ICARDA

Animal Nutrition and Product Quality Laboratory Manual

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Foreword

Livestock production is the major source of livelihoods for millions of households worldwide. Small ruminants (sheep and goats) are critically important in dry areas, and a key focus of ICARDA's research. The Center's livestock research covers many areas, two of which relate to this manual: development of low-cost alternative feeds to improve animal nutrition, and value-added products such as cheese and yogurt, to increase farmers' income. Both aspects require field research as well as laboratory analyses.

Feed analyses are necessary to determine the nutritional value of different feed products in terms of dry matter, crude protein, crude fiber, crude fat, crude ash, minerals, tannins and other parameters. Similarly, laboratory tests are needed to analyze the composition and quality of milk and milk derivatives in terms of protein, fat, lactose, total solids, acidity, pH, density, conductivity and other parameters including hygiene aspects.

ICARDA's Animal Nutrition Laboratory is well equipped, with highly trained staff. It provides analytical services to support livestock research by ICARDA and national research centers in many countries; and helps develop research capacity through training programs for researchers, farmers and extension staff, student internships and other means.

This manual describes detailed procedures for conducting a wide range of laboratory analyses. These procedures are based on international standards, and have been refined through many years of use and experience at ICARDA. The manual will be freely available in printed and on-line versions. It will be a useful guide for livestock technicians and researchers in developing countries worldwide. It will also serve as a reference for developing-country laboratories seeking to establish their own standards and protocols.

Mahmoud Solh
Director General, ICARDA

Preface

ICARDA's livestock research aims to develop appropriate technologies to improve small ruminant production by resource-poor households. Technologies – particularly for nutrition, feeding and milk processing – are tested under controlled conditions at the Animal Research Laboratory at ICARDA's headquarters. The laboratory also runs training programs for national researchers, extension staff and farmers; and supports graduate students in their research.

The Animal Research Laboratory, named after the late Dr Giro Orita, holds an experimental flock of 400 Awassi sheep, a hall for indoor feeding experiments, an animal nutrition laboratory, an animal health laboratory, and a milk processing plant that is connected to an automatic milking parlor.

The flock is used for research on animal nutrition, husbandry, reproduction and breeding. The milk processing plant supports research on and production of dairy products, and helps develop practical solutions to farmers' problems in milking hygiene, processing and product diversification. The animal nutrition laboratory conducts quality analysis of feeds, milk and milk products, conducting about 20,000 analyses of feed, milk and blood samples every year.

This manual was developed by scientists and technicians at ICARDA's headquarters in Syria, and regional offices in Tunisia. It provides a comprehensive guide to feed and milk quality analyses that are being conducted at ICARDA. It is hoped this information will also contribute to establishing standard and protocols at animal nutrition laboratories in national institutions.

Figure 1. Dr Giro Orita Animal Research Laboratory at ICARDA

Introduction

Animal nutrition is an important factor limiting livestock productivity, and feed costs are considered as the main constraint to raising income from small–scale ruminant production in dry areas. ICARDA's livestock scientists are searching for alternative feed resources to be included in well balanced diets that can lead to improvements in flock productivity and in meat and milk quality. The nutritional value of proposed low cost diets and their effects on product quality need careful evaluation in the laboratory. Therefore, ICARDA's Animal Nutrition Laboratory analyzes feed as well milk and dairy product quality.

This manual covers all analyses conducted in the Animal Nutrition Laboratory at ICARDA, as well as the equipment needed. Special chapters refer to analyses conducted in the newly established laboratories in Libya.

Feed analyses conducted in the laboratory include basic nutritional analyses such as dry matter, crude protein, crude fiber, crude fat, crude ash, minerals and tannins. For these analyses, the laboratory is equipped with Kjeldahl nitrogen analyzer, Soxhlet, Atomic Absorption Spectrophotometer, balances, and centrifuges. The unit also holds donor animals for rumen liquid that is used for two–phase digestibility analysis and Gas Production Tests.

In addition, this manual describes composition and quality analyses of milk and milk derivatives. The laboratory provides analyses for protein, fat, lactose, total solids, acidity, pH, density and conductivity. It also

Figure 2. Animal Nutrition Laboratory at ICARDA

facilitates advanced analyses that use gas chromatography, and HPLC analyzer for detection of organic acids and Volatile Fatty Acids.

One chapter of this manual describes assays to determine the udder health of animals. Mastitis can be determined by either the California Mastitis Test or using somatic cell counter. The blood of sheep can be evaluated for progesterone content using the ELISA assay.

The main purpose of this manual is to provide a guide for technicians in the Animal Nutrition Laboratory, and for technicians and researchers from NARS and other collaborators that come to ICARDA for training or collaborative research projects. Furthermore, it informs scientists from ICARDA and outside ICARDA on the services offered by the laboratory.

1. Feed Analysis

1.1. Sample Receiving and Preparation

1.1.1. Record Incoming Samples

Upon receipt at the laboratory samples are assigned batch numbers. The requester has to fill a form (see Appendix 1) providing sample description, source, requested analyses, costs for each analysis and budget code for charges. This form must be approval by the project manager for charge back payment. The Animal Nutrition Laboratory uses this information to build up a data base on results of analyses and source description of all samples that pass through the laboratory.

1.1.2. Sample Preparation

Sample preparation converts the samples into homogeneous material for the various nutritional analyses. Drying and grinding are the essential operations. Sample preparation is conducted according to sample type and analyses requested.

Reference

- AOAC (Association of Official Analytical Chemists). 1995.

1.1.3. Drying Samples

In case fresh forage samples are wet when received they are dried overnight at 60 °C in an air-circulation oven to obtain air dried samples ready for grinding.

Feces samples are dried at 60 °C in an air-circulation oven (Fig.3) for at least 48 hours prior to grinding.

Milk and milk derivatives are prepared according to the analyses requested (see chapter 3). For dry matter see section 1.2.1.

Figure 3. Air-circulation oven for field samples

Equipment

- Air-circulation oven for large field samples (Fig. 3)

1.1.4. Grinding

Feed samples are ground to 1 mm particle size with a Wiley mill (Fig. 4).

Samples for tannin analyses are ground to 0.5 mm particle size.

Dried or frozen feces are first passed through a 2 mm mesh, then through a 1 mm mesh.

Figure 4. Wiley mill

Dried and ground samples are stored in airtight containers away from heat and light. Caution has to be taken to avoid insect damage.

Equipment

- Wiley mill (Fig. 4)

1.2. Determination of Dry Matter, Ash and Organic Matter

Accuracy, repeatability and comparison of results between laboratories require standardization of methods. The following methods for dry matter, ash and organic matter determination are essential for validating any method for feed analysis. Most of the nutritional analyses of feed are based on dry matter values.

1.2.1. Determination of Dry Matter (DM)

Principle

Dry matter is the portion in forages that is not water. All nutritional analyses are based on dry matter content.

In ICARDA's Animal Nutrition Laboratory the AOAC method for determination of moisture in animal feed has been adapted to the working routine in the laboratory as follows:

Procedure

- Dry empty porcelain crucibles overnight at 105 °C
- Cool in desiccators to room temperature
- Weight oven-dry crucible (W_t)

- Add approximately 2 g of ground sample; record weight (W_s)
- Dry overnight at 105 °C
- Cool in desiccators to room temperature
- Weight oven–dry crucible+sample = (W_0)

For milk and milk derivatives samples

- For dry matter based analyses, 2 ml of milk are dried in crucibles overnight at 105 °C and its weight taken
- For cheese, a 2 g sample is dried overnight at 105 °C and then dry weight taken.

Calculations

$\%DM = [(W_0 – W_t)/W_s] \times 100$

Equipment

- Precision balance (Fig. 5)
- Air-circulation oven (Fig. 6)
- Desiccator (Fig. 7)
- Porcelain crucibles (Fig. 9).

References

- AOAC. 1995.

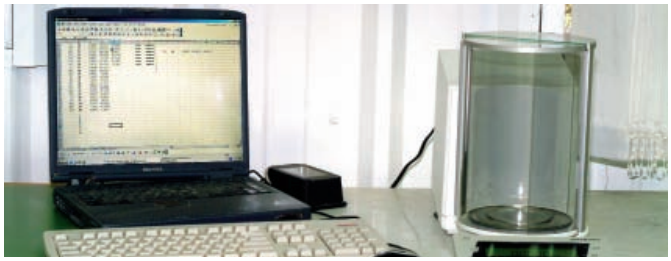


Figure 5. Precision balance

Figure 6. Air-circulation oven

Figure 7. Desiccator

1.2.2. Determination of Ash

Procedure

- Ignite dry matter samples (see 1.2.1) overnight at 550 °C in muffle furnace
- Cool in desiccators to room temperature
- Weigh ignited crucible + sample (W_a).

Calculation

$\%Ash = [(W_a – W_t)/(W_0 – W_t)] \times 100$

Equipment

- Precision balance (Fig. 5)
- Muffle furnace (550 °C) (Fig. 8)
- Desiccator (Fig. 7)
- Porcelain crucibles (Fig. 9).

Figure 8. Muffle furnace

Figure 9. Porcelain crucible

Reference

- AOAC.1995.

1.2.3. Determination of Organic Dry Matter (ODM)

Calculations

$\%ODM = 100 – \%Ash$

1.3. Determination of Crude Protein

Principle
Determination of total nitrogen (crude protein) is conducted using the Kjeldahl method. The sample is digested in sulfuric acid using K_2SO_4/$CuSO_4$/TiO_4 as a catalyst. N is converted into NH_3, then distilled trapped in boric acid and titrated with H_2SO_4.

Procedure
- Add 1g of dry sample in digestion tubes (250 ml)
- Consider blank tube
- Consider standard sample of known nitrogen contents
- Add half a tablet of catalyst
- Add 13 ml of concentrated sulfuric acid (H_2SO_4)
- Insert rack with 20 tubes, including blank and standard sample in digestion block heater under fume hood, and install exhaust manifold connected to water aspirator (Fig.11)
- Keep in digester at 420 °C until liquid becomes transparent
- Remove rack with exhaust manifold from digester and allow to cool to room temperature under fume hood
- Remove exhaust manifold and transfer tubes separately to distillation unit
- Automatic distillation: 65 ml dist. water + 35 ml of 40% sodium hydroxide solution
- Collect condensed liquid in Erlenmeyer flask with 10 ml indicator solution
- Titrate liquid with 0.1142 *N* sulfuric acid until color turns purple.

