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Chemical and Biological Evaluation of Feeds

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Chemical evaluation of feeds and feed material

Due to the rapidity and ease the scheme of proximate analysis of feeds has remained unchanged. Gravimetric methods have been automated and expensive equipments are available today. The underlying principles remain the same.

Moisture and Dry matter (DM)

Proximate composition of feeds are generally expressed on a dry matter basis. For this pre-weighed samples are dried at constant temperature in a hot air oven at 100±5 ^{o}C and the loss of weight is the moisture content. Moisture in prepared feed must be monitored because levels over 8% favour the presence of insects, and over 14% there is the risk of contamination by fungi and bacteria. Since the moisture content is variable generally proximate composition tables are presented on a dry matter basis. If not they have to be converted to dry matter basis as shown below.

As fed nutrient content = % nutrient on DM basis x (100 – moisture content)
Suppose a feedstuff contains 35 % protein on DM basis and its moisture content is 9%
Then, as fed protein content = 35 x (100-9)/ 100 = 38.46
Similarly if a feed stuff contains 32% protein as fed with a moisture content of 20%
Then, % protein on DM basis = 32 x 100/ (100-20) = 40%

Organic matter (OM)

Dry matter fraction of the feed contains all nutrients which can be broadly divided into organic matter and ash. Organic matter contains all the macronutrients and vitamins and ash contains all minerals. Therefore Dry matter – total Ash = Organic matter (OM)

Crude protein

Protein nitrogen in the feed sample is converted into ammonium (NH_4^+) ions by digestion with Con. H_2SO_4 in the presence of a catalytic mixture. Ammonia is determined after steam distillation by capturing it in excess of boric acid (4%) and titrating it with standard acid (HCL or H_2SO_4)**.**

Digestion

$(C_6H_{10}O_{5)+}\ n\ H_2SO_4 \rightarrow 6nC + 5n\ H_2O$

$C + 2\ H_2SO_{4+}\ 4H_2 \rightarrow CO_{2+}\ 2H_2O + 2SO_2$

$4RCOONH_{2+2}\ H_2SO_4 + 4H_2 \rightarrow 2(NH_4)_2SO_4 + RCOOH$

Distillation

$2(NH_4)_2SO_4+4NaOH \rightarrow 2Na_2SO_4+4NH_3+4H_2O$

$4NH_3+4H_2O \rightarrow 4NH_4OH$

$NH_4OH+H_3BO_3 \rightarrow NH_4[B(OH)_4]$ (Ammonium borate)

Titration

$2NH_4[B(OH)_4] + H_2SO_4 \rightarrow (NH_4)_2SO_4+2H_3BO_3+2H_2O$

Reagents: Con. H_2SO_4, 40% solution of boric acid, Indicator 0.1% methyl red +o.2% Bromocresol green in 95% ethanol. Prepare 4% solution of boric acid and mix it with 10 ml indicator. 40 g boric acid in 1 L water and add 5-10 ml indicator solution. Catalyst mixture. $CuSO_4$ and KSO_4 in the ratio 1:9 or 1:10, Standard N/10 H_2SO_4 or HCL.

Procedure

Weight 1-2 grams of feed sample, oven dried and transfer into kjheldhal flask (800 ml). Add to it 2–10 grams of catalyst mixture, followed by 25ml of concentrated H_2SO_4 (increased the amount of concentrated H_2SO_4 by 10 ml for each 2 gram of sample for samples low in N). Kept it on the heater for digestion maintained at 100°C. Continue heating till frothing clears. If frothing comes up to the neck stop heating for some time. During digestion turn the flask every 30 minutes till the solution clears and all the carbon has been oxidized. Change in color indicates completion of digestion. Digestion is completed when the content gives transparent fluish white color. Then cool it at room temperature, transfer the content to 100-250ml volumetric flask as desired and dilute it to the volume required. Then take 5 – 10ml of the above digested samples in the micro kjeldhal distillation flask. Then take 10 ml of boric acid with mixed indicator in a beaker and place it under the condenser tube of the distillation flask, making sure that the condenser tube extends beneath the solution. Add about 20-25 ml of NaOH (40%) solution to the samples in the distillation flask. Start steam distillation, continue distillation until the volume in the receiver beaker is about 100-150 ml then stop distillation while stopping just remove the receiver beaker to avoid the possibility of back suction. Then titrate this excess boric acid us the N/10 H_2SO_4 and find out the CP by % by calculation. %CP=Nx6.25.

