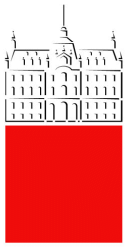

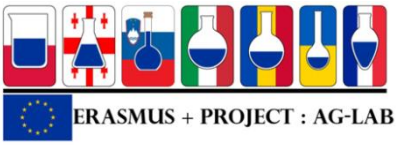

Proximate analysis

Laboratory manual

Course: Basic Animal Nutrition, ZOO16, Agriculture-Animal production, Bologna level 1, year 2

Chair for Nutrition
Animal Science Department
Biotechnical Faculty
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Laboratory Sample Preparation

Subsampling or sample reduction of an unground sample in the laboratory is frequently the largest single source of variation during the analysis procedure and should be avoided whenever possible. Samples too large to be ground in their entirety are first ground through a large mill (Wiley or equivalent) to pass a 4 to 6 mm sieve. The coarse ground sample is then reduced in a gated riffle splitter and ground again to the fineness desired for analysis.

1. Sample Preparation

Scope:
This method is applicable to the preparation of forages for laboratory analysis.

Basic Principle:
Laboratory sample preparation is the process of converting the sample received at the laboratory into a homogeneous material suitable for analysis. This process generally involves drying and/or grinding.
Most forage samples received at a laboratory fall into one of the following categories:
1) those dry enough to grind and analyze immediately (sample 90 to 95% or more dry matter).
2) those dry enough to be coarsely ground (to pass a 4 to 6 mm sieve) but too wet to be finely ground.
3) those samples which need to be partially dried before the sample can be coarsely ground (sample dry matter less than approximately 85%).

Precautions are taken during sample preparation to avoid sample contamination and retain sample identity. All equipment is kept clean and maintained on a regular schedule. Prepared samples are stored in airtight containers away from heat and light.

Preparing samples less than approximately 85% dry matter for grinding
1) Remove sample from shipping container and discard any roots from plants and brush off dirt particles. Note and report removed material and any other sample manipulation.
2) Chop samples of whole plants into about half-inch pieces using either hand clippers or a laboratory forage chopper. Silages and haylages generally have average particle lengths less than 1 inch and do not require chopping.
3) Place the chopped sample into a clean dishpan or on a clean plastic sheet. Mix thoroughly.
4) If the entire sample cannot be dried, reduce the sample size by making a cone of sample and quartering. Save opposite quarters. Repeat mixing, coning and quartering until the volume is reduced to an appropriate size. Make certain that representative ratios of stem and leaf occur in each pile.
5) Transfer reduced sample to a tared container for drying.
7) Dry the reduced sample using either the forced-air
8) Grind the partially dried sample to fineness desired for analyses (< 1 mm) in appropriate grinder.
9) Thoroughly mix the ground sample. Transfer to an airtight container and label immediately.

2. Dry Matter Determination

Dry matter can be determined either by residual weight following drying or with a near infrared reflectance (NIR) spectrophotometer using equations based on such reference techniques. Errors arise if the sample is insufficiently dried prior to taking the final weight or if the sample is overheated and additional compounds are volatilized (e.g. volatile acids and alcohols from haylages and silages). Room humidity for areas where dry matter is being determined should be below 60% RH.
Definition of Terms: "as fed," "as is," or "as received" refers to the forage or feed as it is consumed by the animal. On a laboratory analysis report, these terms refer to the moisture content of the feed at the time of analysis.
Moisture content of a forage at analysis vary from the true dry matter of the original lot if drying occurred between sampling and analysis.
"air dry" refers to a sample that has been allowed to dry in air, without aid of an oven or other drying device. In air of less than 60% relative humidity most air-dry samples will contain about 90% ± 2% dry matter.
"partially dry" refers to the initial dry matter of a sample that has been dried in an oven, usually at 55 to 60^{o}C or in a microwave oven to less than complete dryness. These samples, with 3 to 15% moisture, can be easily ground in most laboratory mills to produce a homogeneous sample for further analysis.
"laboratory dry matter" refers to the (final) dry matter content of the partially dry sample.
dry, dry matter, total dry matter, moisture free refer to a sample that has all of the moisture removed.
Forage and feedstuff analyses should be compared on a dry matter basis because varying moisture contents of "as is" results can alter analysis of other constituents.