Calculations
$\%N = [1.4007 \times (V_a - V_b) \times N]/W$

V_a: volume of acid used for sample titration
V_b: volume of acid used for the blank
N: Normality of acid
W: sample weight in grams
1.4007: conversion factor milliequivalent weight of nitrogen and N percent

Calculation: Percent Crude Protein (CP)
%CP = %N × F
F = 6.25 for all forages
F = 5.70 for wheat grains
F = 6.38 for milk

Using acid normality of 0.1142 one can consider that each ml of above sulfuric acid added at titration corresponds to 1% of crude protein (CP) if the above conversion factor (F) is equal to 6.25.

Reagents / Solutions

Digestion reagents

- Catalyst tablet composition: 5 g K_2SO_4, 0.15 g TiO_2, 0.15 g $CuSO_4.5H_2O$, Foss, cat. No. A15270010
- Concentrated sulfuric acid H_2SO_4 (96-98%).

Distillation solution

40% sodium hydroxide solution:

- 400 g of NaOH (99%)
- 1 l distilled water.

Indicator solution

- Boil 4 l distilled water
- Add 160 g boric acid (H_3BO_3)
- Cool to next day
- Add 2.8 ml methy l red
- Add 4 ml bromocresol green.

Titration solution

- 0.1142 *N* sulfuric acid H_2SO_4 (Merck 1.9981: Titrisol for 1 l, H_2SO_4, 0.5 mol/l)
- Empty Titrisol ampoule into 1 l volumetric flask
- Make up with distilled water to 1 l
- Transfer total contents of volumetric flask into 10 l Nalgene container
- Add 7757 ml of distilled water.

Equipment

- Precision balance (Fig. 5)
- Fume hood (Fig. 12)
- Digestion block heater (Tecator 2020, Foss, Denmark) with exhaust manifold connected to water aspirator for 20 digestion tubes (Fig.11)
- Distillation unit (Vapodest 30,Gerhardt, Germany) (Fig.10)

Figure 10. Automatic distillation unit (Kjeldahl) and automatic burette for titration

Figure 11. Digestion block heater with exhaust manifold

Figure 12. Fume hood

- Automatic burette (Eppendorf, Germany) for titration
- Kjeldahl digestion tubes 6100, 250 ml
- Erlenmeyer flask, 250 ml
- Volumetric flasks,1 l.

References

- AOAC. 1995.
- ISO 2004

1.4. Determination of Nonprotein Nitrogen (NPN) using Trichloro-acetic Acid (TCA)

Principle

Nonprotein nitrogen is the nitrogen remaining after precipitation of true protein. After filtration the insoluble nitrogen can be determined.

Procedure

- Weigh 0.5 g dry sample into a 125 ml Erlenmeyer flask
- Add 50 ml distilled water; allow standing for 30 min
- Add 10 ml 10% trichloroacetic acid; stand for 20-30 min
- Filter by gravity through Whatman #54 or 541 paper
- Rinse twice with TCA solution
- Transfer paper to Kjeldahl flask and determine residual nitrogen.

Calculation

NPN = Total nitrogen – Residual nitrogen

Express NPN as Crude Protein or as % of total nitrogen in feed

Reagents / Solutions

TCA Solution

- Trichloroacetic acid (CCl_3COOH) 10% w/v in water

Reference

- Licitra G, Hernandez TM and VanSoest PJ. 1996.

1.5. Determination of Fat

Principle

The dried feed sample is dissolved with petrol ether and then the ether is evaporated in the Soxhlet apparatus. The residues are crude fat. In the Animal Nutrition Laboratory at ICARDA a Soxtherm apparatus from Gerhardt GmbH is used. The procedure described below follows the instruction for this specific Soxhlet apparatus. In the laboratory the modified method for determination of fat in meat has been adapted.

Procedure

- Fill extraction beakers with boiling stones dried at 103 °C (Fig. 14)
- Cool beakers down to room temperature in desiccator
- Weigh beakers on precision balance (W_0)
- Weigh 5 g of dried samples in extraction thimbles (W_s)
- Cover with fat free cotton
- Insert extraction thimble into extraction beaker
- Add 140 ml of petrol ether and insert beaker in Soxhlet apparatus (Fig. 13)
- Start program
- Program:

1.	Boiling time	30 min
2.	Solvent reduction	5×15 min
3.	Extraction time	80 min
4.	Extraction temperature	150 °C
5.	Solvent reduction	8 min
6.	Solvent reduction	5 min
7.	Solvent reduction interval	3 min
8.	Solvent reduction phase	3 sec

- After termination of program, dry beakers at 105 °C overnight
- Cool beakers down to room temperature in desiccator and weigh (W_e).

Calculations

$\%\text{Crude fat} = [(W_e - W_0)/(W_s \times \%DM)] \times 100$

Reagents / Solutions

Petrol ether (40–60 °C)

Figure 13. Soxhlet apparatus with control unit

Figure 14. Soxhlet extraction beaker unit with thimble and boiling stones inside

Equipment

- Precision balance (Fig. 5)
- Soxhlet (Soxtherm S306 A, Gerhardt GmbH, Germany) (Fig. 13)
- Air-circulation oven (Fig. 6)
- Extraction beakers with boiling stones (Fig. 14)
- Extraction thimbles (Fig. 14)

Reference

- Undersander D, Mertens DR and Thiex N. 1993.
- AOAC. 1995.
- Instruction Manual Soxtherm S306 A. 2000.

1.6. Detergent Fiber Analyses

Important fractions of feed are fibers that affect the digestibility. They consist of cellulose, hemicelluloses and polysaccharides that are bound to protein and phenols, especially to lignin. Detergent fiber is the residue of plant cells after fractionation using detergent solutions for the volatilization of protein and starch.

1.6.1. Determination of Neutral Detergent Fiber (NDF)

Principle

Insoluble fiber in feed is determined as neutral detergent fiber (NDF). Neutral detergent solution recovers its main components cellulose, hemicelluloses and lignin.

Procedure

- Weigh oven-dry glass crucible (W_t)
- Add 0.5–1 g sample (W_s) in 600 ml Berzelius beaker
- Add 100 ml of neutral detergent solution and 0.5 g sodium sulfite (Na_2SO_3)
- Boil for one hour in refluxing apparatus (Fig. 15)
- Pour through glass crucibles (Fig. 17)
- Admit vacuum (Fig. 16)
- Rinse crucibles with approximately 50 ml hot water four times until all traces are removed
- Rinse with acetone repeatedly until drained liquid is cleared.
- Dry at 105 °C overnight
- Cool to room temperature in desiccator
- Weigh sample and crucible (W_0)
- Ash residues for three hours at 550 °C
- Cool to room temperature in desiccators
- Weight crucibles and residues (W_a).

Figure 15. Refluxing apparatus

Calculations

$\%NDF = [(W_0 – W_t)/W_s]\times100$

Cell soluble material = 100 – %NDF

NDF expressed as organic matter:

Ash insoluble in neutral detergent

$\%NDF_{ash} = [(W_s – W_t)/(W_0 – W_t)]\times100$

Neutral detergent solution

- 2 l distilled water.
- 60 g sodium lauryl sulfate ($C_{12}H_{25}O_4S$)
- 37.22 g disodium dihydrogen EDTA ($C_{10}H_{14}N_2Na_2O_8$)
- 13.62 g sodium borate (decahydrate) ($Na_2B_4O_7.10H_2O$)

- 9.12 g disodium hydrogen phosphate (Na_2HPO_4)
- 20 ml 2–ethoxy–ethanol ($C_4H_{10}O_2$)
- Adjust pH 6.9–7.1.

Equipment

- Precision balance (Fig. 5)
- Refluxing apparatus (Fig. 15)
- Vacuum filtering system with trap in line (Fig. 16)
- Desiccator (Fig. 7)
- Berzelius beakers (600 ml)
- Sintered glass crucibles (coarse porosity 1) (Fig. 17).

References

- Goering HK and VanSoest PJ. 1970.
- Van Soest PJ, Robertson JB and Lewis BA.1991.

1.6.2. Determination of Acid Detergent Fiber (ADF)

Principle

Acid detergent fiber is determined as the residue remaining after adding an acidified solution. It is the NDF without the hemicelluloses. Cell soluble, hemicelluloses and soluble minerals dissolve. Cetyl trimethyl-ammonium bromide (CTAB) separates proteins from the remaining cellulose and lignin, and minerals (ash). The acid detergents solution recovers cellulose and lignin.

ADF determination is a preparation step for lignin determination.

Procedure

- Add 0.5–1 g sample (W_s) in 600 ml Berzelius beaker
- Add 100 ml of acid detergent solution
- Boil for one hour on refluxing apparatus

Figure 16. Vacuum filtering system with trap in line

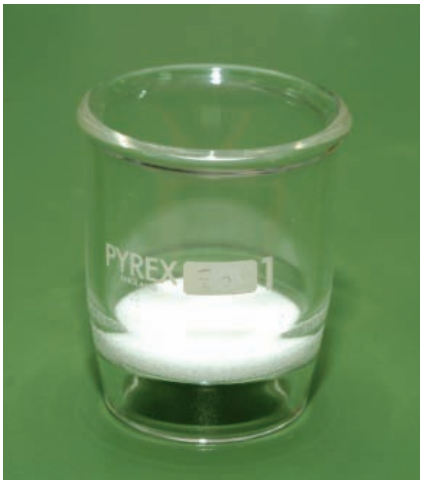


Figure 17. Glass crucible, porosity 1

- Weigh oven–dry glass crucible (W_t)
- Pour through glass crucibles
- Admit vacuum
- Rinse crucibles with approximately 50 ml hot water four times until all traces are removed
- Rinse with acetone repeatedly until drained liquid is cleared
- Dry at 105 °C overnight
- Cool to room temperature in desiccator
- Weight sample and crucible (W_0)
- Ash residues for three hours at 550 °C
- Cool to room temperature in desiccator
- Weight crucibles and residues (W_a).