$$\%N = \frac{\text{Vol. of N/10 } H_2SO_4 \text{ used x dilution factor x 0.0014 x 100}}{\text{Vol. of aliqout taken x weight of sample digested}}$$

Ether Extract:

This represents the fraction consisting of substances such as glycerides of fatty free fatty acids, sterols, phospholipids, chlorophyll, alkaloid substances, volatile oils, waxes, resins etc. The fraction is extracted from moisture free samples with petroleum ether. Ether is continually volatilized condensed and allowed to pass through the samples, extracting ether soluble materials. After completion of the process ether is recovered, remaining crude fibre is dried and weighed.

Apparatus:

Soxhlet extraction apparatus, Extraction thimbles and hot plate.

Reagents:

Petroleum ether (BP 40°C - 60°C) if anhydrous ether is not available proceeds as follows. Wash commercial ether with 2 – 3 portions of water, add solid NaOH or KOH and let it stand still till most of the water is absorbed from the ether, decant into a dry bottle, add small pieces of carefully cleared metallic sodium and let it stand until hydrogen evolution causes. Keep the ether thus dehydrated over metallic sodium in loosely stoppered bottles (AOAC 1970 11th edition).

Procedure:

Weigh 2 grams of oven dried sample and transfer it into the ether extraction thimble. If the sample is not dried dry it by weight thimble overnight at 105°C. Place the thimble in the soxhlet extractor filled with a condenser and a pre-weighed flask containing anhydrous ether approximately 2 siphons, heat the flask on a hot plate with temperature regulated for about 8 hours. The extraction is complete when a drop of ether taken from the dripping of the extractor has no greasy stain on a filter paper.

After the extraction is complete, lower the heater and allows the thimble to drain empty. Remove the thimble weigh the sample from extraction apparatus and pour it back into the container. Dry the flask completely evaporates, dry the flask at 105°C in explosion proof oven for about 30 minutes. Cool in a desiccator to room temperature and weigh. Increase in weight of the flask is the estimate of ether extract. Alternately the ether extract can also be estimated by recording the loss in weight of moisture free sample following its extraction with anhydrous ether and drying it completely in the drying oven

Calculation:

$$\% \text{ EE} = \frac{\text{Weight of EE}}{\text{Weight of sample}} \times 100$$

Crude fibre:

Crude fibre is the loss on ignition of dry residue remaining after digestion of sample with 1.25% H_2SO_4 and 1.25% NaOH, under specific conditions crude fibre represents a fraction which is composed of substances making up the frame work of plants and include cellulose, hemi cellulose and lignin of the cell wall. This portion of the feed is supposed to be in digestible and hence the estimations based on treating the moisture and fat free sample with dilute acid and dilute alkali thus imitating the gastric and intestinal action in the process of digestion.

Apparatus:

Digestion apparatus consisting of individual controlled heaters and water condensers designed to maintain constant volume of the solution throughout digestion. Digestion containers 600 ml or 1 litre beaker, muslin cloth, aluminium dish or sintered crucible.

Procedure:

Take 2 grams of moisture free fat free sample in a beaker fitted with a round bottomed condenser. Add 200 ml of 1.25 % H_2SO_4 and boil half an hour on a hot plate, with a temperature regulator fitted

through a muslin cloth and make the residue acid free using a suction pump. If needed, make the residue acid free with hot water washings and suction if needed. Transfer the residue to the same beaker and add 200 ml of 1.25 % NaOH, again boil for half an hour, again filter through a muslin cloth and make the residue free of alkali… using hot water and then acetone. Transfer the residue to the weighed crucible filter the excess water and dry it at constant temperature at 100°C. Ignite the recidue in a muffle furnace at 600°C and cool the crucible in a dessicator. Crude fibre can be determined by loss of weight due to ignition.

