reducing union and connect it to 1/8" PTFE tubing. Check all fittings for fit and tightness. Run the capillary tubing up the side of the halter and tape it into the place with electrical tape. If the capillary tubing is longer than side of the halter carefully coil it up tape the coil into place.

Parts required

- Cow halter with adjustable chin straps
- Piece of leather with rivets
- 2.0 cm piece of tygon tubing
- 1.0 cm piece of 1/8" PTFE tubing
- 50 micron filter
- 1/8"-1/16" reducing union
- 1/16" stainless steel capillary tubing
- 1/8" PTFE tubing
- 1-male quick connect
- Assorted ferrules and swage lock nuts

Construction of PVC collection canister

- A PVC pipe of 2-2.5" ID and 200 psi pressure (6 kg/cm³ pressure) rating is satisfactory for canister construction.
- PVC end caps (10 kg/cm³ pressure) and a 90° elbow are used to seal the sample canister.
- To construct a canister, a 6 cm ID and 200 psi pressure (6 kg/cm³ pressure) PVC pipe (6 kg/cm³ pressure), should be cut into 31-33 cm pieces.
- Wash the inside and outside of the pipe and fittings with warm soapy water allow them to dry.
- Glue the two end PVC end caps into a 90° elbow and the end caps to the open end of the PVC pipes using PVC glue.
- Wipe of excess glue and dry it for 12-24 hrs.
- Place the entire assembly into 120-135°C ovens for 5-10 minutes. Keep checking the pipe by squeezing it until it is soft and pliable. Remove the pliable pipe and bend the legs into the desired position.
- The final dimensions of the canister depend on the size of the animal being sampled.
- For average sized cows an 8" space between the ends of the pipe is adequate. If the canister does not have the desired shape, reheat it and begin again.
- If the pipe collapses on itself it is not hot enough.



Fig. 18.4 Preparation of canister

- To install gas sampling valve, tap the canister elbow, with 0.25" pipe thread. Install the gas sampling valve by gently screwing it into the threaded hole.
- A short (4") piece of 1/4" teflon tubing is attached to the valve with a female 1/4" quick connect on the upstream end to allow attachment to the halter.
- Wrap the legs in tape for safety purposes against the possibility of implosion.
- After assembling, the canister (Fig. 18.4) can be checked for leaks by pressurizing it with compressed air or nitrogen to 0.4 bar and then submerging it under water.

Parts required

- Two 31-33 cm length pieces of PVC pipes (2.0-2.5" ID & 6 kg/cm³ pressure)
- 2 end caps(10 kg/cm³ pressure, 2")
- 90° elbow
- 1 litre PVC Glue
- Hot air oven (120-135°C)
- Gas sampling valve
- Swage lock fittings
- Teflon tubing
- Female quick connect
- Tape, packaging
- compressed air or nitrogen
- Water tub
- Araldite –one Tube

Dilution system

- A dilution system is necessary to pressurize the sample with nitrogen gas.
- In order to begin sampling, the canister should be in negative pressure, so that a steady breath sample is taken inside the canister.
- First evacuate the canister and create negative pressure by using a vacuum pump and record the pressure.
- At the time of collection, a halter fitted with a capillary tube is placed on the animals head and connected to an evacuated canister. As the vacuum in the sampling canister slowly dissipates, a steady breath sample is taken inside the canister.
- After the collection period is over, record the pressure of canister.
- Then connect the canister to the dilution system and slowly add nitrogen gas until the pressure in the canister is increased to about 1.2 atmospheric pressure and record the final pressure and calculate the dilution factor. (Sample dilution is done to bring the contents of the canister under positive pressure which enables easy transfer of an aliquot of the sample to GC systems).
- Attach canister directly to the gas sampling valve in the GC via quick connect fittings. Simply opening the canister valve will allow sample transfer to the fixed volume loop in the GC.

Required parts

- Nitrogen gas cylinder with regulator
- Regulator valve
- Pressure gauge
- Copper tubing
- Male quick connect
- Vacuum pump

Methane analysis

- Methane concentration in the sampling canister is determined by gas chromatography (Fig. 18.5).



Fig. 18.5 Breath sample analysis using GC

- The GC system consists of a 1 ml sampling loop attached to a low dead volume gas sampling valve, a 1/8" x 4' stainless steel packed with porapack N and flame ionization detector (FID).
- GC oven is maintained at about 50°C and detector at 150°C for methane analysis.
- Each analysis can be completed in less than one minute.
- Triplicate analysis should exhibit reproducibility of less than 2% or more.
- The GC oven should be conditioned to 150°C for several hours (overnight) prior to the next analysis period.

SF₆ analysis

- SF₆ is measured using a gas chromatograph equipped with an electron capture detector (ECD).
- The GC system consists of a 1.0 ml sampling loop attached to a low dead volume gas sampling valve, a 1/8" x 6' stainless steel packed with molecular sieve 5A column and a electron capture detector (ECD).
- With a column temperature of 50°C, SF₆ elutes in less than one minute and prior to oxygen peak.

Calculations

The tracer method utilizes SF₆ to account for dilution as gases exiting the cow's mouth mixed with ambient air. It is assumed that the SF₆ emission exactly stimulates the CH₄ emission and thus the dilution rates of SF₆ and CH₄ are identical. The methane emission rate (Q_{CH_4}) can then be calculated from measured CH₄ and SF₆ concentrations and the known release rate of SF₆ (Q_{SF_6}):

$$Q_{CH_4} = Q_{SF_6} \times [CH_4] / [SF_6]$$

Background concentrations of CH₄ and SF₆ should be subtracted from the concentration measured in the sampling canister.

Reference: Johnson, K. A., Huyler, M.T., Westberg, H. H., Lamb, B. R. and Zimmerman, P.1994. Measurement of methane emissions from ruminant livestock using a SF₆ tracer technique. *Environmental Science and Technology*, 28:359-362.; Johnson, K. A., Westberg, H. H., Michal, J. J. and Cossalman, M. W. 2007. The SF₆ tracer technique: methane measurements from ruminants. In: *Measuring Methane Production from Ruminants*, (eds. Makkar, H. P. S. and Vercoe, P. E.), IAEA, Vienna, pp. 33-67.

REFERENCES

- Acharya N. G. Ranga Agricultural University, Hyderabad. A book on Feed Processing. National Agricultural Technology Project (ICAR), New Delhi.
- Applied Animal Nutrition Research Technique by Raman Malik and S. K. Sirohi. Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal – 132 001, Haryana (India).
- Ashes J. R., Mangan J. L. and Sidhu G. S. 1984. Nutritional availability of amino acids from protein cross-linked against degradation in the rumen. *The British Journal of Nutrition* 52, 239-247.
- Association of Official Analytical Chemists, 1980. *Official Methods of Analysis*. 12th Edition, AOAC, Box 540, Benjamin Franklin Station, Washington DC 20044.
- Benke, K. C. 1994. Factors affecting pellet quality. Proc. Maryland Nutrition Conference. Dept. of Poultry Science and Animal Sciences, College of Agriculture, University of Maryland, College Park.
- BIS, Bureau of Indian Standards, New Delhi, India.
- C. Reed Richardson. The Centre for Feed Industry Research and Education. Department of Animal Science and Food Technology, Texas Tech University, Lubbock, Texas.
- Current issues in feed manufacturing (II): Conditioning controls pellet quality. *Feed Tech* 10.6.2006, www.Agreworld.nl.
- Feed manufacturing technology: Current issues and challenges by Keith C. Behnke, Kansas State University, Manhattan, KS.
- Ferguson K. A., Hemsley J. A. and Reis P. J. 1967. Nutrition and wool growth. The effect of protecting dietary protein from microbial degradation in the rumen. *Australian Journal of Science*, 30: 215-217.
- Improving pellet quality and efficiency by Matthias Franke and Andreas Rey, Buhler AG Uzwil, Switzerland, *Feed Tech* 10.3.2006, www.Agreworld.nl.
- Jim Balthrop, Benedikt Brand, Richard A. Cowie, Jugen Danier, Johan De Boever, Leon de Jonge, Felicity Jackson, Harinder P. S. Makkar and Chris Piotrowski. 2011. *FAO Animal Production and Health manual on quality assurance for animal feed analysis laboratories*.
- Khajarn, J. and Khajarn, S. 1998. Quick quality tests for protein meals. *Technical Bulletin American Soybean Association*.
- Laboratory manual for nutrition research by Gopal Krishna and S. K. Ranjhan. 1980.
- Off-the-shelf output transforms into tailor-made production by Harm Klein, Tebodin CCE, Consultants and Engineers, Deventer, the Netherlands. *Feed Tech* 10.10.2006, www.AllAboutFeed.net
- Processing: Moisture control in practice by Dick Ziggers, *Feed Tech* 12.4.2008, www.AllAboutFeed.net
- Source: http://www.hydrnix.com/applications/moisture_in_animal_feed.php
- Tilley, J. M. A. and Terry, R. A. (1963). *J. Brit. Grassld. Soc.* 18: 104-111.
- Uppal, D. S., Ilyas, S. M. and Sikka, S. S. *Quality and Safety of animal feed in India*.