Calculations
$\%ADF = [(W_0 – W_t)/W_s] \times 100$
ADF expressed as organic matter:
$\%ADF_{ash} = [(W_a – W_t)/(W_0 – W_t)] \times 100$

Acid detergent solution
- 20 g cetyl trimethylammonium bromide (CTAB)
- 1l of 1.0 *N* sulfuric acid (H_2SO_4).

Equipment
- Precision balance (Fig. 5)
- Refluxing apparatus (Fig. 15)
- Vacuum filtering system with trap in line (Fig. 16)
- Desiccator (Fig. 7)
- Berzelius beakers (600 ml)
- Sintered glass crucibles (coarse porosity 1) (Fig. 17).

References

- Goering HK and VanSoest PJ. 1970.
- AOAC. 1995.
- Undersander D, Mertens DR and Thiex N. 1993.

1.6.3. Determination of Acid Detergent Lignin (ADL)

Principle
Lignin is the indigestible non-carbohydrate component of forages. Residues from ADF determination are treated with sulfuric acid. Lignin represents the indigestible NDF fraction.

Procedure
- Transfer crucibles with residues from ADF to a flat container or tray
- Cover the contents of the crucible with cooled 72% sulfuric acid (H_2SO_4)
- Stir with glass rod, breaking all lumps

- Fill crucible about half full with sulfuric acid and stir
- Refill three times at room temperature
- Leave for three hours stirring every 15 min with glass rod
- Filter under vacuum
- Rinse twice with 400 ml hot water
- Dry overnight at 105 °C
- Cool in desiccator and weigh (W_0)
- Ash at 550 °C for three hours
- Cool in desiccator and weigh (W_a).

Calculations
$\%ADL = [(W_a - W_0)/W_s] \times 100$

Reagents / Solutions
72% sulfuric acid solution
- 735 ml concentrated sulfuric acid (98%)
- Add 265 ml water.

Equipment
- Precision balance (Fig. 5)
- Muffle furnace (550 °C) (Fig. 8)
- Sintered glass crucibles (coarse porosity 1) (Fig.17)
- Desiccator (Fig. 7).

References

- Goering HK and VanSoest PJ. 1970.
- AOAC. 1995.

1.6.4. Determination of Acid–Detergent Insoluble Nitrogen (ADIN)

Principle
ADIN is determined as the nitrogen remaining in ADF residue. In this manual the percentage of Acid Detergent Insoluble Nitrogen (ADIN) on DM basis is determined from the total ADF residue that is trapped in filter paper.

Procedure
- Dry filter paper overnight at 100 °C and weigh (W_0)
- Weigh 1 g air dry sample in 600 ml Berzelius beaker (W_s)
- Add 100 ml acid detergent solution
- Boil for 1 h on refluxing apparatus
- Filter onto filter paper using funnel with vacuum suction
- Wash beaker with hot distilled water to remove all fiber particles into filter paper
- Use blanks (filter paper washed with ADF solution) to estimate nitrogen in paper and reagents
- Soak twice with boiling water after filling funnel with vacuum off for 2 minutes

- Rinse twice with acetone by filling funnel with vacuum off for 2 minutes before vacuuming dry
- Leave folded filter paper with residues in oven at 105 °C for 8 h or overnight
- Weigh hot filter paper with residues (W_r)
- Transfer paper residue into Kjeldahl digestion tube
- Add additional 5 ml sulfuric acid to digest filter paper
- Determine nitrogen in residue by standard Kjeldahl
- Titrate with 0.01 *N* standard acid.

Calculation

$\%ADIN = [(\%N \text{ in } W_t - \%N \text{ in } W_0)/(W_s \times \%DM)] \times 100$

Reagents / Solutions

- Standard ADF solution (see 1.6.2.)
- For titration 0.01 *N* sulfuric acid (H_2SO_4) (see 1.6.2.).

Equipment

- Precision balance (Fig. 5)
- Refluxing apparatus (see Fig. 15)
- Vacuum filtering system with trap in line (Fig. 16)
- Air-circulation oven (Fig. 6)
- Berzelius beakers (600 ml)
- Sintered glass crucibles (course porosity 1) (Fig. 17)
- Büchner funnel
- Whatman filter paper number 54 or 541.

Crucible cleaning

The sintered glass crucibles (coarse porosity1) that are used for detergent fiber analyses (1.6.) need to be cleaned regularly as follows:

- Place crucibles on a metal tray and pull washing solution to cover the bottom of the crucibles
- Keep overnight for 18 hours
- Rinse with distilled water until clear
- Dry crucibles in oven at 105 °C.

Washing solution

Dissolve 6 g of either Sodium dichromate or Potassium dichromate in 100 ml distilled water and then add 100 ml of concentrated sulfuric acid

References

- Licitra G, Hernandez TM and VanSoest PJ. 1996.

1.7. Fiber Analysis using the Semi–automated ANKOM Filter Bag Technique

Conventional methods (Goering and VanSoest, 1970; AOAC, 1990) require a great deal of preparatory and analytical technician time. Semi–automated equipment for fiber analysis (ANKOM Technology Corp., Fairport, NY) requires less technician time and increases sample handling capacity. Furthermore, the semi–automated method helps reduce technician interaction with harmful chemicals and alleviate errors that can occur due to variation in crucible filtration. This technique is used in the Animal Nutrition Laboratories in Libya.

1.7.1. Acid Detergent Fiber (ADF) in Feeds using Filter Bag Technique

Principle
This method determines Acid Detergent Fiber, which is the residue remaining after digesting with H_2SO_4 and CTAB. The fiber residues are predominantly cellulose and lignin.

Scope
This method is applicable to grains, feeds, forages and all fiber–bearing material.

Acid detergent solution
Add 20 g cetyl trimethylammonium bromide (CTAB) to 1 l of 1 *N* H_2SO_4 previously standardized. Agitate and heat to aid solution.

Preparation of sample
Grind samples in a centrifugal mill with a 2 mm screen, or cutter type (Wiley) mill with a 1 mm screen. Samples ground finer may have particle loss from the filter bags and result in low values.

Procedure

- Use a solvent resistant marker to label the filter bags. Weigh filter bag (W_1) and zero balance. Do not pre–dry filter bags; any moisture will be accounted for by the blank bag correction
- Weigh 0.45–0.55 g of prepared sample (W_2) directly in filter bag Avoid placing the sample on the upper 4 mm of the bag
- Completely seal the upper edge of the filter bag within 4 mm of the top with the heat sealer encapsulating the sample. Use sufficient heat to completely seal the filter bag and allow enough cooling time (2 sec) before removing the bag from the heat sealer
- Weigh one blank bag and include in run to determine blank bag correction (C_1)
- **Pre-extraction of fat–rich products (>5 % fat)**. Extract samples by placing 24 bags with samples into a container with a top Pour enough acetone into container 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

- Exception – **Roasted soybean**: Due to the processing of roasted soya, a modification is required at the extraction stage. Place roasted soy samples into a container with a top. Pour enough acetone into container to cover bags and secure top. Shake the container 10 times and pour off acetone. Add fresh acetone and allow samples to soak for twelve hours. After soaking time, pour out acetone and place bags on a wire screen to air–dry
- Place a maximum of 24 bags into the Bag Suspender. All nine trays should be used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated to 120 degrees. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the Bag Suspender weight on top to keep it submerged. Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cold water and exhaust
- When processing 24 sample bags, add 1900–2000 ml of ambient temperature AD solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of AD solution (use a minimum of 1500 ml to ensure Bag Suspender is covered).
- Turn Agitate and Heat ON and confirm agitation. Set timer for 60 min and close lid
- At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid. 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
- After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900–2000 ml of (70–90 °C) rinse water. Turn Agitate on and rinse for 5 min. The lid may be sealed with the Heat On or left open with the Heat Off. Repeat hot water rinses a total of four times or until water is of neutral pH
- When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker, add enough acetone to cover bags and soak for 3–5 min
- Remove bags from acetone and place on a wire screen to air–dry Completely dry in oven at 105±2 °C (most ovens will complete drying within 2–4 hours). Do not place bags in the oven until the acetone has completely evaporated
- Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags (W_3). Do not use conventional desiccator container.

Caution

- Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. Always add acid to water and not the reverse
- CTAB will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical
- Acetone is extremely flammable. Avoid static electricity and use a fume hood when handling.

Calculations

%ADF (as-received basis) = [W3 – (W1×C1)/W2]×100

Where:
W1 = Bag tare weight
W2 = Sample weight
W3 = Dried weight of bag with fiber after extraction process
C1 = Blank bag correction (final oven-dried weight divided by original blank bag weight)

Equipment

- Analytical balance, capable of weighing down to 0.1 mg
- Oven capable of maintaining a temperature of 105±2 °C
- ANKOM200, 65 rpm agitation, digestion instrument capable of performing digestion at 100 ± 0.5 °C, and maintaining a pressure of 10–25 psi. The instrument must also be capable of creating a similar flow around each sample to ensure uniformity of extraction (Fig. 18)
- Filter bags, constructed from chemically inert and heat resistant filter media, capable of being heat sealed closed and able to retain 25 micron particles while permitting rapid solution penetration (F57, ANKOM Technology) (Fig. 19)
- Heat sealer – sufficient for sealing the filter bags to complete closure (1915, ANKOM Technology) (Fig. 19)
- Desiccator pouch, collapsible sealable pouch with desiccant inside that enables the removal of air from around the filter bags (Moisture Stop Weigh Pouch, ANKOM Technology)
- Marking pen – solvent and acid resistant (F08, ANKOM Technology).

Figure 18. ANKOM200

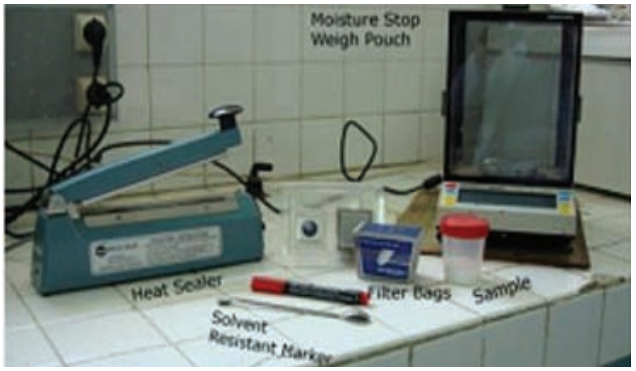


Figure 19. ANKOM bags, sealer, balance

References

- Goering HK and VanSoest PJ. 1970.
- Anonymous. 1995.
- AOAC. 1990.