2.1 Two Step Total Dry Matter Determination of Wet Samples

For wet samples (less than approximately 85% dry matter) it is necessary to partially dry the sample in forced-air or microwave oven prior to grinding. The goal is to dry the unground sample while keeping sample temperature below 45-55^{o}C so that chemical composition is affected minimally. Drying at low temperature (less than 60^{o}C) does not remove all water from the sample; therefore (initial) partial drying does not represent the dry matter of the sample.
Following drying, the sample is ground and analyzed for (final) laboratory dry matter (the remaining 3 to 15% moisture) when other chemical constituents are determined.
Therefore, a two-step procedure for determining dry matter is recommended:
Step 1: Determine partial dry matter of sample (if less than 85% dry matter).
Step 2: Determine laboratory dry matter on ground sample and multiply partial dry matter times laboratory dry matter to determine total dry matter.
Step (1) may be skipped if dry matter ≥85% and the sample can be ground without drying.

2.1.1 Partial Drying of Wet Samples

Scope
This procedure is applicable to all types of forages and is intended for initial, partial drying of wet samples (less than 85% dry matter, greater than 15% moisture). The procedure has minimal effect on chemical composition, allowing samples to be subsequently analyzed for fiber, lignin, or acid detergent insoluble nitrogen analysis.

Basic Principle
Moisture is evaporated from sample and partial dry matter is determined gravimetrically as the residue remaining after oven drying. Some moisture remains in the sample because drying at this temperature does not remove all water. Sample should be equilibrated at room temperature for 2 to 4 hrs before measuring partial dry matter to minimize the potential change in moisture that can occur during grinding and storage. Drying at higher temperatures (greater than 60^{o}C) causes chemical changes in the sample that affect subsequent fiber, lignin, or acid detergent insoluble nitrogen analysis.

Equipment
Forced-air drying oven set at 55° C
Top loading electronic balance, accurate to 0.5 g
Pans, sufficient in size to hold 250 to 500 g coarse forage

Safety Precautions
Use standard precautions when working with electrical equipment or glassware.

Procedure
2) Weigh paper on a top loading balance and record weight (W1) to nearest 0.01 g.
3) Tare paper to zero and weigh constant volume of coarse forage into pans
recording weight to nearest 0.01 g (W2)
4) Dry in forced-air drying oven at 45-55° C for 16 to 24 hr.
5) Air-equilibrate samples for 2 to 4 hr and weigh the sample and pan recording weight to nearest 0.01 g (W3).

Calculation
Partial Dry Matter (pDM), expressed as ratio of (w/w, g/kg) of dry matter to total weight.

$$pDM(g/kg) = \frac{(m_3 - m_1)}{(m_2 - m_1)} \cdot 1000$$

m_1 = weight of paper, on which sample was dried (g)
m_2 = weight of sample and paper before drying (g)
m_3 = weight of sample and paper after drying (g)

Quality control
Maximum allowed differences between parallel determinations:

moisture (g/kg)	Allowed differences
150 or less	± 3.0 g/kg (A)
More than 150	± 2% (R)

2.1.2 Determination of moisture (dry matter)

Purpose and Scope
This method makes it possible to determine the moisture content of feed. In case of feed containing volatile substances, such as organic acids, it is to be observed that also significant amount of volatile substances are determined together with the moisture content.

Principle
The sample is desiccated under specified conditions which vary according to the nature of the feed. The loss in weight is determined by weighing. It is necessary to carry out preliminary drying when dealing with solid feed which has high moisture content.

Equipment
Forced-air drying oven at 103 - 105°C, capable of maintaining temperature at ±1oC
Glass dish with lid
Desiccator
Top loading electronic balance, accurate to 0.5 mg

Procedure

Weigh a dried (in a desiccator) and cleaned glass container with its lid to the nearest 1 mg. Weigh into the weighed container, to the nearest 1 mg, about 5 g of the sample and spread evenly. Place the container, without its lid, in the oven preheated to 103 ^{o}C. To prevent the oven temperature from falling unduly, introduce the container as rapidly as possible. Leave to dry for 3 hours reckoned from the time when the oven temperature returns to 103 ^{o}C. Replace the lid on the container, remove the latter from the oven, leave to cool in the desiccator and weigh to the nearest 1 mg.