PART - III

APPENDICES

APPENDIX - I

IS 2052 : 2012

Indian Standard

COMPOUNDED FEEDS FOR CATTLE – SPECIFICATION

(Fifth Revision)

Sr. No.	Characteristic	Requirement		
		Type I	Type II	Type III
1	Moisture, percent by mass (Max.)	11	11	11
2	Crude protein (N x 6.25), percent by mass (Min.)	22	20	18
3	Crude fat, percent by mass (Min.)	4.0	2.5	2.0
4	Crude fibre, percent by mass (Max.)	10	12	15
5	Acid insoluble ash, percent by mass (Max.)	3.0	4.0	5.0
6	Salt (as NaCl), percent by mass (Max.)	1.0	1.0	1.0
7	Calcium (as Ca), percent by mass (Min.)	0.8	0.8	0.8
8	Total phosphorus, percent by mass (Min.)	0.5	0.5	0.5
9	Available phosphorus, percent by mass (Min.)	0.25	0.25	0.20
10	Urea, percent by mass (Max.)	1.0	1.0	1.0
11	Vitamin A, IU/kg (Min.)	7000	7000	7000
12	Vitamin D ₃ IU/kg (Min.)	1200	1200	1200
13	Vitamin E, IU/kg (Min.)	30	30	30
14	*Aflatoxin B ₁ (ppb) (Max.)	20	20	20

Note: The values for requirements at Sr. No. 2 to 14 are on moisture-free basis.

For routine analysis, the characteristics mentioned at Sr. No. 1 to 5 may be tested by near infrared analyzer. However, in case of dispute, the method given in IS 7874 (Part I) shall be the referee's method.

*In case of dispute, HPLC method shall be the referee's method.

APPENDIX - II

IS 1664 : 2002

Indian Standard**MINERAL MIXTURES FOR SUPPLEMENTING CATTLE FEEDS – SPECIFICATION**

(Fourth Revision)

Sr. No.	Characteristic	Requirement	
		Type 1	Type 2
1	Moisture, percent by mass (Max.)	5	5
2	Calcium, percent by mass (Min.)	16	20
3	Phosphorus, percent by mass (Min.)	9	12
4	Magnesium, percent by mass (Min.)	4	5
5	Salt (chlorine as NaCl), percent by mass (Min.)	22	-
6	Iron, percent by mass (Min.)	0.3	0.4
7	Iodine (as KI), percent by mass (Min.)	0.020	0.026
8	Copper, percent by mass (Min.)	0.078	0.100
9	Manganese, percent by mass (Min.)	0.10	0.12
10	Cobalt, percent by mass (Min.)	0.009	0.012
11	Fluorine, percent by mass (Max.)	0.05	0.07
12	Zinc, percent by mass (Min.)	0.64	0.80
13	Sulphur, percent by mass	1.4 - 2.3	1.8 – 3.0
14	Acid insoluble ash, percent by mass (Max.)	2.4	3.0
15	Lead (as Pb), mg/kg (Max.)	16	20
16	Arsenic (as As ₂ O ₃), mg/kg (Max.)	5	7
17	Total ash, in moisture-free sample, percent by mass	81 – 88	78 – 85
18	Presence of proteinous/ organic impurities	Shall pass the test	Shall pass the test

Note: The values for requirement given in sr. No. 2 to 17 are on moisture-free basis.

APPENDIX - III

IS 5560 : 1970
(Reaffirmed 1980)
(Reaffirmed 2004)

Indian Standard

SPECIFICATION FOR COMPOUNDED FEEDS FOR YOUNG STOCK

Sr. No.	Characteristic	Requirement	
		Calf starter meal	Calf growth meal
1	Moisture, percent by weight (Max.)	10	10
2	*Crude protein (N x 6.25), percent by weight	23-26	22-25
3	Crude fat, percent by weight (Min.)	4.0	--
4	Crude fibre, percent by weight (Max.)	7	10
5	Total ash, percent by weight (Max.)	5.0	5.0
6	Acid insoluble ash, percent by weight (Max.)	2.5	3.5
7	Common salt, percent by weight (Max.)	1.0	1.5
8	Calcium (as Ca), percent by weight (Min.)	1.2	1.2
9	Phosphorus (as P), percent by mass (Min.)	0.8	0.8
10	Vitamin A, IU/kg	10000	5000
11	Total digestible nutrients, percent by weight (Min.)	70	65
12	Digestible crude protein, percent by weight (Min.)	17	16.5

Note: The values for requirement given in Sr. No. 2 to 12 are on moisture-free basis.

*While analyzing for crude protein, it should be ensured that the nitrogen has not been derived from urea or other ammonium salts.

*Indian Standard***BYPASS PROTEIN FEED FOR CATTLE – SPECIFICATION**

(First Revision)

Sr. No.	Characteritic	Requirement
1	Moisture, percent by mass (Max.)	11
2	Crude protein (N x 6.25), percent by mass (Min.)	22
3	Crude fat, percent by mass (Min.)	3.5
4	Crude fibre, percent by mass (Max.)	10
5	Acid insoluble ash, percent by mass (Max.)	3.0
6	Rumen undegradable protein (UDP), percent by mass (Min.)	14
7	Common salt (as NaCl), percent by mass (Max.)	1.5
8	Calcium (as Ca), percent by mass (Min.)	1.0
9	Available phosphorus (as P), percent by mass (Min.)	0.25
10	Vitamin A, IU/kg (Min.)	7000
11	Vitamin D ₃ IU/kg (Min.)	1200
12	Vitamin E, IU/kg (Min.)	30
13	*Aflatoxin B ₁ ppb (Max.)	20

Note: The values for requirement given in sr. No. 2 to 9 are on moisture-free basis.

*Aflatoxin limits shall be reviewed from year to year on production of Indian and international data.

APPENDIX - V

IS 13451 : 1992

Indian Standard

UREA MOLASSES BLOCK AS RUMINANT FEED SUPPLEMENT – SPECIFICATION

Sr. No.	Characteristic	Requirement
1	Moisture, percent by mass (Max.)	3.5
2	Crude protein (N x 6.25), percent by mass (Min.)	52.0
3	Crude fibre, percent by mass (Max.)	2.0
4	Total ash, percent by mass (Max.)	34.0
5	Acid insoluble ash, percent by mass (Max.)	3.0
6	Calcium (as Ca), percent by mass (Min.)	4.0
7	Phosphorus (as P), percent by mass (Min.)	1.5
8	Sulphur, percent by mass (Min.)	1.0
9	Urea, percent by mass (Max.)	15.0

Note: The values specified for characteristics 2 to 9 are on moisture-free basis.