1.7.2. Acid Detergent Lignin (ADL) in Feeds using Filter Bag Technique

Scope and preparation of sample

See 1.7.1.

Procedure

- After completion of ADF analysis, return filter bags to the Isotemp oven to ensure complete removal of moisture before applying acid
- Remove filter bags containing ADF residues from the Isotemp oven and place them in a desiccator and allow cooling for 20 min
- After cooling, place filter bags into the ANKOM Daisy II Incubator vessel (3.7 l) with 72% H_2SO_4. The volume of acid added to the vessel is 40 ml/sample or 480 ml for 12 samples (which is the same volume per sample of acid added to each crucible in the conventional crucible method using recycled acid)
- After 3 hours of submersion and rotation, remove samples from the vessel and place them in a 385×200 mm acid resistant, plastic tub filled with 3 l of cold distilled water. Submerge filter bags and circulate by hand (use acid resistant gloves) through the container for 5 min. Three rinses are required and acid–contaminated water must be discarded in an acid waste container between rinses. The final pH of the rinse water should be ≥6
- When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker and add enough acetone to cover bags. Soak for 3–5 min
- Remove bags from acetone and place on a wire screen to air–dry.Completely dry in oven at 105±2 °C (most ovens will complete

drying within 2–4 h). Do not place bags in the oven until acetone has completely evaporated
- Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags (W3). Do not use conventional desiccator container.

Calculations
%ADL (as-received basis) = [W3 – (W1×C1)/W2]×100

Where:
W1 = Bag tare weight
W2 = Sample weight
W3 = Dried weight of bag with fiber after extraction process
C1 = Blank bag correction (final oven–dried weight divided by original blank bag weight)

Equipment
- See Acid detergent fiber (1.7.1)

References

- Goering HK and VanSoest PJ.1970.
- Anonymous. 1995.
- AOAC. 1990.

1.7.3. Neutral Detergent Fiber (NDF) in Feeds using Filter Bag Technique

Principle
This method determines Neutral Detergent Fiber, which is the residue remaining after digesting in a detergent solution. The fiber residues are predominantly hemicelluloses, cellulose, and lignin.

Scope
This method is applicable to grains, feeds, forages and all fiber–bearing material.

Reagents / Solutions
- Neutral Detergent Solution: Add 30 g sodium lauryl sulfate, USP; 18.61 g Ethylenediamine-tetraacetic disodium salt, dihydrate; 6.81 g sodium tetraborate decahydrate; 4.56 g sodium phosphate dibasic, anhydrous; and 10 ml triethylene glycol, in 1 l distilled H_2O Check pH range to 6.9 to 7.1. Agitate and heat to aid solution
- Alpha-amylase: Heat-stable bacterial alpha-amylase: activity = 17,400 Liquefon Units/ml (FAA, ANKOM Technology)
- Sodium sulfite: Na_2SO_3, anhydrous.

Preparation of sample
Grind samples in a centrifugal mill with a 2 mm screen, or cutter type (Wiley) mill with a 1 mm screen. Samples ground finer may have particle loss from the filter bags and result in low values.

Procedure
- Use a solvent resistant marker to label the filter bags. Weigh filter bag (W1) and zero balance. Do not pre-dry filter bags; any moisture will be accounted for by the blank bag correction
- Weigh 0.45–0.55 g of prepared sample (W2) directly in filter bag. Avoid placing the sample on the upper 4 mm of the bag
- Completely seal the upper edge of the filter bag within 4 mm of the top, with the heat sealer encapsulating the sample. Heat sufficiently to completely seal the filter bag and allow enough cooling time (2 sec) before removing the bag from the heat sealer
- Weigh one blank bag and include in run to determine blank bag correction (C1)
- **Pre-extraction of fat-rich products (>5% fat)** (as for Acid detergent fiber)
- Place a maximum of 24 bags into the Bag Suspender. All nine trays should be used regardless of the number of bags being processed Place three bags per tray and then stack trays on center post with each level rotated to120 degrees. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the weight on top to keep it submerged. Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cold water and exhaust
- When processing 24 sample bags, add 1900–2000 ml of ambient ND solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of ND solution (use a minimum of 1500 ml to ensure Bag Suspender is covered). Add 20 g (0.5 g/50 ml of ND solution) of sodium sulfite to the solution in the vessel
- Turn Agitate and Heat on, and confirm agitation. Set timer for 75 min and close lid
- At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid. 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
- After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900 ml of (70–90 °C) rinse water, and 4 ml of alpha–amylase to the first and second rinses. Turn Agitate on and rinse for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat hot water rinses a total of three times
- When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker, add enough acetone to cover bags and soak for 3–5 min.

- Remove bags from acetone and place on a wire screen to air-dry. Completely dry in oven at 105±2 °C (most ovens will complete drying within 2–4 h). Do not place bags in the oven until acetone has completely evaporated
- Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags (W3). Do not use conventional desiccator container.

Calculations
%NDF (as–received basis) = [W3 – (W1×C1)/W2]×100

Where:
W1 = Bag tare weight
W2 = Sample weight
W3 = Dried weight of bag with fiber after extraction process
C1 = Blank bag correction (final oven-dried weight divided by the original blank bag weight)

Caution
- Powdered chemicals will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical
- Acetone is extremely flammable. Avoid static electricity and use a fume hood when handling.

Equipment
- As for Acid detergent fiber.

References

- Goering HK and VanSoest PJ. 1970.
- Anonymous. 1995.
- AOAC. 1990.

1.7.4. Crude Fiber Analysis in Feeds by Filter Bag Technique

Principle
This method determines crude fiber which is the organic residue remaining after digesting with 0.255 *N* H_2SO_4 and 0.313 *N* NaOH. The compounds removed are predominantly protein, sugar, starch, lipids and portions of both the structural carbohydrates and lignin.

Scope
This method is applicable for all feed materials such as grains, meals, pet foods, mixed feeds, forages and the following oilseeds: corn and soybeans.

Reagents / Solutions
- Sulfuric acid solution: Density 0.255±0.005, 1.25 g H_2SO_4/100 ml.

Concentration must be checked by titration. Sulfuric acid is a strong acid and will cause severe burns. Protective clothing should be worn when working with this acid. Always add acid to water and not the reverse

- Sodium hydroxide solution: 0.313±0.005 *N*, 1.25 g NaOH/100 ml. Concentration must be checked by titration. Sodium hydroxide can severely burn skin, eyes and respiratory tract. Protective clothing should be worn. Always add caustic material to water and not the reverse.

Preparation of sample

As for Acid detergent fiber.

Procedure

- Use a solvent and acid resistant marker to label the filter bags. Weigh filter bag (WI) and zero balance. Do not pre-dry filter bags; any moisture will be accounted for by the blank bag correction
- Weigh 0.95–1.0 g of prepared sample (W2) directly in filter bag Avoid placing the sample on the upper 4 mm of the bag
- Completely seal the upper edge of the filter bag within 4 mm of the top with the heat sealer encapsulating the sample. Use sufficient heat to seal the filter bag and allow enough cool time (2 sec) before removing the bag from the heat sealer
- Weigh one blank bag and include in run to determine blank bag correction (C1)
- Extract fat from samples by placing all bags into a 350 ml container. Add enough petroleum ether to cover bags and soak for 10 min. Pour off solvent and allow bags to air–dry. Spread sample uniformly inside the filter bag by shaking and flicking the bag to eliminate clumping
- Place a maximum of 24 bags into the Bag Suspender. All nine trays must be used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated to 120 degrees. Insert the Bag Suspender with bags into the fiber analyzer vessel and place the Bag Suspender weight on top of the empty 9th tray to keep it submerged. Prior to inserting the Bag Suspender, if the vessel temperature is warm from a previous run, add cool water and exhaust
- When processing 24 sample bags, pour 1900–2000 ml of ambient temperature acid (0.255 *N* H_2SO_4) solution to the fiber analyzer vessel. If processing less than 20 bags, add 100 ml/bag of acid solution (use minimum of 1500 ml to ensure Bag Suspender is covered)
- Turn Agitate and Heat on and confirm that Bag Suspender is agitating properly. Close and completely seal the lid of vessel. Extract samples for a total of 40 min
- At end of extraction, turn Heat and Agitate off. Open the drain valve (slowly at first) and exhaust hot solution before opening lid

- Note: 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
- After the solution has been exhausted, close the exhaust valve and open the lid. Add 1900 ml of (50–85 °C) rinse water and agitate for 5 min. The lid may be sealed with the Heat on or left open with the Heat off. Repeat hot water rinse (total of two rinses)
- When the rinsing process is complete remove the samples. Gently press out excess water from bags. Place bags in a 250 ml beaker, add enough acetone to cover bags and soak for 3–5 min.
- Remove bags from acetone and place on a wire screen to air–dry. Completely dry in oven at 105±2 °C (most ovens will complete drying within 2–4 h). Do not place bags in the oven until acetone has completely evaporated
- Remove bags from oven, place directly into a collapsible desiccant pouch and flatten to remove air. Cool to ambient temperature and weigh bags
- Ash the entire bag/sample in pre-weighed crucible for 2 h at 600±15 °C, cool in desiccator and weigh to calculate loss of weight of organic matter (W3).

Calculations
% Crude Fiber = [W3–(W1×C1)/W2]×100

Where:
W1 = Bag tare weight,
W2 = Sample weight
W3 = Weight of Organic Matter (Loss of weight on ignition of bag and fiber)
C1 = Ash corrected blank bag factor (Loss of weight on ignition of blank bag/original blank bag)

Equipment
As for Acid detergent fiber.

References

- Goering HK and VanSoest PJ. 1970.
- Anonymous. 1995.
- AOAC. 1990.

1.8. Determination of Macro and Micro Minerals

Principle
Samples are dried and ashed. The ash is dissolved in acid and diluted with water. The minerals in the diluted ash are determined.