Calculation:

$$\frac{\text{Lose of weight on ignition}}{\text{Weight of sample}} \times 100 = \text{CF\%....}$$

Ash:

Ash is the inorganic residue in the forage sample left after igniting the sample at about 60°C in a muffle furnace. Though in the feeds crude ash do not seem to have any direct nutritional use but it is to be determined in proximate analysis when NFE is determined by difference or when the figure of total organic matter is required.

Ash from plant materials is a poor index of inorganic constituents/nutrients the reason for this may be that the ash component of these materials is highly variable, not only in the total amount but in its component parts also. One of the specific examples is of silica which is of no nutritional value but never the less may be a factor in the total crude ash reported in the sample. Especially in forages, this figure may be much magnified because of adhering sand or soil etc.

Apparatus:

Muffle furnace, dessicator, porcelain crucibles and metal tongs.

Procedure:

Place new or clean crucible in a dessicator and cool to room temperature, weigh them immediately to prevent moisture absorption.

Take 5-10ml of oven dried finely powdered sample in a weighed crucible. Char the sample on a burner till the smoke is removed and then keep it in the muffle furnace at about 600°C for about 3 hours. Remove the crucibles, cool in a dessicator and weigh. Calculate the percentage of ash from the increase in weight of the crucible.

Calculation:

$$\text{Ash \%} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

Nitrogen free Extract (NFE)

Nitrogen free extract is determined by substracting the sum of percentage of moisture, EE, CF, CP and contents of a sample from 100. The value obtained is again in percent. However, it will be affected by the chemical errors in the analysis of all the five separate fractions.

This fraction comprises of starch and sugars of the sample plus some hemicellulose and much of lignin through originally considered as representing the highly indigestible carbohydrates of the feed, this assumption is incorrect as in many cases it has been shown that this fraction has lower digestibility in ruminanat animals than even the CF fraction of the same feed if results partially from extraction of indigestible hemi cellulose in fibre determination. Nevertheless it is still practically a useful index of non cellulose portion of the feed carbohydrates.

Calculation:

NFE % = 100 - (CP %+CF %+EE %+Ash %)

PROTEIN – Folin – Ciocalteu phenol method, Lowry *et al*. (1951)

Tissue preparation (ovary, liver & muscle)

1. 25 mg tissue + 1 ml 10% TCA
2. Homogenized & centrifuged at 3000 rpm for 15 mts.
3. The resultant supernatant (clear solution) for carbohydrate estimation & precipitate for protein estimation

Standard

4. 25 mg BSA standard crystals + 5ml 1N NaOH = Standard
5. 0.3 ml standard + 0.7ml 1N NaOH = ie. 1 ml total
6. This 1 ml + 5ml alkaline mixture (freshly prepared) + 0.5ml Folin-ciocalteu phenol reagent (2N stock solu.diluted1:1), (Conc. of protein in this dilution = 1.5 mg/1ml NaOH)
7. Adjust OD blank to 'zero'
8. OD = approximately 0.7 (660nm)

Estimation (ovary, liver & muscle)

9. Precipitate + 5ml of 1N NaOH (digestion)
10. 1ml from the above + 5ml alkaline mixture* (freshly prepared)
11. Mix well and keep it for 10minutes
12. Add 0.5 ml 1N Folin-phenol reagent
13. OD measured after 30 mts. at 660nm (blue colour)

*Alkaline mixture = 50ml of 2% Na_2CO_3 in 0.1N NaOH + 1ml of 0.5% $CuSO_4$. 5H2O in 1% sodium potassium tartarate

$$\text{Mg protein / 100 mg tissue (mg\%)} = \frac{\text{OD sample}}{\text{OD standard (0.7)}} \times 5 \times 1.5 \times 100 \times \frac{}{25}$$

Estimation (serum)

0.1 ml serum + 1ml 10% TCA (precipitate - on sample preparation, steps 2,3,9 -13 are the same)

$$\text{mg protein /100ml serum} = \frac{\frac{\text{OD sample}}{\text{OD standard (0.7)}} \times 5 \times 1.5 \times 100}{0.1}$$