APPENDIX - VI

IS 1162 : 1958

Indian Standard**SPECIFICATION FOR CANE MOLASSES**

Sr. No.	Characteristic	Requirement		
		Grade I	Grade II	Grade III
1	Density, in degree Brix at 27.5°C, Min.	85	80	80
2	Ash, sulphated, percent by mass (calculated for 100° Brix), Max.	14.0	17.5	17.5
3	Total reducing matter as invert sugar, percent by mass, Min.	50	44	40

APPENDIX - VII

Indian Standard

SPECIFICATION FOR LIVESTOCK FEED INGREDIENTS

Sr. No.	Livestock Feed Ingredients	IS Number	Reaffirmed Date
1	Cottonseed oil cake (second revision)	IS 1712:1982	Mar 2009
2	Decorticated groundnut oil cake (second revision)	IS 1713:1986	Apr 2011
3	Mustard and rape seed oil cake (second revision)	IS 1932:1986	Apr 2011
4	Sesamum oil cake (first revision)	IS 1934:1982	Mar 2009
5	Linseed oil cake (first revision)	IS 1935:1982	Mar 2009
6	Maize germ oil cake (first revision)	IS 2151:1985	Mar 2009
7	Maize gluten (first revision)	IS 2152:1972	Mar 2009
8	Maize bran (first revision)	IS 2153:1985	Mar 2009
9	Coconut oil cake (second revision)	IS 2154:1986	Apr 2011
10	Wheat bran (first revision)	IS 2239:1971	Mar 2009
11	Tur chuni	IS 3160:1965	Mar 2009
12	Gram chuni	IS 3161:1965	Mar 2009
13	Rice polish	IS 3163:1965	Mar 2009
14	Solvent extracted linseed oil cake (meal) (first revision)	IS 3440:1985	Mar 2009
15	Solvent extracted groundnut oil cake (meal) (first revision)	IS 3441:1982	Mar 2009
16	Solvent extracted coconut oil cakes (meal) (second revision)	IS 3591:1985	Mar 2009
17	Solvent extracted decorticated cottonseed oil cake (meal) (second revision)	IS 3592:1985	Mar 2009
18	Solvent extracted rice bran (second revision)	IS 3593:1979	Mar 2009
19	Rice bran (first revision)	IS 3648:1975	Mar 2009
20	Guar meal (first revision)	IS 4193:1986	Apr 2011

Sr. No.	Livestock Feed Ingredients	IS Number	Reaffirmed Date
21	Tur husk	IS 5063:1969	Mar 2009
22	Dicalcium phosphate, animal feed grade - (first revision)	IS 5470:2002	Mar 2008
23	Solvent extracted nigerseed oil cakes (meal)	IS 5862:1970	Mar 2009
24	Solvent extracted undecorticated safflower oil cake (first revision)	IS 6242:1985	Mar 2009
25	Parboiled rice bran	IS 9867:1981	Mar 2009
26	Mango seed kernels	IS 10671:1983	Mar 2010
27	Ambadi (<i>Hibiscus cannabinus</i>) cake	IS 10741:1983	Mar 2009
28	Malt sprouts	IS 11586:1986	Apr 2011
29	Mango seed kernel (solvent extracted)	IS 12829:1989	Mar 2009
30	Sunflower oil cake	IS 14702:1999	Mar 2009

APPENDIX - VIII

LIST OF CATTLE FEED PLANTS UNDER DAIRY COOPERATIVES

S.N.	Plant address	Milk Union / Federation
Gujarat		
1	Manager Cattle Feed Plant, Itola – 391 240, Dist: Vadodara Phone: 0265-2830052; Email: alpesh@barodaunion.coop	Managing Director Baroda District Cooperative Milk Producers Union Ltd., Baroda Dairy, Makarpura Road, Vadodara – 390 009 Phone: 2644422, 2641988; Fax: 2641206 E mail: amit@barodaunion.coop
2	Manager Sumuldan Factory, Chalthan, Surat – 394 305 Phone: 02622-281162/64 Email: mhk@sumul.coop (M): 075748 67522	Managing Director Surat District Cooperative Milk Producers' Union Ltd., Sumul Dairy, PB No. 501, Surat – 395 008 Phone: 0261-2537693-4/ 2538717, 2531666, 2531137; Fax: 0261-2533572 E mail: root@sumul.coop
3	Manager Cattle Feed Plant, Hazipur, P.B. No. 21, Himatnagar – 383 001, Dist: Sabarkantha	Managing Director Sabarkantha District Cooperative Milk Producers' Union Ltd., Sabar Dairy, Sub Post Boriya, Himatnagar – 383 001
4	Manager Cattle Feed Plant, Mehsana District Cooperative Milk Producers' Union Ltd., Mehsana-A'bad Highway, P.O. Jagudan, Ta. & Dist.: Mehsana – 382 710.	Managing Director Mehsana District Cooperative Milk Producers' Union Ltd., Dudhsagar Dairy, P.B. No. 1, Mehsana – 384 002 Phone: 02762-53201 to 05; Fax: 53422 E mail: amulmeh@guj.nic.in
5	Manager Cattle Feed Plant, Ubkhal, Ta: Vijapur, Dist: Mehsana	
6	Manager Cattle Feed Plant Banas Dairy, P.B. No. 20, Palanpur – 385 001	Managing Director Banaskantha District Cooperative Milk Producers' Union Ltd., Banas Dairy, PB No. 20, Palanpur – 385 001 Phone: 02742-253881 to 253885 Fax: 02742-252723
7.	Manager Cattle Feed Plant Katarva, Tal: Deesa, Dist.: Banaskantha	
8	Manager Cattle Feed Plant, Kanjari – Boriavi, Dist: Anand – 387325, Phone: 0268-278588	Managing Director Kaira District Cooperative Milk Producers' Union Ltd., Amul Dairy Road, Anand – 388 001 Phone: 02692-256124
9.	Manager Cattle Feed Plant, Kapdivav, Tal. Kapadvanj, Dist.: Anand	
10	Manager Cattle Feed Plant Sarkhej, Nr. Bavla Rly. Crossing, Dist: Ahmedabad Phone: 079-26891057	Managing Director Ahmedabad District Cooperative Milk Producers' Union Ltd., Uttam Dairy, Gomtipur, Ahmedabad – 380 021 Phone: 079-22749575
11	Manager Cattle Feed Plant, Khandia, Lunawada Road, Dist: Godhra Phone: 02670-225305 Email: ncp@panchmahalunion.coop (M): 099789 93955	Managing Director The Panchmahal District Cooperative Milk Producers' Union Ltd., Lunawada Road, PB No. 37, Godhra – 389 001 Phone: 260255,261782,83,84; Fax: 260327 E mail: root@panchamrut.guj.nic.in
12	Manager Cattle Feed Plant, Sagbara, Alipur – 396 409, N.H. No. 8, Ta: Chikhali, Dist: Navsari Phone: 02634-232763, 232846 Fax: 02634-23334	Managing Director Valsad District Cooperative Milk Producers' Union Ltd., Alipur – 396 409, N.H. No. 8, Ta: Chikhali, Dist: Navsari Phone: 02634-232423, 232761, 232762 E mail: vasudhara@vasudhara. Guj.nic.in