Procedure

Sample preparation

- Mix and homogenize the samples
- Weigh about 1 g of the sample to nearest mg in crucible (Vycor® dish)
- Dry at least for 1 h in 105 °C air forced oven
- Ash in furnace overnight (16 h) at 550 °C
- Cool in desiccator
- Add 1 ml HNO_3 to dissolve the ash
- Transfer to 250 ml volumetric flask and make up volume with H_2O.

Equipment

- Vycor® dish (Fig. 20)
- Volumetric flask 250 ml
- Desiccator (Fig. 7)
- Air forced oven (Fig. 6)
- Muffle furnace (Fig. 8).

Figure 20. Vycor®

1.8.1. Determination of Phosphorus

Standards

- Pipet 0, 5, 10, 15, 20, 25, 30, and 35 ml of diluted stock solution into a series of 50 ml volumetric flasks.
- Add 10 ml molybdovanadate reagent and mix
- Make up volume to 50 ml with H_2O

Sample

- Pipet 10 ml sample solution into 50 ml volumetric flask
- Add 10 ml molybdovanadate reagent and mix
- Make up volume to 50 ml with H_2O.

Measurements

- Set spectrophotometer at wavelength 400 nm.
- Adjust to 0 absorbance with 0 µg/ml standard, and run test samples and standards under the same conditions.
- Determine ppm phosphorus sample solution vs. 4 or 5 standards of respective material.
- Use smaller portion of sample solution if absorbance of sample solution is beyond range of curve.
- The correlation of standard linear regression should yield a coefficient of ≥0.999.

1.8.2. Determination of Potassium and Sodium

Standards

Use 0, 1, 2, 3, 4, 6, 8 and 10 ppm standards.

Sample
Pipet 5 ml sample solution into 50 ml volumetric flask.
Make up to volume.

Measurements
- Adjust flame photometer to 0 absorbance with 0 ppm standard and run test portions along with standards under the same conditions
- Determine ppm minerals sample solution vs 4 or 5 standards of respective material
- Use smaller portion of sample solution if absorbance of sample solution is beyond range of curve
- The correlation of standard linear regression should yield a coefficient of ≥0.999
- Linearity is harvested using standards from 0 to 10 ppm; beyond 10 ppm the standard curve is not linear.

1.8.3. Determination of Calcium and Magnesium

Standards
- Pipet 0, 2.5, 5, 7.5, 10, 12.5 and 15 ml of diluted stock solution into a series of 50 ml volumetric flasks
- Add 5 ml La stock solution, and dilute to volume. You will obtain 0–6 µg/ml of Ca and 0–0.3 µg/ml of Mg in 0.1% La.

Samples
- Pipet 5 ml sample solution into 50 ml volumetric flask
- Add 5 ml La stock solution and dilute to volume.

Measurements
- Set the optimum response of AAS system (Table 1)
- Adjust to 0 absorbance with 0 µg/ml standard, and run test samples and standards under the same conditions
- Determine ppm minerals sample solution vs. 4 or 5 standards of respective material
- Use smaller portion of sample solution if absorbance of sample solution is beyond range of curve
- The correlation of standard linear regression should yield a coefficient of ≥0.999.

Note: All glassware should be rinsed in 1:1 HCl and then rinsed with distilled water.

Reagents / Solutions
Preparation of chemicals for concentrated stock solution
- Calcium carbonate $CaCO_3$, dry overnight at 200 °C
- Ammonium dihydrogen phosphate $(NH_4)H_2PO_4$, dry overnight at 110 °C

- Sodium chloride NaCl. Dry overnight at 110 °C
- Potassium chloride KCl. Dry overnight at 120 °C
- Magnesium stock solution (1000 mg Mg/l)
  - Magnesium metal. Mg 9.95%
  - 1000 mg pure Mg metal in 50 ml H_2O
  - Add slowly 10 ml HCl
  - Make up to 1000 ml
- HCl stock solution(1:3).
  - 75 ml distilled water
  - Add slowly 25 ml HCl

Concentrated standard stock solution

- 500 mg Ca/l
- 300 mg P/l
- 25 mg Mg/l
- 1000 ml volumetric flask
- Add 1.249 g $CaCO_3$
- 1.114 g $(NH_4)H_2PO_4$
- 25 ml Mg stock solution
- Add 30 ml HCl stock solution
- Mix until dissolved and then make up to volume.

Diluted standard stock solution (20 mg Ca/l, 12 mg P/l, and 1 mg Mg/l)

- Add 40 ml concentrated stock solution in 1000 ml volumetric flask
- Make up to 1000 ml with distilled water.

Molybdovanadate solution

While stirring gradually add molybdate solution to vanadate solution.

Molybdate solution

- Weigh 1.5 g ammonium metavanadate
- Add 690 ml hot H_2O
- Add 300 ml HNO_3
- Cool to 20 °C
- Make up to 1000 ml with distilled water.

Vanadate solution

- Weigh 60 g ammonium molybdate tetrahydrate
- Add 900 ml hot H_2O
- Cool to 20 °C
- Make up to 1000 ml with distilled water.

Note: Store at room temperature in polyethylene or glass–stopper Pyrex bottle. Reagent is stable in Pyrex bottle. Discard reagent if precipitate forms.

Lanthanum stock solution (1% La)

- Weigh 11.73 g La_2O_3 or 26.74 g $LaCl_3.7H_2O$
- Add slowly 25 ml HNO_3
- Make up solution to 1 l.

Note: Reagents for mineral standards must be ultra–pure (99.95%). Opened reagents should be resealed and stored in desiccator.

Calculations

Plot absorbance vs. µg/ml minerals using Microsoft Excel. Use the Forecast function of Microsoft Excel to calculate mineral concentration (*C*) based on standard data.

Calculate the content of minerals in mg /100 g as follows:
Minerals,mg/100 g = (C×D)/W
Where:
C = µg/ml Ca or Mg in the assay solution
W = g weight of sample
D = dilution factor×factor for transforming to mg/100 g
D = [(250×50)/10]×(100/1000) = 125

Table 1. Setting AAS for different elements

Element	Wavelength (nm)	Lamp current (mA)	Flame	Burner height (mm)	Support gas flow	Fuel gas flow
Ca	422.7	10	air-acetylene	10	10	2.6
Mg	285.2	10	air-acetylene	5	10	2.4
Cu	324.7	10	air-acetylene	4	10	2.3
Fe	248.3	10	air-acetylene	4	10	2.5
Co	240.7	10	air-acetylene	6	10	2.5

Equipment

- Shimadzu, Atomic Absorption & Flame Emission Spectrophotometer AA–630–12, Japan (AAS). Determine minerals as shown in table 1. Allow the device to warm up for 10 min with flame and source lamp lit (Fig. 21).
- Spectrophotometer, Hitachi U 2000, Tokyo, Japan. The device is equipped with automatic sampler unit and 1 cm cuvette holder unit. Determine P at 400 nm. Allow the device to warm up for 10 min with source lit (Fig. 23).
- Flame photometer, Gallenkamp, UK. The device is equipped with filters to determine Na, K and Li (Fig. 22).

References

- AOAC. 2000.

Figure 21. Atomic absorption and flame emission spectrophotometer

Figure 22. Flame photometer

Figure 23. Spectrophotometer

1.9. Determination of Tannins

Many agro-industrial by-products that play an increasing role as supplementary feed contain tannins. The measurement of the anti–nutritional effects of tannins is, therefore, an important analysis in the nutrition laboratory. In this manual the analyses for determination of tannins that can be deducted at the laboratory are described.

1.9.1. Total Phenolics and Tannins

Principle

This section describes the method for determination of tannins in plant samples by binding with polyvinyl polypyrrolidone (PVPP). PVPP binds tannins. First the total phenols are measured. In a second test the tannins are precipitated together with the PVPP. The results are then subtracted from the total phenols to determine the tannins as a percentage of tannic acid equivalent in dry matter.

Procedure

Sample extraction

- Grind dry material and pass through 2 mm sieve
- Mix ground sample well, grind again and pass through 0.5 mm sieve
- Weigh 200 mg of sample
- Dissolve in 10 ml 70% acetone
- Keep for 35 minutes in ultrasonic water bath
- Centrifuge at 4 °C, 3000 rpm for 10 min
- Take supernatant as original extract (oE)
- Keep oE in refrigerator.

Preparation of standard curve

- Use 100×12 mm test tubes
- Use aliquots of tannin acid solution as in below table
- Add distilled water, Folin-Ciocalteu reagent and sodium carbonate solution as in Table 2
- Vortex after each step
- After adding last component (sodium carbonate solution) keep tubes in darkness for 40 minutes
- Read absorbance for levels of concentration of tannic acid at 725 nm.

Preparation and reading of samples

- Add 450 µl of distilled water to 50 µl of oE
- Vortex
- Add 250 µl of Folin-Ciocalteu reagent
- Vortex
- Add 1250 µl of sodium carbonate solution

Table 2. Standard curve for tannic acid

Tannic acid solution (0.1 mg/ml) µl	Distilled water µl	Folin Ciocalteu reagent µl	Sodium carbonate solution µl	Tannic acid µg
0	500	250	1,250	0
20	480	250	1,250	2
40	460	250	1,250	4
60	440	250	1,250	6
80	420	250	1,250	8
100	400	250	1,250	10

- Vortex
- Keep tubes in darkness for 40 minutes
- Read absorbance and concentration at 725 nm
- Dilute samples (oE) with absorbance >0.600 and read again.

Preparation and reading of samples with PVPP

- Weigh 100 mg of PVPP in 100×12 mm test tubes
- Add 1 ml of distilled water
- Vortex
- Add 1 ml of oE sample
- Vortex
- Keep at 40 °C for 10 min
- Centrifuge at 4 °C, 3000 rpm for 10 min
- Take 100 µl of supernatant to new test tube
- Add 400 µl of distilled water
- Vortex
- Add 250 µl of Folin-Ciocalteu reagent
- Vortex
- Add 1250 µl of sodium carbonate solution
- Vortex
- Keep tubes in darkness for 40 minutes
- Read absorbance and concentration at 725 nm.