Calculation of results:

$$DM(g/kg) = \frac{(m_3 - m_1)}{(m_2 - m_1)} \cdot 1000$$

m_1 = weight of glass container (g)
m_2 = weight of sample and glass container before drying (g)
m_3 = weight of sample glass container after drying (g)

Quality control

Maximum allowed differences between parallel determinations:

moisture (g/kg)	Allowed differences
150 or less	± 3,0 g/kg (A)
More than 150	± 2% (R)

3. Nitrogen Determination by Kjeldahl

Scope
The methods described are applicable for determination of nitrogen (N) in forages.

Basic Principle
The Kjeldahl method is the standard method of nitrogen determination dating back to its development in the late 1800's. The method consists of three basic steps:
1) digestion of the sample in sulfuric acid with a catalyst, which results in conversion of nitrogen to ammonia;
2) distillation of the ammonia into a trapping solution; and
3) quantification of the ammonia by titration with a standard solution.
The sample is digested by sulphuric acid in the presence of a catalyst. The acid solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected an excess of boric acid solution, followed by titration with standard sulphuric acid solution.

Equipment
Kjeldahl flasks, 500 to 800 mL
Kjeldahl digestion unit with fume removal manifold
Kjeldahl distillation apparatus - Kjeldahl flask connected to distillation trap by rubber stopper. Distillation trap is connected to condenser with low-sulfur tubing.
Erlenmeyer flask, 250 ml
Analytical balance, sensitive to 0.5 mg

Reagents
Sulfuric acid, concentrated, 95-98%, reagent grade
Sodium hydroxide
Potassium sulfate (K_2SO_4), anhydrous
Copper sulfate ($CuSO_4$), anhydrous
boric acid, 2% (w/v)
standardized HCl (C= 0.1000 mol/l)

Safety Precautions
Handle acid safely: use acid resistant fumehood. Always add acid to water unless otherwise directed in method. Wear face shield and heavy gloves to protect against splashes. If acids are spilled on skin, immediately wash with large amounts of water. Sulfuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely.
Wear heavy rubber gloves and face shield to protect against concentrated acid or alkali.
Use effective fume removal device to protect against acid fumes or alkali dusts or vapors. Always add concentrated sulfuric acid or sodium hydroxide pellets to water, not vice versa. Concentrated sodium hydroxide can quickly and easily cause blindness. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.
Digests must be cool before dilution water is added to avoid a violent reaction during which the acid can shoot out of the flask. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

Procedure

Digestion
Weigh 0.5 g of the sample to the nearest 0.001 g and transfer the sample to the Kjeldahl flask of the digestion apparatus. Add 15g of mix of potassium sulphate and catalyst (copper (II) sulphate pentahydrate), 12 ml of sulphuric acid and mix.

Heat the Kjeldahl flask moderately at first, swirling from time to time if necessary until the mass has carbonized and the foam has disappeared; then heat more intensively until the liquid is boiling steadily. Heating is adequate if the boiling acid condenses on the wall of the flask. When the solution becomes clear and light green continue to boil for another two hours, then leave to cool.

Distillation into boric acid

Where the distillation unit is fully automated to include titration of the ammonia content of the distillate, follow the manufacturer's instructions for operation of the distillation unit. In a semi-automatic distillation unit, the addition of excess sodium hydroxide and the steam distillation are performed automatically.

Place a collecting flask containing 60 ml of the boric acid solution under the outlet of the condenser in such a way that the delivery tube is below the surface of the excess boric acid solution. Operate the distillation unit in accordance with the manufacturer's instructions and distil off the ammonia liberated by the addition of the sodium hydroxide solution. Collect distillate in the boric acid receiving solution.

Titration

Titrate the contents of the collecting flask with the sulphuric acid standard volumetric solution using a burette and read the amount of titrant used.

When colorimetric end-point detection is applied, the end-point is reached when color of the solution changes from green to red. Estimate the burette reading to the nearest 0, 01 ml.

Blank test

To confirm that the reagents are free from nitrogen, carry out a blank test (digestion, distillation and titration) using only reagents (no sample is added).