CARBOHYDRATE ESTIMATION – DUBOIS *et al.* (1956) (phenol-sulphuric acid method)

Standard

1. 20 mg D.Glucose/100 ml saturated Benzoic acid = Standard
2. 0.2ml Standard + 0.8ml Satu. Ben. Acid + 1ml 5% phenol +5ml conc. H_2SO_4
3. Read at 490 nm after 30 mts.(OD approximately 0.47)

Estimation (ovary, liver & muscle)

0.2 ml supernatant (tissue sample) + 0.8 ml Ben. Acid + 1ml 5% phenol+5ml conc. H_2SO_4

Read at 490 nm after 30 mts. (orange- yellow colour)

$$\text{Mg carbohydrate/100mg tissue} = \frac{\frac{\text{OD sample}}{\text{OD Standard}} \times 0.04^* \times 100}{25}$$

* conc. of standard

Estimation (serum)

0.1 ml serum + 1ml 10% TCA (on sample preparation)

1ml clear solution (supernatant) + 1ml 5% phenol + 5 ml conc. H_2SO_4

$$\text{mg protein /100ml serum} = \frac{\frac{\text{OD sample}}{\text{OD standard}} \times 0.04 \times 100}{0.1}$$

CHOLESTEROL (Henly's method)

Standard

Stock = 1mg pure cholesterol in 1ml Glacial Acetic Acid (Std.)
Working solution = 1ml Stock + 24ml $FeCl_3$: CH_3COOH mixture (0.05% $FeCl_3.6H_2O$ in GAA, ie. 0.2 mg/ 5ml $FeCl_3$)
This 25 ml + 3ml conc. H_2SO_4 (reddish brown colour)
Read at 560 nm after 20 mts. (OD approximately 0.55)

Blank

5ml $FeCl_3$: CH_3COOH mixture + 3 ml conc. H_2SO_4

Estimation (ovary,liver & muscle)

25mg tissue + 10 ml $FeCl_3$: CH_3COOH mixture
Mix well – overnight in refrigerator
Centrifuge at 3000 rpm – 15 mts. – proteins precipitate
5 ml supernatant transferred to a glass stoppered centrifuge tube (keep it on ice)
Add 3 ml conc. H_2SO_4
read after 20 mts. at 560 nm

$$\text{Mg cholesterol / 100mg tissue} = \frac{\text{OD sample X 100 X 0.2}}{\text{OD standard X 12 .5}}$$

Estimation (serum)

0.1 ml + 10 ml 10 ml $FeCl_3$: CH_3COOH mixture

$$\text{mg cholesterol/100ml serum} = \frac{\text{OD sample X 100 X 0.2}}{\text{OD standard X 0.05}}$$

TOTAL LIPIDS – Sulphophosphovanillin method of Barnes & Blackstock (1973)

Standard

1. Stock solu. = 8mg cholesterol in 10ml 2:1 V/V $CHCl_3$:CH_3OH (equivalent to 10mg total lipid/10ml 2:1 $CHCl_3$:CH_3OH)
2. Working standard solu. = 0.2 ml stock + 0.3 ml, 2:1 V/V $CHCl_3$:CH_3OH (conc. of liquid = 0.2 mg/0.5ml)
3. This 0.5 ml working standard is mixed well
4. Dry in vacuum over silica gel in a desiccator
5. Add 0.5ml conc. H_2SO_4 (content 0.2mg/0.5 ml H_2SO_4)
6. Shake well and plug with non-absorbent cotton wool
7. Heat exactly for 10mts. at 100^0C in boiling water bath
8. Cool rapidly in running water to room temp.
9. Take 0.1 ml of acid digest in pipette &pour to clean test tube (0.4mg/0.1ml)
10. Add 2.5ml of vannilin reagent and mix well in a cyclo mixer (pink colour)
11. Read after 30-60mts. at 520nm (OD approximately 0.6)

Blank

0.5 ml, 2:1 $CHCl_3$:CH_3OH solution.
Read at 520 nm after 30-60 minutes.