In case samples show absorbance greater than 0.600 repeat extraction as follows:

- Increase the water– oE ratio keeping the PVPP constant at 100 mg
- Extract by refrigerated centrifuging as above described
- Take 100 µl of supernatant to new test tube
- Add 400 µl of distilled water
- Vortex
- Add 250 µl of Folin-Ciocalteu reagent
- Vortex
- Add 1250 µl of sodium carbonate solution

- Vortex
- Keep tubes in darkness for 40 minutes
- Read absorbance and concentration at 725 nm.

Calculations

Calculation of total phenols

1. Reading of samples as per the procedure described above gives a concentration (conc.) of tannic acid in µg/µl for 50 µl of the original extracted sample (oE)
2. Concentration for 1 ml of oE in µg can be calculated dividing by 0.05
3. Transfer result into mg tannic acid
4. 200 mg of sample were extracted in 10 ml solvent (20 mg/ml). Therefore 100 mg content 5 times mg of tannic acid
5. Transfer result into g of tannic acid per 100 g of sample.

Step 1–5 can be therefore calculated as:
x = [(conc/0.05)/1000]×5
Express as percentage of tannic acid in dry matter.

Calculation of non–tannin phenols

1. Reading of samples as per above described procedure gives concentration (conc.) of tannic acid in µg/ µl for 100 µl of the supernatant.
2. Concentration for 1 ml of supernatant in µg can be calculated dividing by 0.1.
3. Transfer result into mg tannin acid to get per 10 mg sample since extract is diluted 2–fold.
4. Multiply by 10 to get concentration of tannic acid in 100 mg of sample.
5. Step 1– 4 can be therefore calculated as:
   y = [(conc/0.01)/1000]×10
   Express as percentage of tannic acid in dry matter.
6. Calculate tannin in sample as tannic acid equivalent subtracting the non–tannin phenols from total phenols: x–y % in dry matter

Reagents / Solutions

70% acetone

- Prepare 70% aqueous acetone (v/v).

Folin–Ciocalteu reagent (1N)

- Dilute commercial Folin–Ciocalteu reagent (2*N*) with equal volume of distilled water
- Keep in brown bottle at 4 °C; watch golden color. If color changes to green, discard.

Sodium carbonate (20%)

- Weigh 40 g sodium carbonate ($Na_2CO_3.10H_2O$) in 200 ml distilled water

Figure 25. Ultrasonic water bath

Figure 26. Cooled centrifugebath

Standard tannin acid solution (always prepare fresh)

- Dissolve 13 –15 mg tannic acid in equal volume of dist water (13–15 ml)
- Dilute 1:10 with distilled water and use this as 0.1mg/ml working solution.

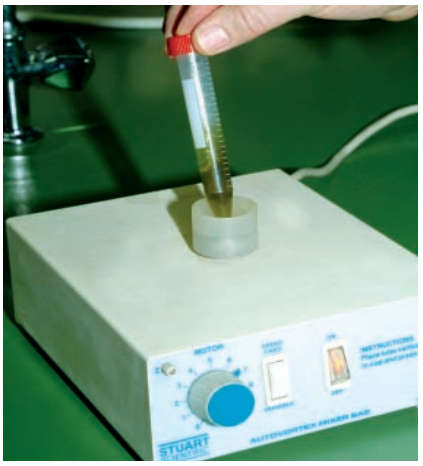


Figure 24. Vortex

Equipment

- Precision balance (Fig. 5)
- Ultrasonic water bath (Fig. 25)
- Cooled centrifuge (Fig. 26)
- Vortex (Fig. 24)
- Spectrophotometer (Fig. 23)
- Eppendorf pipettes of different volumes (Fig. 28)
- Cuvettes
- Test tubes 100×10 mm
- Centrifuge tubes, 13 ml
- Centrifuge tubes, 50 ml.

References

- Makkar HPS. 2003.

1.9.2. Condensed Tannins

Principle

The butanol-HCl-iron method is used to ascertain the presence of con-

densed tannins in feeds. At temperature around 95 °C the interflavan bonds in mineral acids are broken in alcoholic solutions to pink colored anthocyanidins (Makkar 2003). These can be measured at 550 nm. However, Makkar (2003) recommends that the method should be used with caution as a quantitative assay.

Procedure

Sample extraction

- Grind dry material and pass through 2 mm sieve
- Mix ground sample well, grind again and pass through 0.5 mm sieve
- Weigh 200 mg of sample
- Dissolve in 10 ml 70% acetone.
- Keep for 35 minutes in ultrasonic water bath
- Centrifuge at 4 °C, 3000 rpm for 10 min
- Take supernatant as original extract (oE)
- Keep oE in refrigerator.

Preparation and reading of samples

- Use three replications of test tubes 100×10 mm for each sample
- Prepare samples as:
  - 250 µl of oE
  - 1500 µl of Butanol-HCl reagent
  - 50 µl of Ferric reagent
- Cover tubes with glass marbles
- Heat two replications of each sample in heating block or water bath at 97–100 °C
- Use one (unheated) replication as blank
- Read absorption at 550 nm against blank for each sample
- Dilute samples with absorbance greater than 600 nm as follows: dilute oE with 70% acetone, keeping total volume of oE and acetone as 250 µl
- Repeat reading absorption at 550 nm.

Calculations

A: Absorption at 550 nm
F: Dilution factor (if sample diluted because of absorption greater than 600)
DM: dry matter
CT: Condensed tannin (as leucocyanidin equivalent) in dry matter
CT = (A×78.26×F)/(%DM)

Reagents/ Solutions

70% acetone

Prepare 70% aqueous acetone (v/v)

Butanol–HCl reagent

Butanol–HCl 95/5 v/v
Mix 950 ml n–butanol with 50 ml concentrated HCl (37%).

Figure 27. Heating block

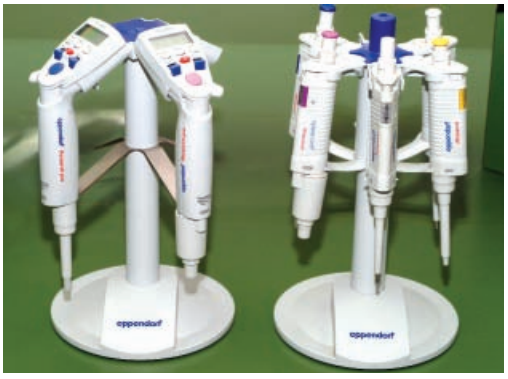


Figure 28. Eppendorf pipettes (different volumes)

Figure 29: Sample extraction

Ferric reagent

- Make 2*N* HCl: make 16.6 ml concentrated HCl up to 100 ml with distilled water
- Add 2 g ferric ammonium sulfate to 100 ml of 2*N* HCl solution
- Store in dark bottle.

Equipment

- Precision balance (Fig. 5)
- Ultrasonic water bath (Fig. 25)
- Cooled centrifuge (Fig. 26)
- Heated water bath or heating block (Fig. 27) set at 97–100 °C
- Vortex (Fig. 24)
- Spectrophotometer (Fig. 23)
- Eppendorf pipettes of different volumes (Fig. 28)
- Cuvettes
- Test tubes 100×12 mm
- Centrifuge tubes, 13 ml
- Centrifuge tubes, 50 ml.

References

- Makkar HPS. 2003.

1.9.3. Bioassay using PEG

Principle (see 1.9.2)

Tannin activity is measured as the percent increase of gas produced in the gas production test (Hohenheim test), on addition of polyethylene glycol (PEG) compared to samples without PEG. PEG binds tannins and inactivates them. With this test the level of Small chain fatty acids (SCFA) can be predicted from the gas production values as an indicator of available energy in the feed.

Procedure

- Weigh 375 mg of feed sample (1 mm ground) and insert carefully in 100 ml calibrated glass syringe
- Weigh 375 mg feed (Ws) and 750 mg polyethylene glycol (peg, mw 4000) in equal number of 100 ml calibrated glass syringes
- Include blank syringes without feed
- Include syringes with standard feed (concentrate or/and hay)
- Keep syringes overnight in circulating water bath at 39 °C
- Prepare fermentation buffer solution, except rumen fluid and reducing solution, in 2 l.Woulff bottle
- Keep bottle in water bath at 39 °C with stirrer overnight

Next morning:

- Keep under CO_2
- Add reducing solution and wait 20 minutes until color changes from blue to purple to colorless
- Add rumen fluid
- Keep stirring under CO_2 for 10 minutes
- Fill 30 ml into glass syringes
- Incubate syringes in 39 °C
- Shake syringes every 30 min during first 4 hours, then once every hour

- Record gas production at 8 hours and push back piston to 30 ml if gas production exceeds 70 ml
- Record gas production at 24 hours and terminate experiment.

Calculations for tannin activity

$G_n = [(V_{24}–V_0–G_0)×F_{St}×0.375]/W_s$

if piston pushed back to 30 ml after 8 hours use:

$G_n = [(V_{24}–V_0+V_8–30–G_0)×F_{St}×0.375]/W_s$

$F_{St} = G_{St}/G_{St\ measured}$

Reference value (F_{St}) is gas production G_{St} of the standard sample (e.g. hay and/or concentrate) as per supplier (e.g. Landesarbeitskreis. Fütterung, Baden Württemberg e.V. (LAF), Hohenheim, 70578 Stuttgart, PF 7200220) compared to the measured gas production in the test ($G_{St\ measured}$).

G_n = gas production value in ml
G_0 = gas production of blank syringes (ml)
V_0 = volume in ml at begin
V_8 = volume in ml at 8 hours
V_{24} = volume in ml at 24 hours
W_s = weight of dried sample in mg

%Tannin activity = $G_{PEG}– G_{noPEG}$

G_{PEG} = Gas production with PEG
G_{noPEG} = gas production without PEG

Prediction of Small Chain Fatty Acids (SCFA)

Relationship between SCFA production (nmol) and gas volume (ml) after 24 hours incubation (example of tannin containing browses) (Makkar, 2003):

$SCFA_{noPEG} = –0.0601+0.0239×G_{24}$
$R^2 = 0.953$; N = 39; P <0.001

$SCFA_{PEG} = 0.0521+0.0207×G_{24}$
$R^2 = 0.925$; N = 37; P <0.001

overall SCFA = $–0.00425+0.0222×G_{24}$
$R^2 = 0.935$; N = 76; P <0.001

Reagents/ Solutions

Rumen fluid

- Collect rumen fluid before morning feeding from 2 rams
- Filter through two–layer cheese cloth into thermos container
- Keep at 39 °C and under carbon dioxide (CO_2).