S.N.	Plant address	Milk Union / Federation
13.	Manager Cattle Feed Plant, Amreli Amreli District Cooperative Milk Producers' Union Ltd., Amar Dairy, Amreli 365 601	Managing Director Amreli District Cooperative Milk Producers' Union Ltd., Amar Dairy, Amreli 365 601
14.	Manager Cattle Feed Factory Khandheri, Rajkot-Jamnagar Road, Rajkot-360001	
Maharashtra		
15	Manager Mahalaxmi Cattle Feed Plant Gadmodshingi, Ta: Karvir, Dist: Kolhapur Phone: 0231-2615061, 2615062 Dr VD Patil (M): 094212 02788 Email: vdpatilgokul@yahoo.in	Managing Director Kolhapur Zilha Sahakari Dudh Utpadak Sangh Ltd., Gokul Prakalp, B-1, MIDC Area, Gokul Shirgaon, Kolhapur – 416 234 Phone: 2672311 to 2672315; Fax: 0231-2672374 E mail: kpr_gokul@sancharnet.in
16.	Manager Cattle Feed Plant B-3, Kagal-Hatkanangale 5 Star MIDC, Village: Halsvade, Ta: Karveer, Dist: Kolhapur	
17	Assistant Manager Vikas Cattle Feed Plant Nashirabad, Opp. Bhadli Rly. Station, Dist: Jalgaon - 425309 Phone: 0257-2356222	Managing Director Jalgaon Jilha Sahakari Dudh Utpadak Sangh Maryadit, PB No. 32, Shivaji Nagar Road, Jalgaon – 425 001 Phone: 0257-2226645; Fax: 0257-2225611 E mail: jjsdus_jal@sancharnet.in
18	Manager Cattle Feed Plant Shivamrut Dudh Utpadak Sahakari Sangh, Akluj, Dist: Solapur Phone: 0218-2522566, 2522126	Managing Director, Shivamrut Dudh Utpadak Sahakari Sangh Maryadit, Akluj, At: Vijayanagar (Vizori), P.O. Yeshwantnagar, Tal: Malshiras, Akluj – 413 118, Dist: Solapur Phone: Direct MD 522914; Fax: 0218-522617
19	Manager Cattle Feed Plant. Warananagar :416113, Kolhapur Phone: 02328-224181 to 187	Managing Director Shri Warana Sahakari Dudh Utpadak Prakriya Sangh Ltd., Tatyasaheb Korenagar, Warananagar – 416 113, Dist: Kolhapur
20	Manager Cattle Feed Plant Rajarambapu Patil Sahakari Dudh Sangh Ltd., Plot No. A/16 to 19, M.I.D.C., Islampur-415 409, Tal: Walwa, Dist: Sangli Phone: 02342-220136, 220179 Email: cattlefeed@krushnadudh.com	Managing Director Rajarambapu Patil Sahakari Dudh Sangh Ltd., Plot No. A/16 to 19, M.I.D.C., Islampur – 415 409, Tal: Walwa, Dist: Sangli
21.	Manager Cattle Feed Plant, Solapur Milk Union, Barshi, Dist.: Solapur-413401	Managing Director Solapur Zilha Sahakari Dudh Utpadak Sangh Maryadit, 24/1-A, Murarji Peth, Near Navbharat Ice Factory, Solapur – 413001
22.	Cattle Feed Plant, Malegaon Opposite Malegaon Engineering College, Tal: Baramati-413115 Dist: Pune, Tel: 02112-254258 E-mail: baramati_doodh@yahoo.co.in	Managing Director Baramati Taluka Sahakari Dudh Utpadak Sangh Maryadit, Kasba, Karbhari Circle, Nira Road, Baramati- 413 102, Dist.: Pune
Goa		
23	Manager Cattle Feed Plant, Marvasada, Usgaon, Goa Cooperative Milk Producers' Union Ltd., Curti Ponda – 403 401, Goa	Managing Director Goa State Cooperative Milk Producers' Union Ltd., Curti Ponda – 403 401, Goa Phone: 0832-2312247, 2313790, Fax: 2313790, E mail: goadairy@sancharnet.in

S.N.	Plant address	Milk Union / Federation
Madhya Pradesh		
24	General Manager Cattle Feed Plant, Manglia, Indore – 453 771 Phone: 0731-2806221, 4021405 Email: sudana_manglia@rediffmail.com	Managing Director Indore Dudh Sangh Sahakari Maryadit, Chanda Talawali, Indore – 453 771 Phone: 0731-2802535/2802554/2802553/4021401 Fax: 0731-2802559
25	Manager Cattle Feed Factory, Pachama, Dist: Sehore – 466 001 Phone: 07562-221132/222783/ 221135 (M): 094069 00330 Email: cff.pachama@gmail.com	Managing Director Bhopal Dugdh Sangh Sahakari Maryadit, Habibganj, Bhopal – 462 024 Phone: 0755-466011/12/13/14 Fax: 0755-2580896
26	Manager Cattle Feed plant C/o-Sanchi Milk Chilling Plant, Sanchi Dugdh Dairy, Dist.: Bandol, Dist: Seoni-480 882 (MP), Email: cff.bandole@gmail.com	
27.	Manager Cattle Feed Factory Guna Naka, A.B. Road, Shivpuri (MP)-473 551 Mr Bharat Arora (M): 9406900751 E-mail: cffshivpuri@gmail.com	
28	Manager Cattle Feed Plant, Sironja, Sagar-470 002 Tel: 07582-272468 Email: cfpsagar@gmail.com	
Uttar Pradesh		
29	General Manager Cattle Feed Plant, Ramnagar, Ramnagar Industrial Estate, Varanasi-221008 Phone: 0542-2666327	Managing Director Pradeshik Cooperative Dairy Federation Ltd., (PCDF) 29, Park Road, Lucknow – 226 001 Phone: 0522-2236465, 2236035 Fax: 0522-2236472
30	General Manager Cattle Feed Plant, Gangol Road, Partapur, Meerut-250103, Phone: 0121-2514413	
31	Manager Cattle feed plant, Khanjanpur-262801 Dist: Ambedkar Nagar	
Uttarakhand		
32	Manager Cattle Feed Factory, Kichha Bye Pass Road, Rudrapur (U. S. Nagar)-263153 Phone: 05944-242552	Chief General Manager Uttaranchal Cooperative Dairy Federation Ltd., Magal Parao Haldwani, Nainital
Haryana		
33	General Manager Hafed Cattle Feed Plant, Nr. Sukhpura Chowk, Rohtak – 124 001, Phone: 01262-276908	Managing Director Hafed Cooperative Office Building, Sector-5, Panchkula – 134 109, Haryana
34	General Manager Hafed Cattle Feed Plant, Saktakhera, Sanaria Road, Dabwali, Sirsa 125 104	
Punjab		
35	Manager Cattle Feed Plant, Bhattian (Khanna) – 141 401, G. T. Road, P.B. No. 8, Dist: Ludhiana Phone: 01628-230752, 2231059/60	Managing Director The Punjab State Cooperative Milk Producers' Fed. Ltd., SCO No. 153-155, Sector 34-A, New City Centre, Chandigarh – 160 022
36	Manager Cattle Feed Plant, Ghania-Ke-Banger, P.B. No. 28, Batals – 143 505, Dist:Gurdaspur Phone: 01871-266449, 266741	