Estimation (ovary, liver & muscle)

1. 10mg wet tissue in 1ml, V/V 2:1 $CHCl_3$:CH_3OH
2. Mix well using a glass rod in cold
3. Kept overnight in tightly stoppard test tubes in refrigerator for complete extraction
4. Mix well once again &centrifuge at 3000 rpm for 15 mts

5. Take 0.5 ml of extract into clean dry test tubes (supernatant)
6. Dry in vacuum over silica gel in a dessicator
7. Add 0.5 ml conc. H_2SO_4 & shake well (content 5.0 mg/0.5 ml H_2SO_4)
8. Plug the tubes with non-absorbent cotton wool
9. Heat for 10mts at 100^0C in boiling water bath
10. Cool rapidly to room temperature under running tap water
11. Take 0.1 ml of this acid digest into a clean dry test tube (1mg/0.1ml)
12. Add 2.5 ml phosphovanillin reagent & mix well in a cyclomixer (pinkish red colour)
13. Read after 30 mts. at 520 nm

Total lipid mg% (mg/100mg tissue) $= \frac{\text{OD sample}}{\text{OD Std.}} \times \frac{0.04}{0.1} \times \frac{0.1}{1} \times 100$ or $\frac{\text{OD sample}}{\text{OD Std.}} \times 4$

Estimation (serum)

0.1mlserum + 1ml 2:1 V/V $CHCl_3$:CH_3OH (steps 2-13 is the same)

Total lipid in mg/100ml serum $= \frac{\text{OD sample}}{\text{OD Std.}} \times \frac{0.04}{0.1} \times \frac{0.1}{0.01} \times 100$ or $\frac{\text{OD sample}}{\text{OD Std.}} \times 400$

TOTAL LIPIDS (Bligh & Dyer with Folch extraction)

1. 100mg tissue ground in a mortar & pestle with 10ml distilled water (in case of serum 0.5 or 1ml)
2. pulp transferred to 250ml conical flask or test tube
3. add 3ml $CHCl_3$:CH_3OH (2:1, V/V)
4. mix well & keep overnight in refrigerator in Stoppard tube in dark
5. add 0.5ml of 0.9 % NaCl, allow to separate into two phases in a separating funnel
6. lower $CHCl_3$ phase with lipids collected carefully
7. make upto 3ml with $CHCl_3$
8. pour into a clean dry preweighed beaker
9. carefully evaporate in a vacuum desiccator (covered with a dark paper)
10. weight difference of beaker = calculated as mg total lipids/100mg tissue and mg total lipids/ 100 ml serum

TOTAL CAROTENOIDS, OLSON (1979)

Standard

Crystalline â – carotene (E-Merck)

Estimation

1. Quickly remove 1g tissue from animals to screw capped vial + 2.5g anhydrous sodium sulphate

2. Gently mash the sample with a glass rod against the side of the vial until it is reasonably well-mixed (never grind it to a fine powder)
3. Add 5 ml CHCl3 over the caked residue
4. Seal the vial & place it at 0^0C overnight (8-12 hrs.)
5. $CHCl_3$ should form a clear 1-2 cm layer above the caked residue
6. Take 0.3 ml (extract) + 2.7 ml ethanol
7. read at 290, 350, 380, 450, 475, & 500 nm
8. plot the readings on a graph

Result

$$\text{mg carotenoids/gm tissue} = \frac{\text{absorption at 450 nm X dilution factor}}{0.25 \text{ X sample wt. (g)}}$$

0.25 = extinction coefficient

Biological Evaluation of feeds

Evaluation of feeds is a very important component in feed development. After the analysis of feed material, feed formulation and feed making or production are the steps which follow logically. After the feed production certain physical parameters like water stability (hydrostability) of the feeds can be assessed using standard procedures which are available in Goldbaltt et al., 1980, Jayaram and Shetty, 1981, Ruscoe et al. 2005. Bulk density is another physical parameter which can be looked into because the final product property and packaging designs are based on this physical property as shown in the Table below

Nutritional evaluation of aquatic feeds can be done in different ways depending upon the objectives of the study. First let us look at the indoor wet laboratory situation where the culture system is either static, recirculating or flow through. In all these systems water quality should not interfere with the response to nutritional actors. Therefore the basic water quality parameters specified for normal aquatic life has to be maintained. There is a wide choice of filteration, oxygenation and disinfection systems available. Water with abnormally high levels of hardness (in the case of freshwater), iron and alkalinity should not be used. Pesticides, heavy metals, ammonia and nitrite depress growth.