Calculation of results

Nitrogen content in the sample:

$$m_N(g/kg) = \frac{(V_a - V_b) \cdot c_{HCl} \cdot M_N}{m_{vz}} \cdot 1000$$

V_a = volume of standard HCl solution when titrating sample (l)
V_b = volume of standard HCl solution when titrating blank (l)
c_{HCl} = concentration of HCl (mol/l)
M_N = nitrogen molar mass (g/mol)
m_{vz} = weight of sample (g)

Crude protein (CP) in sample:

$$CP(g/kg) = m_N(g/kg) \cdot F$$

m_N = nitrogen content in sample (g/kg)
F = factor (6.25 for feed samples)

Quality control

Maximum allowed differences between parallel determinations:

Crude protein content (g/kg)	Allowed differences
less than 160	± 4.0 g/kg (A)
160.0 – 320.0	± 2.5% (R)
more than 320.0	± 8.0 g/kg (A)

4. Crude Fat (Ether Extract) in Forages

Purpose and scope
This method is for the determination of crude oils and fats in feed (feed materials of plant origin).

Equipment
Analytical balance, sensitive to 0.1 mg
Forced-air drying oven set at 98^{o} C
Automated fat extraction system
glass extraction cups
thimbles (paper)

Reagents
petroleum ether

Procedure
The sample is extracted with petroleum ether. The solvent is distilled off and the residue dried and weighed.
Weigh 2-3 g of the sample to the nearest 1 mg, transfer it to an extraction thimble and cover with a fat-free wad of cotton wool. Weigh clean and dry extraction flask to the nearest 1 mg.
Place the thimble in an extractor and extract for 80 min with light petroleum. Extraction procedure is automated. Dry the residue maintaining the flask for one hour in the drying oven at 98^{o}C. Leave to cool in a desiccator and weigh.

Calculation of results

$$CF(g/kg) = \frac{(b-a)\cdot 1000}{c}$$

b – weight of dried and cooled flask and extract after extraction (g)
a – weight of dried and cooled flask (g)
c – weight of sample (g)

Quality control
Maximum allowed differences between parallel determinations:

Crude fat content (g/kg)	Allowed differences
4.0 – 100.0	±4.0 (g/kg) (A)
101.0 – 200.0	± 4% (R)
more than 200.0	± 8 (A)

5. Determination of crude ash

Purpose and Scope
This procedure is applicable for the determination of ash in all types of dried, ground forages and feeds. It is not applicable for ash determination in liquid feeds or feeds high in sugar content.This method makes it possible to determine the crude ash content of feed.

Principle
The sample is ashed at 520 to 550 ^{o}C; the inoganic residue is weighed.

Equipment:
Porcelain crucibles
Muffle furnace
Analytical balance, sensitive to 0.1 mg
Desiccator

Procedure
Weigh out to the nearest mg clean and dried porcelain crucible than weigh into the crucible approximately 4 g of the sample, record the weight. Put the crucible into the muffle furnace set at 520 -550 ^{o}C. Keep at this temperature until white, light grey or reddish ash is obtained which appears to be free from carbonaceous particles. Place the crucible in a desiccator, leave to cool and weigh immediately.

Calculation of results

$$CA(g/kg) = \frac{(m_c - m_a)}{(m_b - m_a)} \cdot 1000$$

m_a= weight of porcelain crucible (g)
m_b= weight of porcelain crucible and sample (g)
m_c= weight of porcelain crucible and ash (g)

Quality control
Maximum allowed differences between parallel determinations:

Crude ash (g/kg)	Allowed differences
2.0 – 40.0	± 10% (R)
41.0 –100.0	± 4.0 g/kg (A)
101.0 – 150.0	± 4% (R)
151.0 – 200.0	± 0.6 g/kg (A)
more than 201.0	± 3% (R)

6. Determination of crude fibre

Purpose and scope
This method makes it possible to determine fat-free organic substances in feed which are insoluble in acid and alkaline media and are conventionally described as crude fibre. Feed samples, containing more than 5 % of fat, should be de-fatted using appropriate fat extraction method, prior to analysis of crude fibre.