S.N.	Plant address	Milk Union / Federation
Rajasthan		
37	Manager Cattle Feed Plant, Rajasthan Cooperative Dairy Fed. Ltd., MIA, 2 nd Phase, Bansi, Jodhpur – 342 003, Phone: 0291-2741080	Managing Director Rajasthan Cooperative Dairy Federation Ltd., Saras Sankul, Opp. MRE College, JLN Marg, Jaipur – 302 017
38	Dy. Manager Cattle Feed Plant, Lalgarh, P.O.R.C.P., Bikaner-334002, Phone: 0151-2251615	
39	Manager Cattle Feed Plant, Beawar Road, Tabiji, Ajmer – 305 003 Phone: 0145-2681643, 2440945	
40	Dy. Manager Cattle Feed Plant, Nadbai Town, Dist: Bharatpur – 321 602, Phone: 05643-223530	
41	Cattle Fee Plant Lambiya Kalan, Bhilwara-311024 Tel: 01482-265228, (M): 094141 95932	
42	Manager Cattle Feed Plant, Kaladera SP 34 & 34A, RICCO Industrial Area – Kaladera – 303801, Dist.: Jaipur, (M): 095719 36000 (Dr RN Agarwal) Email: cfp.kaladera@gmail.com	Managing Director Jaipur Zila Dugdh Utpadak Sahakari Sangh Ltd., Near Gandhi Nagar Railway Station, Jaipur 302015
Andhra Pradesh		
43	Manager Cattle Feed Plant, K.V.R., Tenali Taluk, Vadlamudi – 522 213, Dist: Guntur, Phone: 08644-258226	Managing Director Guntur District Milk Producers' Mutually Aided Cooperative Union Ltd., Sangam dairy, Vadlamudi – 522 213
44	Manager Cattle Feed Plant, Bhongir, Nalgonda – 509 116 Phone: 08685-242538	Managing Director Nalgonda Ranga Reddy District Cooperative Milk Producers' Union Ltd., Mother Dairy, Hayatnagar, Hyderabad
45	Manager Feed Mixing Plant, Budhavaram – 521 101, Via: Vijayawada, Dist: Krishna	Managing Director The Krishna District Milk Producers Mutually Aided Cooperative Union Ltd., Vijayawada 520009, Phone: 0866-568461-67 Fax: 0866-564817, E mail: vijdairy@Satyam.net-in
46	Manager Feed Mixing Plant, Amadalawalasa – 532 185, Dist: Srikakulam	Managing Director Sri Vijayavishakha District Milk Producers' Mutually Aided Cooperative Union Ltd., Visakha Cooperative Dairy campus, Visakhapatnam – 530 012
47	Manager Feed Mixing Plant, Gudlavalleru-521356, Dist: Krishna	
48	Manager Feed Mixing Plant, Padmanagar, Karimnagar-505002	
49	Manager Feed Mixing Plant, Anakapalle-531001, Dist: Visakhapatnam	

S.N.	Plant address	Milk Union / Federation
50	Manager Feed Mixing Plant, Kovvuru-534350 Dist: West Godavari	
Tamil Nadu		
51	General Manager Cattle Feed Plant, Chennimalai Road, Erode – 638 001 Phone: 0424-2275358, Fax: 0424-2534150 E mail: cfp_erode@sancharnet.in	Managing Direct The Erode District Cooperative Milk Producers' Union Ltd., E.D. 296, Erode – 638 001
Karnataka		
52	General Manager Cattle Feed Plant, Rajanukunte, Singanayakapalli P.O., Bangalore – 560 064 Phone: 080-28468293, 28468295, 28468314 Telefax: 28468293, E mail: kmfcfpr@vsnl.net	Managing Director Karnataka Cooperative Milk Producers' Fed. Ltd., KMF Complex, Dr. Marigowda Road, Bangalore – 560 029
53	Manager Cattle Feed Plant, Gubbi, Nr. Rly. Station, Tumkur-572 216, Phone: 08131-222239, Fax: 08131-222239	
54	Manager Cattle Feed Plant, Rayapur, Dharwad – 580 009 Phone: 0836-2322671, 2322639. Fax: 0836-2322639	
55	General Manager Cattle Feed Plant, Post Box # 155, Gandhi Nagar, Holenarasipur Road, M. Hosakoppalu, Hassan – 573201 Phone: 08172-243369 Fax: 08172- 243172 E mail: kmfcfph@sancharnet.in	
56	Manager Cattle Feed Plant Shikaripura, Shivamogga District Co-operative Milk Producers' Societies' Union Limited. Shivamogga Dairy, Machenahalli, Nidige Post, Shivamogga - 577 222	
Kerala		
57	Manager Cattle Feed Plant, Malampuzha, Palakkad – 678 651 Phone: 0491-2815107, 2815170 Fax: 0491-2815176, E mail: cfm@milma.co.in	Managing Director Kerala Cooperative Milk Marketing Fed. Ltd. (MILMA), Milma Bhavan, Pattom palace-P.O., Thiruvananthapuram – 685 004 Phone: 0471-2555980 to 2555985 Fax: 2555992 E mail: milma@milma.com
58	General Manager Cattle Feed Plant, Pattankkad P.O., Alappuzha - 688 531 Phone: 0478-2562314, 2562355, 2565034 Fax: 2564125, E mail: cfp@milma.co.in	
59	Manager Cattle Feed Plant Kerala Feeds Limited., Kallettumkara 680 683, Thrissur District, Kerala Email: qc@keralafeeds.com	Managing Director Kerala Feeds Limited. Kallettumkara 680 683, Thrissur District, Kerala E-mail: kflproject@gmail.com

S.N.	Plant address	Milk Union / Federation
Pondicherry		
60	Manager Cattle Feed Plant, Pondicherry Cooperative Milk Producers' Union, Pondicherry-605 010 Phone: 0413-2371607;2371992	Administrator Pondicherry Cooperative Milk Producers' Union Ltd., Kurumampet, Pondicherry – 605 009
West Bengal		
61	Manager Cattle Feed Plant, Malaguri, P.O. Pradhannagar, Siliguri – 734 003, Phone: 0353-2517496	
62	Manager Cattle Feed Plant, Opp. MIT, P.O. Cossim Bazar, Raj, Berhampore, Dist: Murshidabad Phone: 03482-254517, 252407 Email: karmakarnirmal@ymail.com	Managing Director Bhagirathi Cooperative Milk Producers' Union Ltd., Feeder Dairy, Panchanantala PO, Berhampore – 742 101
63	Manager Kalyani Feed Milling Plant D-23, Industrial Area, Kalyani – 741 235 Dist:- Nadia (West Bengal)	
Bihar		
64	Office Incharge Cattle Feed Plant, Jagdeopath, Patna – 800 014, Phone: 0612-2223180	Patna Dairy Project Patna - 14
65	Manager Cattle Feed Plant Tirhut Dugdh Utpadak Sahkari Sangh Ltd. Near Railway Crossing, PO : Kanti Muzaffarpur – 843109	Tirhut Dugdh Utpadak Sahkari Sangh Ltd. Muzaffarpur Dairy, P.O. – Kolhua Paigambarpur, Muzaffarpur- 843108 Phone No- 7070992952, 7070992977
Jharkhand		
66	Manager Cattle Feed Plant, P.O. NeoriVikas Hotwar, Ranchi – 835 217 Phone: 0651-2275817;2275562 E mail: supersudha@cpran.com	Managing Director Bihar State Cooperative Milk Producers' Fed. Ltd., P.O. NeoriVikas Hotwar, Ranchi – 835 217
Odisha		
67	Manager Cattle Feed Plant, P.O.: Radhadamodarpur, At Radhakishorepur, Via: Khuntani, Athagarh, Dist: Cuttack – 754 029 Phone: 06723-232428/ (M): 094370 52603, Email: cattlefeedplantomfed@yahoo.in	Managing Director Cuttack District Cooperative Milk Producers' Union Ltd., 25-B, Mahanandi Vihar, Naya Bazar P.O., Cuttack – 753 004
68	Manager Cattle Feed Plant, Khurda OMFED Training & Demonstration Centre, AT-PO Jagannathpur, Via-Balianta, Dist.- Khurda,- 752 101 (M): 094371 47377 (Sh JK Das); 0671-2356627 Email: omfedomtdc@yahoo.com	
Assam		
69	Manager Cattle Feed Plant, Changsari, Guwahati – 781 101	Managing Director WAMPC Union Ltd., Khanapara Narengi Road, Panjabari, Guwahati – 781 022
70	Manager Cattle and Poultry Feed Unit HP Agro-Industries Corporation Ltd., Plot No.1, Parwanoo 173 220, Dist: Solan	(A Govt. of HP undertaking)