Fermentation buffer solution

- 630 ml of bicarbonate buffer
- 315 ml of macromineral solution
- 0.16 ml of micromineral solution
- 1.6 ml of resazurine solution
- 945 ml distilled water
- 60 ml of fresh prepared reducing solution
- 660 ml rumen fluid.

Bicarbonate buffer

- 35 g sodium bicarbonate ($NaHCO_3$)
- 4 g ammonium carbonate
- Dissolve in 500 ml distilled water and then make up to 1 l.

Macromineral solution

- 6.2 g potassium dihydrogen phosphate (KH_2PO_4)
- 5.7 g disodium hydrogen phosphate (Na_2HPO_4)
- 0.6 g magnesium sulphate ($MgSO_4.7H_2O$)
- Dissolve in 500 ml distilled water and then make up to 1 liter.

Micromineral solution

- 10 g manganese chloride ($MnCl_2.4H_2O$)
- 13.2 g calcium chloride ($CaCl_2.2H_2O$)
- 1 g cobalt chloride ($CoCl_2.6H_2O$)
- Dissolve in 50 ml distilled water and then make up to 100 ml.

Resazurine

- 0.1 g resazurine in 100 ml distilled water

Reducing solution

- 996 mg sodium sulphide ($Na_2S.9H_2O$)
- Dissolve in 94 ml distilled water
- 6 ml 1 N sodium hydroxide solution (NaOH)
- 1 N NaOH = 4 g NaOH in 100 ml distilled water.

Equipment

- Precision balance (Fig. 5)
- Circulating water bath (rotating syringe holder) (Fig. 30)
- Circulating water bath with holder for syringes (Fig. 31)
- Heating plate with stirrer (Fig. 32)
- CO_2 cylinder (Fig. 33)
- Woulff bottle (Fig. 32)
- Dispenser (Fig. 32)
- 100 ml special syringes for GPT (Fig. 34).

Reference

- Makkar HPS. 2003.

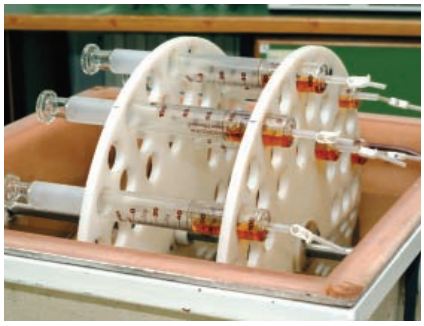


Figure 30. Circulating water bath (rotating syringe holder)

Figure 31. Circulating water bath with holder for syringes

Figure 32. Heating plate with stirrer, Woulff bottle, and dispenser

Figure 33. CO_2 cylinder

Figure 34. Syringe for GPT & weighing spatula

1.10. Digestibility Determination

In vivo (in animal) and *in vitro* (in test tube) procedures are used to evaluate forage quality. Utilization of dry matter is measured as digestibility of feed.

Castrated rams with an external opening into the rumen (*canula*) are used to collect rumen fluid for artificial inoculation (*in vitro*) of feeds in the laboratory (Fig. 35, 36). For the *in situ* or in *sacco* method small nylon bags containing feeds are inserted into the rumen to measure digestion. The donor animals are fed with similar diets to the forages being evaluated.

Figure 35. Collecting rumen fluid

Figure 36. Thermos for rumen fluid

1.10.1. *In vitro* Digestibility

Principle

The two steps procedure described is used for in vitro determination of digestibility of forages. In the first step, dried ground forages are incubated in test tubes with rumen fluid for a given period of time. The tubes also contain buffer solution, macro-minerals, trace-minerals, nitrogen sources, and reducing agents to maintain pH and provide nutrients required for growth of rumen bacteria. Because oxygen is toxic to rumen bacteria, solutions are gassed with carbon dioxide to maintain anaerobic conditions, and temperature is held at 39 °C (body temperature) during the incubation. In the second step, after 48 hours of incubation, an enzyme solution is added to simulate digestion in the small intestine.

Procedure

- Add 0.5 g of ground sample in centrifuge tubes (W_s), include blanks
- Mix buffer solution and rumen fluid as follows (volumes per one sample):
  - 53 ml of buffer solution and adjust to ph 6.9 gazing with carbon dioxide.
  - Add 13 ml of rumen fluid.
- Add 66 ml of mixture to sample in centrifuge tubes
- Keep in water bath at 36.5–38 °C for 48 hours (stir 2–3 times per day)
- Then add 5 ml of pepsin solution carefully in 0.5 ml doses
- Keep in water bath at 36.5–38 °C for another 48 hours (stir once a day)
- Centrifuge for 10 min at 4000 rpm
- Take off excessive liquid under vacuum using sinta glass gas distribution tube
- Transfer residues to pre-weight oven–dry crucibles, rinsing with small amount of distilled water
- Dry crucibles overnight at 105 °C and weigh (W_t)
- Cool to room temperature in desiccators
- Weigh sample in crucible (W_0)
- Ignite at 550 °C in muffle furnace for four hours
- Cool to room temperature in desiccators
- Weigh sample in crucible (W_a).

Calculations

$$\% \text{ DMD} = [(W_s \times (\%DM/100) - (W_0 - W_t) + \text{blank}) \times 100] / [(W_s \times \%DM)/100)]$$

$$\% \text{ OMD} = [(W_s \times (\%DM)/100) \times (\%OM/100) - (W_t - W_a) + \text{blank}) \times 100] / [(W_s \times \%DM)/100]$$

Reagents/ Solutions

Rumen liquor

Rumen fluid has been taken through a fistula from the castrated ram. The fluid should be kept in a thermos container to maintain the body temperature of the animal (38–39 °C). Liquor filtered through a 2–fold layer of muslin into a flask while passing CO_2 to the flask to displace air from above the fluid.

Mixed chloride solution

- Make up to 2 l with distilled water
- 47 g sodium chloride (NaCl)
- 57 g potassium chloride (KCl)
- 12 g magnesium chloride ($MgCl_2.6H_2O$)
- 4 g calcium chloride ($CaCl_2.2H_2O$).

Stock solution

- Make up to 2 l with distilled water
- 37 g di–sodium orthophosphate (Na_2HPO_4)
- 98 g sodium hydrogen–carbonate ($NaHCO_3$)
- 200 ml mixed chloride solution.

Buffer solution

- 10.4 ml stock solution
- 41.6 ml distilled water
- 1 ml ammonium sulphate (66.07g/l) ($(NH_4)_2SO_4$).

Pepsin solution

- 720 ml distilled water
- 280 ml hydrochloric acid fuming 36.5% conc. (HCl)
- 24 g pepsin.

Equipment

- Precision balance (Fig. 5)
- Centrifuge (Fig. 37)
- Air-circulation oven (Fig. 6)
- Water bath (Fig. 38)
- Vacuum extraction unit (Fig. 16)
- Muffle furnace (Fig. 8)
- Desiccator (Fig. 7)
- Sinta glass gas distribution tubes (Fig. 39)

Figure 37. Centrifuge with centrifuge tubes

Figure 38. Water bath with centrifuge tubes, adding pepsin

Figure 39. Sinta glass gas distribution tubes

- Nalgene centrifuge tubes 28 mm×160 mm (Fig. 37)
- Crucibles (Fig. 9).

References
- Tilley JMA and Terry RA. 1963.

1.10.2. Gas Production Test (Hohenheim Method)

Principle
The gas production test is based on the association between rumen fermentation and gas production.
The *in vitro* gas production method can be used to measure the metabolizable energy of feeds and to quantify utilization of nutrients.

Procedure
- Weigh 200 mg of feed sample (1 mm ground) and insert carefully in 100 ml calibrated glass syringe
- Include blank syringes without feed
- Include syringes with standard feed (concentrate or/and hay)
- Keep syringes overnight in circulating water bath at 39 °C
- Prepare fermentation buffer solution except rumen fluid and reducing solution in 2 l Woulff bottle
- Keep bottle in water bath at 39 °C with stirrer overnight.

Next morning
- Keep under CO_2
- Add reducing solution and wait 20 minutes until color changes from blue to purple to colorless
- Add rumen fluid
- Keep stirring under CO_2 for 10 minutes
- Fill 30 ml into glass syringes
- Incubate syringes in 39 °C
- Shake syringes every hour during first four hours, then twice every hour
- Record gas production at 8 hours and push back piston to 30 ml if gas production exceeds 70 ml
- Record gas production at 24 hours (V_{24}) and terminate experiment.

Reagents / Solutions
Rumen fluid
- Collect rumen fluid before morning feeding from 2 rams
- Filter through two-layer cheese cloth into thermos container
- Keep at 39 °C and under carbon dioxide (CO_2).

Fermentation buffer solution
- 630 ml of bicarbonate buffer
- 315 ml of macromineral solution

- 0.16 ml of micromineral solution
- 1.6 ml of resazurine solution
- 945 ml distilled water
- 60 ml of fresh prepared reducing solution
- 660 ml rumen fluid.

Bicarbonate buffer

- 35 g sodium bicarbonate ($NaHCO_3$)
- 4 g ammonium carbonate
- Dissolve in 500 ml distilled water and then make up to 1litre.

Macromineral solution

- 6.2 g potassium dihydrogen phosphate (KH_2PO_4)
- 5.7 g disodium hydrogen phosphate (Na_2HPO_4)
- 0.6 g magnesium sulphate ($MgSO_4.7H_2O$)
- Dissolve in 500 ml distilled water and then make up to 1 l.

Micromineral solution

- 10 g manganese chloride ($MnCl_2.4H_2O$)
- 13.2 g calcium chloride ($CaCl_2.2H_2O$)
- 1 g cobalt chloride ($CoCl_2.6H_2O$)
- Dissolve in 50 ml distilled water and then make up to 100 ml.

Resazurine

- 0.1 g resazurine in 100 ml distilled water.

Reducing solution

- 996 mg sodium sulphide ($Na_2S.9H_2O$)
- Dissolve in 94 ml distilled water
- 6 ml 1 *N* sodium hydroxide solution (NaOH)
- 1 *N* NaOH = 4 g NaOH in 100 ml distilled water.