Principle
The sample, defatted where necessary, is treated successively with boiling solutions of sulphuric acid (1.25 %) and potassium hydroxide (1.25 %). The residue is separated by filtration on a sintered-glass filter washed, dried, weighed and ashed within a range of 520 to 550 ^{o}C. The loss of weight resulting from ashing corresponds to the crude fibre present in the test sample.

Equipment:
Analytical balance, sensitive to 0.5 mg
600 ml glass beaker
Hot plate
Condenser
Fritted glass crucubles
Desiccator
Manifold for vacuum filtration
Forced-air drying oven, set at 103-105^{0}C
Muffle furnace

Reagents
Suphuric acid, 1.25 %
Sodium hydroxide, 1.25 %
Sea sand, extra pure, washed with acid
Acetone

Procedure
Weigh out 3 g of the prepared sample to the nearest 10 mg and place it in the glass beaker, add 200 ml of sulphuric acid (1.25 %) and cover the baker with condenser. Bring the liquid to the boil within 5 ± 2 minutes and boil vigorously for exactly 30 minutes.
Open the tap to the discharge pipe and, under vacuum, filter the suspension of sample in 1.25 % sulphuric acid through the fritted glass crucible (containing cca 1.5 cm of sea sand) and wash the residue with three consecutive 30 ml portions of boiling water, ensuring that the residue is filtered dry after each washing. Put the sample from fritted glass crucible back to glass beaker (with minimal proportion of the sea sand) and pour 200 ml of potassium hydroxide solution (1.25 %) in the beaker and cover the baker with condenser. Bring the liquid to boiling point within 5 ± 2 minutes and boil vigorously for exactly 30 minutes. Filter and repeat the washing procedure used for the sulphuric acid step.
After the final washing and liquid aspiration, wash the residue in the crucible with acetone and water ensuring that the residue is filtered dry after each washing.
Dry the crucible to constant weight in the oven at 103 ^{o}C. After drying, cool in the desiccator and weigh rapidly. Place the crucible in a muffle furnace and ash at 520 to 550 ^{o}C overnight.
After heating cool first in the furnace and then in the desiccator before weighing.

Calculation of results

$$CF(g/kg) = \frac{(m_a - m_b)}{m_{vz}} \cdot 1000$$

m_a = weight of crucible and fibre after drying (g)
m_b = weight of crucible and fibre after ashing (g)
m_{vz} = weight of sample (g)

Quality control

Maximum allowed differences between parallel determinations:

Crude fiber content (g/kg)	Allowed differences
4.9 – 100.0	± 4.0 g/kg (A)
more than 100.0	± 4% (R)

7. Analytical report

The result given in the analysis report is the average value obtained from two determinations, carried out on separate portions of the sample.

Average value is calculated according to:

$$\overline{X} = \frac{x_1 + x_2}{2}$$

Absolute error (A) is calculated according to:

$$A = \overline{x} - x_1$$

Relative error (R) is calculated according to:

$$R(\%) = \frac{\overline{x} - x_1}{\overline{x}} \cdot 100$$

8. References:

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7. VDLUFA Methodenbuch. 1993. Die Chemische Untersuchung von Futtermitteln, 3. Ergaenzunglieferung. Darmstadt, VDLUFA-Verlag

Data and measurements – Proximate analysis

Name and surname:_____
Sample laboratory number:_____
Description of sample:_____

Partial dry matter (pDM)	Duplicate 1	Duplicate 2
Weight of paper (g)		
Weight of paper and sample (g)		
Weight of partialy dry paper and sample (g)		
Partial dry matter (g/kg)		
Moisture (g/kg)		

Izračun/calculation:

Average partial dry matter (g/kg)	
Average moisture (g/kg)	
Asolute error(g/kg)	
Relative error (%)	
Is analysis done correctly?	

Remarks:_____
_____

Dry matter (DM)	Duplicate 1	Duplicate 2
Weight of glass dish (g)		
Weight of glass dish and sample (g)		
Weight of glass dish and sample after drying (g)		
Dry matter (g/kg)		
Moisture (g/kg)		

Calculation:

Average dry matter (g/kg)	
Average moisture (g/kg)	
Absolute error(g/kg)	
Relative error (%)	
Is analysis done correctly?	