APPENDIX - IX

ABBREVIATIONS

ADF	Acid Detergent Fibre
ADIN	Acid Detergent Insoluble Nitrogen
BIS	Bureau of Indian Standards
CF	Crude Fibre
CFP	Cattle Feed Plant
CP	Crude Protein
DCP	Digestible Crude Protein
DIP	Degradable Intake Protein
DM	Dry Matter
DMI	Dry Matter Intake
EE	Ether Extract
GE	Gross Energy
HCN	Hydrocyanic Acid
IVDMD	<i>In vitro</i> Dry Matter Digestibility
LCF	Least Cost Feed Formulation
Mcal	Mega calorie
ME	Metabolizable Energy
MMT	Million Metric Tonne
MT	Metric Tonne
NDF	Neutral Detergent Fibre
NFE	Nitrogen Free Extract
NIRS	Near Infrared Reflectance Spectroscopy
NPN	Non-Protein Nitrogen
NPS	Non-Protein Sources
NRC	National Research Council
ppb	Parts Per Billion
ppm	Parts Per Million
RDP	Rumen Degradable Protein
RUP	Rumen Un-degradable Protein
TDN	Total Digestible Nutrients
TMR	Total Mixed Ration
UIP	Un-degradable Intake Protein

APPENDIX - X

GLOSSARY OF TERMS

Additive

An ingredient or combination of ingredients added to the basic feed mix or parts thereof to fulfil a specific need. Usually used in micro quantities and requires careful handling and mixing.

Artificially dried (process)

Moisture having been removed by other than natural means.

Balanced

A term that may be applied to a diet, ration or feed having all known required nutrients in proper amount and proportion based upon recommendations of recognized authorities in the field of Animal Nutrition such as the National Research Council (NRC) for a given set of physiological animal requirements. The species for which it is intended and the functions such as growth, maintenance or maintenance plus production shall be specified.

Blending (process)

To combine two or more ingredients or feeds. It does not imply a uniformity of dispersion.

Blocked, Blocking (processing)

Having agglomerated individual ingredients or mixtures into a large mass.

Blocks (physical form)

Agglomerated feed compressed into a solid mass cohesive enough to hold its form and most generally weighing from 13.6-22.7 kg.

Brand name

Any word, name symbol or device or any combination thereof identifying the commercial feed of a distributor and distinguishing it from that of others.

Bricks

Agglomerated feed compressed into a solid mass cohesive enough to hold its form and weighing less than 0.91 kg.

By-product

Secondary products produced in addition to the principal products.

Cake (physical form): The mass resulting from the pressing of seeds in order to remove oils, fats or other liquids.

Canned (process)

A term applied to a feed, which has been processed, packaged and sterilized for preservation in cans or similar containers.

Carrier

An edible material to which ingredients are added to facilitate uniform incorporation of the latter into feeds. The active principles are absorbed, impregnated or coated onto or into the edible material in such a way as to physically carry the active ingredients.

Chaff (part)

Glumes, husks or other seed coverings together with other plant parts separated from seed in threshing or processing.

Chipped, Chipping (process)

Reduced in particle size by cutting with knives or other edged instruments.

Cleaned or cleaning (process)

Removal of material by such methods as scalping, aspirating, magnetic separation or by any other method.

Cleanings (part)

Chaff, weed seeds, dust and other foreign matter removed from cereal grains.

Complete feed

A nutritionally adequate feed for animals other than man, by specific formula is compounded to be fed as the sole ration and is capable of maintaining life and / or promoting production without any additional substance being consumed except water.

Concentrate

A feed used with another to improve the nutritive balance of the total and intended to be further diluted and mixed to produce a supplement or complete feed.

Condensed or condensing (process)

Reduced to denser form by removal of moisture.

Tempered/ tempering, conditioned or conditioning (process)

Having achieved pre-determined moisture characteristics and / or temperature of ingredients or a mixture of ingredients prior to further processing.

Cooked or cooking (process)

Heated in the presence of moisture to alter chemical and (or) physical characteristics to sterilize.

Crimped or crimping (process)

Rolled by use of corrugated rollers. It may entail tempering or conditioning and cooling.

Crumbled or crumbling (process)

Pellets reduced to granular form.

Crumbles (physical form)

Pelleted feed reduced to granular form.

Flaked, flaking, crusted, crushing, rolled, rolling (process)

Having changed the shape and / or size of particles by compressing between rollers. It may entail tempering or conditioning.

Cubes, pellets (physical form)

Agglomerated feed formed by compacting and forcing through die openings by a mechanical process (similar terms: pelleted feed, hard pellets).

Dehulled or dehulling (processing)

Having removed the outer covering from grains or other seeds.

Dehydrated or dehydrating (processing)

Having been freed of moisture by thermal means.

Diluent

An edible substance used to mix with and reduce the concentration of nutrients and/or additives to make them more acceptable to animals, safe to use and more capable of being mixed uniformly in feed (it may also be carrier).

Dressed or dressing (process)

Made uniform in texture by breaking or screening of lumps from feed and/ or the application of liquid(s).

Dried or drying (process)

Materials from which water or other liquid has been removed.

Dust (part)

Fine, dry pulverized particles of matter usually resulting from the cleaning or grinding of grain.

Emulsifier (part)

A material capable of causing fat or oils to remain in liquid suspension.

Evaporated or evaporating (process)

Reduced to a denser form, concentrated as by evaporation or distillation.

Expanded, expanding (process)

Subjected to moisture, temperature and pressure to gelatinize the starch portion. When extruded its volume is increased due to abrupt reduction in pressure.

Extracted mechanical (process)

Having removed fat or oil from materials by heat and mechanical pressure.

Similar terms (old process)

Expeller extracted, hydraulic extracted.

Extracted, solvent (process)

Having removed fat or oil from materials by organic solvents.

Extruded (process)

A process by which feed has been processed, pushed, or protruded through constrictions under pressure.

Feed, feed stuff

Edible materials which are consumed by animals and contribute energy and/ or nutrients to the animals' diet.

Fines (in crumbles or pellets part)

Any materials which will pass through a screen whose openings are immediately smaller than the specified minimum crumble size or pellet diameter.

Flakes (physical form)

An ingredient rolled or cut into flat pieces with or without prior steam conditioning.

Flour (part)

Soft, finely ground and bolted meal obtained from the milling of cereal grains, other seeds or products. It consists essentially of the starch and gluten of the endosperm.

Formula feed

Two or more ingredients proportioned, mixed and processed according to specifications.

Gelatinized, gelatinizing (process)

Having had the starch granules completely ruptured by a combination of moisture, heat and pressure and in some instances by mechanical shear.

Grits (part)

Coarsely ground grain from which the bran and germ have been removed. Usually screened to uniform particle size.

Groats (part)

Grains from which the hulls have been removed.

Ground, grinding (process)

Reduced in particle size by impact, shearing or attrition.

Heat processed, heat processing (process)

Subjected to a method of preparation involving the use of elevated temperatures with or without pressure.

Hulls (part)

Outer covering of grain or other seed.

Ingredient

The term "Feed ingredient" means each of the constituent material making up a commercial feed.