Final Product Bulk Density Correlation with Buoyancy Properties

Pellet buoyancy	**Sea water @ 20ºC (3% salinity)**
Fast sinking	> 640 g/l
Slow sinking	580-600 g/l
Neutral buoyancy	520-540 g/l
Floating	<480 g/l

Experimental organisms

1. Feeding history should be known otherwise probability of obtaining highly inconsistent data increases
2. Nutritional status should be standardized by a conditioning period which is longer for adult animals and short for juveniles
3. Preferably select organisms from a single brood if hatchery bred stock is used
4. Age specific requirements for a particular nutrient exists

Experimental diets

1. Since the role of nutrition has been overlooked for too long standard diets and standard experimental protocols are necessary
2. Standard reference diets (SRD) recommended by NRC for penaeid shrimp has been successfully used in many nutrient requirement studies
3. Experimental feeds should normally contain small number of ingredients
4. For requirement studies purified ingredients should be used
5. Ingredient nomenclature should conform with published guidelines of International Union of Nutritional Sciences

Experimental diets

1. Factors influencing mixing of dietary ingredients are particle size, density, static charge, hygroscopicity and adhesiveness
2. Manufacturing process should be directed towards minimizing loss of heat labile nutrients
3. Cold extrusion is preferable, cold air drying is preferred and freeze drying is the most conservative and safe method

Interactions

1. Alginates and phytic acid reduced bioavailability of divalent metal ions such as Ca
2. Some carbohydrates are found to reduce the uptake of proteins and amino acids
3. Alginates and guar gum reduces digestibility of protein in rainbow trout

Digestibility

1. Markers are used in aquatic nutrition studies
2. Procedure dependant errors should be minimized
3. Markers do not pass though the gut homogenously due to sequestering
4. If concentration of digestibility indicator in the faecal material is increasing, total collection of faeces would be the accurate procedure
5. Each ingredient under investigation should be added at levels of both 15 and 30%
6. Remaining 70-80% consists of the base formulation
7. All experimental animals should be fed a conditioning diet for at least one week
8. Faeces from experimental feeding must be collected from two independent feedings

9. Approximately one hour after feeding all uneaten food should be removed and faeces collected 3-5 h later
10. Entire faecal production for each day is then pooled for three consecutive days to compensate variability in faecal production (quantity generally) among days

Energy content

1. Digestible energy value (DE) of feedstuffs used are not determined
2. Gross energy (GE) values are determined by calorimetry
3. Apparent DE can be determined from the difference between GE and faecal energy
4. Apparent DE should be based upon calorific equivalents

Evaluation of nutrient requirements

1. The responses should minimally consist of weight gain, survival, tissue levels and related biochemical indices whenever possible
2. A minimum of 4 dietary levels or more should be used in any experiment devoted to determining the quantitative requirement
3. If less than four levels are tested then fitting the data to a descriptive response curve cannot be conducted to a high degree of confidence
4. Desired and actual levels of nutrients should be equivalent
5. Actual availability of the nutrient to the organism should be attempted

Experimental period

1. Tissue levels of nutrient under investigation need to be determined prior to the experiment
2. High tissue level of a specific nutrient at the initiation of an experiment can influence a response elicited
3. Deficiency signs may escape detection simply because duration of the experiment was insufficient to allow depletion
4. 300% increase in weigh has to be recorded in shrimp
5. Duration will thus depend on the nutrient under investigation

The next scenario is outdoor systems in which photoperiod cannot be controlled. These evaluations are more expensive than indoor evaluations because all the requirements more. Finally, on-farm evaluations are the ultimate where level of controls decrease but the results obtained would be realistic. On-farm research involves evaluations at multiple locations also.

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