Remarks
_____
_____

Crude ash (CA)	Duplicate 1	Duplicate 2
Weight of porcelain crucible (g)		
Weight of porcelain crucible and sample (g)		
Weight of ash and porcelain crucible (g)		
Weight of sample (g) – calculate		
Weight od ash (g) – calculate		
Crude ash (g/kg)		

Calculation:

Average ash content (g/kg)	
Absolute error(g/kg)	
Relative error (%)	
Is analysis done correctly?	

Remarks

_____
_____

Crude protein (CB)	Duplicate 1	Duplicate 2
Weight of sample (g)		
Concentration of HCl (mol/l)		
Volume of standard HCl needed to titrate blank (ml)		
Volume of standard HCl needed to titrate sample (ml)		
Crude protein (g/kg)		

Calculation:

Average content of crude protein (g/kg)	
Absolute error(g/kg)	
Relative error (%)	
Is analysis done correctly?	

Remarks

_____
_____

Crude fat (CM)	Duplicate 1	Duplicate 2
Weight of extraction beaker (g)		
Weight of sample in thimble		
Weight of extract and extraction beaker after extraction		
Weight of extract – calculate (g)		
Crude fat (g/kg)		

Calculation:

Average content of crude fat (g/kg)	
Absolute error(g/kg)	
Relative error (%)	
Is analysis done correctly?	

Remarks

_____
_____

Crude fiber (CF)	Duplicate 1	Duplicate 2
Weight of sample (g)		
Weight of glass crucible and solid residue after drying (g)		
Weight of glass crucible and solid residue after ashing (g)		
Weight of crude fiber – calculate (g)		
Crude fiber (g/kg)		

Calculation:

Average crude fibre content (g/kg)	
Absolute error(g/kg)	
Relative error (%)	
Is analysis done correctly?	

Remarks:_____
_____
_____

Calculations:

In dry matter (g/kg DM)

In wet sample (g/kg wet sample)

ANALYTICAL REPORT

Sample number:	
Sample description:	
Date of analysis:	
Analyst:	

RESULTS

Analysis	In partialy dry (analysed) sample (g/kg pDM)	In (wet) sample (g/kg)	In dry matter (g/kg DM)
Dry matter			
Crude protein			
Crude fat			
Crude ash			
Crude fiber			
Nitrogen free extract			

REMARKS:

TEST
Course: Basic animal nutrition (laboratory practise proximate analysis)

Name and surname:_____
Enrolment number: _____

Do not forget units!

1. In which analysis (proximate analysis) have we used following reagents and why:
Analysis_____
concentrated H_2SO_4_____
2 % water solution of H_3BO_3 _____
K_2SO_4_____
(1 point)

2. Using proximate analysis we have analysed hay sample and determined following parameters: partial dry matter 503,0 g/kg, in partialy dryed sample we have determined 938,0 g/kg dry matter, 332,9 g/kg crude fiber, 84,0 g/kg crude ash, 20,2 g/kg crude fat in 96,4 g/kg crude protein. Calculate composition of fresh and dry sample in % and g/kg! (3 points)

3. In silage sample we have determined crude fibre content. We weighted 3,0000 g of sample, made hidrolysis in acidic solution, we filtered solid organic residue through crucible containing quartz sand, hydrolised solid residue in alkaline solution and repeated filtration through the same crucible with quartz sand. Mass of crucible, quartz sand and organic matter (fiber) after drying was 73,2146 g. What is the mass of crucible, quartz sand and anorganic matter (ash) after drying, if we know that sample contains 579,8 g/kg crude fibre? (2 points)

4. Explain (in short) procedure for determination of crude fat! Which data are needed for calculation of crude fat in sample? (2 points)

5. In feed sample we analysed crude protein using Kjeldahl method. After digestion of sample, distillation of ammonia using water wapour and and titration of ammonia using standard HCl (c= 0,1025 mol/l) solution, we calculated following nitrogen (N) content in sample:
1. determination: 12.67 gN/kg sample
2. determination: 12.78 gN/kg sample.
Calculate content of crude protein in your sample, if you know, that proteins in sample contain 14.7 % of nitrogen! (2 points)