Kibbled, kibbling (process)

Cracked or crushed baked dough or extruded feed that has been cooked prior to or during the extrusion process.

Mash (physical form)

A mixture of ingredients in meal form. Similar terms mash feed.

Meals (physical form)

An ingredient which has been ground or otherwise reduced in particle size.

Micro-ingredients

Vitamins, minerals, antibiotics, drugs and other materials normally required and measured in small units.

Mill by-product (part)

A secondary product obtained in addition to the principal product in milling practice.

Mill dust (part)

Feed particles, which escape from mill equipment.

Mill run (part)

The state in which a material comes from the mill, upgraded and usually inspected.

Mixing (process)

To combine by agitation two or more materials to a specific degree of dispersion.

Pearled, pearling (process)

Dehulled grains reduced by machine brushing into smaller smooth particles.

Pellets, soft (physical form)

Pellets containing a large percentage of liquids requiring immediate dusting and cooling.

Pelleted or pelleting (process)

Having agglomerated feed by compacted and forced through die openings.

Premix

A uniform mixture of one or more micro ingredients with diluents and/ or carrier. Premixes are used to facilitate uniform dispersion of the micro ingredients in a larger mix.

Premixing (process)

The preliminary mixing of ingredients with diluents and carriers.

Product (part)

A substance produced from one or more other substances as a result of chemical or physical change.

Range cubes (physical form)

Large pellets designed to be fed on the ground.

Ration

The amount of total feed, which is provided to one animal over a 24 hour period.

Scalped, scalping (processing)

Having removed larger materials by screening.

Scratch (physical form)

Whole cracked or coarsely cut grain. Similar term scratch grain, scratch feed.

Screened or screening (process)

Having separated various size particles by pressing over and / or through screens.

Separating (process)

Having treated ingredient with steam to alter physical and/ or chemical properties. Similar term steam cooked, steam rendered, tanked.

Toasted (process)

Browned, dried, or parched by exposure to a fire or gas or electric heat.

Wafered, wafering (process)

Having agglomerated a feed of a fibrous nature by compressing into a form usually having a diameter or cross section measurement greater than its length.

Wafers (physical form)

A form of agglomerated feed based on fibrous ingredients in which the finished form usually has a diameter or cross section measurement greater than its length.

APPENDIX - XI

CONVERSION FACTORS USED IN GENERAL ANALYSIS WORK

To convert from	To	Multiply by
%	mg/kg	100000
kcal/g	kcal/kg	1000
kcal/100g	kcal/kg	10
mg/100g	mg/kg	10
g/100g	%	1
ppm	mg/kg	1
IU/100g	IU/g	0.01
μg/100g	mg/kg	0.01
g/kg or mg/g	%	0.1
IU/kg	IU/g	0.001
mg/100g	%	0.001
mg/kg	%	0.0001
ppm	%	0.0001
kcal/lb	kcal/kg	2.2046
mg/lb	mg/kg	2.2046
g/lb	%	0.22046
IU/lb	IU/g	0.002205
μg/lb	mg/kg	0.002205
mg/lb	%	0.00022
μg crystalline vita. A alcohol/kg	IU/g	0.00333
μg crystalline vita. A alcohol/100g	IU/g	0.0333
μg crystalline vita. A alcohol/g	IU/g	3.33
μg vita. A acetate/kg	IU/g	0.002906
μg vita. A acetate/100g	IU/g	0.02906
μg vita. A acetate/g	IU/g	2.906

μg vita. A palmitate/kg	IU/g	0.001818
μg vita. A palmitate/100g	IU/g	0.1818
μg vita. A palmitate/g	IU/g	1.818
μg β -carotene/kg	IU/g	0.001667
μg β -carotene/100g	IU/g	0.01667
μg β -carotene/g	IU/g	1.667
% CaO	% of Ca	0.715
% Fe ₂ O ₃	% of Fe	0.699
% K ₂ O	% of K	0.830
% MgO	% of Mg	0.6033
% Na ₂ O	% of Na	0.742
% P ₂ O ₅	% of P	0.436
% SO ₂	% of S	0.500

μ is the Greek letter. When used alone it means 1 micron; when used as a prefix it means micro.

APPENDIX - XII

WEIGHT-UNIT CONVERSION FACTORS

To convert from	To	Multiply by
lb	g	453.6
lb	kg	0.4536
Oz	g	28.35
kg	lb	2.2046
kg	mg	1,000,000
kg	g	1000
g	mg	1000
g	μ g	1,000,000
mg	μ g	1000
mg/g	mg/lb	453.6
mg/kg	mg/lb	0.4536
μ g/kg	μ g/lb	0.4536
Mcal	Kcal	1000
kcal/kg	kcal/lb	0.4536
kcal/lb	kcal/kg	2.2046
ppm	μ g/g	1
ppm	mg/kg	1
mg/kg	%	0.0001
ppm	%	0.0001
mg/g	%	0.1
g/kg	%	0.1

APPENDIX - XIII

WEIGHT AND OTHER MEASUREMENT EQUIVALENTS

$$1 \text{ lb} = 453.6\text{g} = 0.4536\text{kg} = 16\text{Oz}$$

$$1 \text{ Oz} = 28.35\text{g}$$

$$1 \text{ kg} = 1000\text{g} = 2.2046 \text{ lb}$$

$$1 \text{ g} = 1000 \text{ mg}$$

$$1 \text{ mg} = 1000 \mu\text{g} = 0.001 \text{ g}$$

$$1 \mu\text{g} = 0.001 \text{ mg} = 0.000001 \text{ g}$$

1 $\mu\text{g/g}$ or 1 mg/kg or mg/litre is the same as ppm

1 mg/g , mg/ml means parts per thousand

$$1 \text{ cm} = 0.3937 \text{ inch}$$

$$1 \text{ inch} = 2.5399 \text{ cm}$$

$$1 \text{ metre} = 3.2808 \text{ feet}$$

$$1 \text{ feet} = 0.3048 \text{ metres}$$

$$1 \text{ cu. cm} = 0.0610 \text{ cu. inch}$$

$$1 \text{ litre} = 0.2199 \text{ imp. gallons}$$

$$1 \text{ Oz} = 28.3495 \text{ g}$$

$$1 \text{ millimicron (m}\mu) = 10^{-9} \text{ m} = 10^{-7} \text{ cm} = 10 \text{ angstroms (A}^0)$$

$$1 \text{ angstrom (A}^0) = 10^{-10} \text{ m} = 10^{-8} \text{ cm} = 100 \text{ micromicrons (}\mu\mu)$$

Temperature Equivalents

To convert Fahrenheit temperature into Centigrade: subtract 32 and multiply by 5/9

To convert Centigrade temperature into Fahrenheit: multiply by 9/5 and add 32.

APPENDIX - XIV

ATOMIC WEIGHTS

Name	Symbol	Atomic number	Atomic weight*
Actinium	Ac	89	(227)
Aluminium	Al	13	26.9815
Americium	Am	95	(243)
Antimony	Sb	51	121.75
Argon	Ar	18	39.948
Arsenic	As	33	74.9216
Astatine	At	85	(210)
Barium	Ba	56	137.34
Berkelium	Bk	97	(247)
Beryllium	Be	4	9.0122
Bismuth	Bi	83	208.980
Boron	B	5	10.811
Bromine	Br	35	79.909
Cadmium	Cd	48	112.40
Calcium	Ca	20	40.08
Californium	Cf	98	(249)
Carbon	C	6	12.01115
Caesium	Cs	55	132.905
Cerium	Ce	58	140.12
Chlorine	Cl	17	35.453
Chromium	Cr	24	51.996
Cobalt	Co	27	58.9332
Copper	Cu	29	63.54
Curium	Cm	96	(247)
Dysprosium	Dy	66	162.50
Einsteinium	Es	99	(254)
Erbium	Er	68	167.26
Europium	Eu	63	151.96
Fermium	Fm	100	(253)
Fluorine	F	9	18.9984
Francium	Fr	87	(223)
Gadolinium	Gd	64	157.25

Name	Symbol	Atomic number	Atomic weight*
Gallium	Ga	31	69.72
Germanium	Ge	32	72.59
Gold	Au	79	196.967
Hafnium	Hf	72	178.49
Helium	He	2	4.0026
Holmium	Ho	67	164.930
Hydrogen	H	1	1.00797
Indium	In	49	114.82
Iodine	I	53	126.9044
Iridium	Ir	77	192.2
Iron	Fe	26	55.847
Krypton	Kr	36	83.80
Lanthanum	La	57	138.91
Lead	Pb	82	207.19
Lithium	Li	3	6.939
Lutetium	Lu	71	174.97
Magnesium	Mg	12	24.312
Manganese	Mn	25	54.9380
Mendelevium	Md	101	(256)
Mercury	Hg	80	200.59
Molybdenum	Mo	42	95.94
Neodymium	Nd	60	144.24
Neon	Ne	10	20.183
Neptunium	Np	93	(237)
Nickel	Ni	28	58.71
Niobium	Nb	41	92.906
Nitrogen	N	7	14.0067
Nobelium	No	102	--
Osmium	Os	76	190.2
Oxygen	O	8	15.9994
Palladium	Pd	46	106.4
Phosphorus	P	15	30.9738
Platinum	Pt	78	195.09
Plutonium	Pu	94	(242)
Polonium	Po	84	(210)
Potassium	K	19	39.102

Name	Symbol	Atomic number	Atomic weight*
Praseodymium	Pr	59	140.907
Promethium	Pm	61	(147)
Protactinium	Pa	91	(231)
Radium	Ra	88	(226)
Radon	Rn	86	(222)
Rhenium	Re	75	186.2
Rhodium	Rh	45	102.905
Rubidium	Rb	37	85.47
Ruthenium	Ru	44	101.07
Samarium	Sm	62	150.35
Scandium	Sc	21	44.956
Selenium	Se	34	78.96
Silicon	Si	14	28.086
Silver	Ag	47	107.870
Sodium	Na	11	22.9898
Strontium	Sr	38	87.62
Sulfur	S	16	32.064
Tantalum	Ta	73	180.948
Technetium	Tc	43	(99)
Tellurium	Te	52	127.60
Terbium	Tb	65	158.924
Thallium	Tl	81	204.37
Thorium	Th	90	232.038
Thulium	Tm	69	168.934
Tin	Sn	50	118.69
Titanium	Ti	22	47.90
Tungsten	W	74	183.85
Uranium	U	92	238.03
Vanadium	V	23	50.942
Xenon	Xe	54	131.30
Ytterbium	Yb	70	173.04
Yttrium	Y	39	88.905
Zirconium	Zr	40	91.22
Zinc	Zn	30	65.37

*Number in () indicate the isotope with the longest half-life. All atomic weight values are based on the atomic mass of $^{12}\text{C}=12$.

APPENDIX - XV

O.D. CONVERSION TABLE

% T	O.D.	% T	O.D.	% T	O.D.
1	2.000	35	0.456	69	0.1612
2	1.699	36	0.444	70	0.1549
3	1.523	37	0.432	71	0.1487
4	1.398	38	0.420	72	0.1427
5	1.301	39	0.409	73	0.1367
6	1.222	40	0.398	74	0.1308
7	1.155	41	0.387	75	0.1249
8	1.097	42	0.377	76	0.1192
9	1.046	43	0.367	77	0.1135
10	1.000	44	0.357	78	0.1079
11	0.959	45	0.347	79	0.1024
12	0.921	46	0.337	80	0.0969
13	0.886	47	0.328	81	0.0915
14	0.854	48	0.319	82	0.0852
15	0.824	49	0.310	83	0.0809
16	0.796	50	0.301	84	0.0757
17	0.770	51	0.2924	85	0.0706
18	0.745	52	0.2840	86	0.0655
19	0.721	53	0.2756	87	0.0605
20	0.699	54	0.2676	88	0.0555
21	0.678	55	0.2596	89	0.0505
22	0.658	56	0.2518	90	0.0458
23	0.638	57	0.2441	91	0.0410
24	0.620	58	0.2366	92	0.0362
25	0.602	59	0.2291	93	0.0315
26	0.585	60	0.2218	94	0.0269
27	0.568	61	0.2147	95	0.0223
28	0.553	62	0.2076	96	0.0177
29	0.538	63	0.2007	97	0.0132
30	0.523	64	0.1939	98	0.0088
31	0.509	65	0.1871	99	0.0044
32	0.495	66	0.1805	100	0.0000
33	0.482	67	0.1739		
34	0.469	68	0.1675		

PERIODIC TABLE OF THE ELEMENTS

Period	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18																																															
	IA	IIA	Key										IIIA	IVA	VIA	VIIA	VIIIA																																																
	Atomic number	Atomic symbol	Element name	Transition metals	Alkali metals	Alkaline earth metals	Gases	Liquids	Metalloids	Noble gases	Other metals	Non metals	Atomic number	Atomic symbol	Element name	Atomic number	Atomic symbol	Element name	Atomic number	Atomic symbol	Element name																																												
1	1	H	Hydrogen										1	H	Hydrogen	2	He	Helium	2	He	Helium																																												
2	3	Li	Lithium										3	Li	Lithium	4	Be	Beryllium	5	B	Boron	6	C	Carbon	7	N	Nitrogen	8	O	Oxygen	9	F	Fluorine	10	Ne	Neon																													
3	11	Na	Sodium										11	Na	Sodium	12	Mg	Magnesium	13	Al	Aluminum	14	Si	Silicon	15	P	Phosphorus	16	S	Sulphur	17	Cl	Chlorine	18	Ar	Argon																													
4	19	K	Potassium										19	K	Potassium	20	Ca	Calcium	21	Sc	Scandium	22	Ti	Titanium	23	V	Vanadium	24	Cr	Chromium	25	Mn	Manganese	26	Fe	Iron	27	Co	Cobalt	28	Ni	Nickel	29	Cu	Copper	30	Zn	Zinc																	
5	37	Rb	Rubidium										37	Rb	Rubidium	38	Sr	Strontium	39	Y	Yttrium	40	Zr	Zirconium	41	Nb	Niobium	42	Mo	Molybdenum	43	Tc	Technetium	44	Ru	Ruthenium	45	Rh	Rhodium	46	Pd	Palladium	47	Ag	Silver	48	Cd	Cadmium																	
6	55	Cs	Cesium										55	Cs	Cesium	56	Ba	Barium	57 to 71	Lanthanide Series (rare earth metals)	72	Hf	Hafnium	73	Ta	Tantalum	74	W	Tungsten	75	Re	Rhenium	76	Os	Osmium	77	Ir	Iridium	78	Pt	Platinum	79	Au	Gold	80	Hg	Mercury																		
7	87	Fr	Francium										87	Fr	Francium	88	Ra	Radium	89 to 103	Actinide Series	104	Rf	Rutherfordium	105	Db	Dubnium	106	Sg	Seaborgium	107	Bh	Berkelium	108	Hs	Hassium	109	Mt	Moscovium	110	Uun	Ununium	111	Uuu	Ununium	112	Uub	Unbinium	113	Uut	Ununtrium	114	Uuq	Unquadium	115	Uup	Unpentium	116	Uuh	Unhexium	117	Uus	Unseptium	118	Uuo	Unoctium



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