Calculations

Calculation for gas production

$G_{24} = [(V_{24} – V_0 – G_0) \times F_{St} \times 200] / W_s$

If pushed back after 8 hours:

$G_{24} = [(V_{24} – V_0 + V_8 – G_0) \times F_{St} \times 200] / W_s$

G_{24} = gas production value (ml/200 mg) at 24 hours
G_0 = gas production of blank syringes (ml)
V_0 = volume in ml at begin
V_8 = volume in ml at 8 hours
V_{24} = volume in ml at 24 hours
W_s = weight of dried sample in mg
$F_{St} = G_{St} / G_{St\ measured}$

Reference value (F_{St}) is gas production G_{St} of the standard sample (e.g. hay and/or concentrate) as per supplier (e.g. Landesarbeitskreis. Fütterung, Baden Württemberg e.V. (LAF), Hohenheim, 70578 Stuttgart, PF 7200220) compared to the measured gas production in the test ($G_{Stmeasured}$).

Calculation for organic matter digestibility (OMD)

$\%OMD = 14.88+0.889\ G_{24}+0.45\ CP$

CP = Crude Protein

Calculation for metabolizable energy (ME)

$ME(MJ/kg\ DM) = 2.2+0.136\ G_{24}+0.057\ CP$

Calculation for net lactation energy (NEL)

$NEL(MJ/kg\ Ws) = 0.0663\ G_{24}+0.095\ CP+0.228\ CF+0.079\ N_{free}- 3.49$

N_{free} = N-free extract in % dry weight

CF = Crude Fat

Equipment

- Precision balance (Fig. 5)
- Circulating water bath with holder for syringes (Fig. 31)
- Heating plate with stirrer (Fig. 32)
- CO_2 cylinder (Fig. 33)
- Woulff bottle (Fig. 32)
- Dispenser (Fig. 32)
- 100 ml glass syringes for GPT (Fig. 34).

References

- Makkar HPS. 2002.
- Menke KH, Raab L, Salewski A, Steingass H, Fritz D and Schneider W. 1979.
- Methodenbuch Bd.1988.

1.10.3. *In sacco* Degradability and Digestibility

Principle

The incubation of feeds in nylon bags inside the rumen is used to measure the degradation of the feedstuff. Degradability is expressed as loss of weight during incubation. Digestibility can be measured by analyzing the residues for NDF.

Procedure for degradability

- Weigh 3 g of dried and ground feed (3 mm mesh size) in each nylon bag (W_s)
- Attach 3–5 bags to each plastic tube and insert in rumen
- Up to 12 bags can be inserted in rumen
- Use at least three animals
- Diet of animals same as feed in nylon bags

- Incubation time: 4, 8, 16, 24, 36, 48, 72 and 96 hours
- Insert bags at different time intervals to take them out at the same time
- Take out bags and put immediately in cold water to stop fermentation
- Wash in washing machine for 20 minutes at 22–25 °C (record revolutions per minutes, rpm)
- Dry bags at 65 °C for 30 hours
- Weigh residues (W_r).

Calculations for dry matter degradability (DM_{deg})
Calculate dry matter as under 1.2.1. for feed sample (DM_s) and residue (DM_r)

$$DM_{deg} = [(DM_s – DM_r)/DM_s]\times 100$$

Procedure for digestibility
- Transfer contents of nylon bag to Berzelius beaker
- Proceed with NDF determination for residue (NDF_r) as under 1.6.1.

Calculation of digestibility (D)
$$D = (NDF_r/DM_r)\times 100$$

Equipment
- Precision balance (Fig. 5)
- Air circulating oven (Fig. 6)
- Muffle furnace (Fig. 8)
- Nylon bags of 9x16 cm made from indigestible material; 40–60 micron mesh size, (Fig. 40)
- Plastic tubes with attachments for nylon bags (Fig. 41).

References

- Ørskov ER, Hovell FD DeB and Mould F. 1980.

Figure 40. Nylon bags

Figure 41. Plastic tubes with attachments for nylon bags

2. Assessment of Silage Nutritional Quality

2.1. Definition and Advantages of Silage

Ensiling is a forage preservation method based on spontaneous acid lactic fermentation under anaerobic conditions. Silage techniques minimize the loss of nutrients right from harvest, to storage. Moreover, they also improve the quality of feed. The epiphytic (existing on plants) lactic acid bacteria that are present on forage crops are involved in the fermentation water-soluble carbohydrates to lactic acid and, to a lesser extent, to acetic acid. As a result the pH level of the ensiled material is reduced so the activity of spoilage microorganisms is inhibited.

The advantage of using silage includes the following (Cowan 2001, Schroeder 2004, Romero et al. 2006):

- As a reserve during times of extreme feed shortage periods, which entails ensiling pasture, crops or high-moisture by-products under optimal conditions and storing them for a long period
- To profit from excess growth. Generally, this excess is considered to be a waste. Ensiling allows excess growth to be stored so that losses due to maturation or decay in situ are avoided
- To enable storage of perishable materials since the ensiling process ensures the feed can be used over an extended period of time, for instance, the ensiling of wet by-products
- To preserve the dry matter content and maintain income potential (palatability, consistency and composition) of the fermented feed
- To balance the nutrient content of the diet. The silage is used to provide nutrients whenever the feeds available are deficient (example, the use of legume silage to complement maize silage).

References

- Cowen T. 2001.
- Romero LA, Mattero J, Comeron EA, Gaggiotti MC and Cuatrin A. 2006.
- Schroeder JW. 2004.

2.2. Sampling

Collecting a representative sample on farm is vital if the laboratory analysis is to reflect what is actually being fed. To help ensure representative samples are taken, the following key procedures are recommended:

Wait at least six weeks after harvesting before sampling silage, to ensure fermentation has completed.

Representative samples should be made by taking 1 to 5 kg of silage from different locations of the freshly open front silo. Avoid taking samples from the periphery of the silo and from moldy or damaged parts. The face of the silo gives a better sample of the silage the animals are actually eating.

Samples should be thoroughly mixed and if the final sample has to be reduced before being sent to the laboratory, it should be prepared by the quartering method outlined below:

- Tip the sample onto a clean surface such as a clean board or worktop. Rough concrete is not suitable
- Thoroughly mix the sample ensuring an even distribution of material
- Using a clean board or card, halve the sample by dividing across the pile and separating into 2 piles. If one pile is approximately 1 kg, carefully place this into a clean plastic bag ensuring all the material in the pile is included
- After placing in the plastic bag, remove as much of the air as possible by squeezing the bag, and seal. This will help reduce silage changes during transport and make your analysis more accurate
- Label the bag clearly with sample number, forage mixture, stage of maturity and date harvested, and store immediately in a cold place, preferably in a freezer to prevent bacterial decay, until analyzed.

2.3. Silage Quality Assessment

Fig. 42 presents the different steps of silage nutritional quality assessment. There are two major procedures: (i) Assessment of fermentation quality and (ii) Conventional feed analysis. The latter has similarities to the general methodology of feed analysis procedures already described. Focus will be made on fermentation quality assessment. Special mention is made for dry matter that needs to be corrected for volatile components including ammonia and volatile organic acids (acetic, propionic, butyric, isobutyric).

2.3.1. Dry Matter (DM)

This is the material left after all water has been removed by drying. Values given are ‘corrected values’ to allow for volatile fatty acids and other components lost during oven drying.
DM = 100–MC

Where
MC: sample moisture content in %
%MC = W_1–W_2
W_{si}: weight of the sample in gram
W_{sf}: weight of the sample after drying at 105 °C in air-circulation oven and up to constant weight in gram
$W_1 = (100 \times W_{sf})/W_{si}$
W_2: Weight of volatile acids in %

2.3.2. Extraction of Silage "Juice"

Silage "juice" extraction is made on fresh or frozen samples. Frozen samples should be kept in tight bags and left in room temperature (15 to 20 °C) overnight. Juice may be extract by pressing or maceration. Maceration is recommended because pressing is not applicable for silages with high dry matter contents. The following procedure is recommended:

- 200 g of wet silage sample are weighed in a 1 l beaker to which 1 l of de–ionized water is added. Mix carefully and leave overnight at 4 °C
- Blend the sample for 2 min and filter through coarse (20–25 µm particle retention) filter paper.

The extract will be used to determine pH, ammonia, volatile fatty acids (Acetic, Proprionic, Butyric, & Iso–butyric acids) and lactic acid.

2.3.3. pH

pH is a key criteria to evaluate silage fermentation. Generally, the lower the pH, the better preserved and more stable is the silage. Haylage

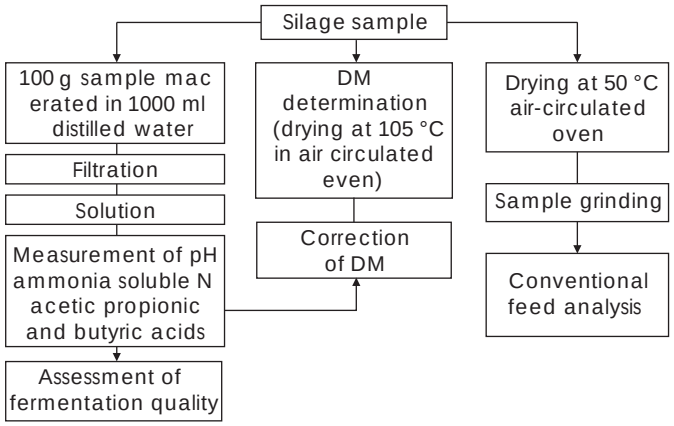


Figure 42. Major steps in silage nutritional quality assessment

should reach a final pH of around 4.5 and corn silage near 4.0. The pH of the forage alone is not a good indicator of the quality of the silage, or the type of fermentation that occurred. Forages ensiled at moisture levels greater than 70% may develop large populations of clostridia bacteria, which produce butyric acid rather than lactic acid. This may result in sour silage with a pH of 5.0 or above.

pH is measured directly using a conventional digital pH meter.

2.3.4. Ammonia

Ammonia Nitrogen (NH_3) N shows the proportion of N (including protein) that has been broken down during ensilage and is the best indicator of silage fermentation. A value <50 g/kg N indicates an excellent fermentation, a stable silage and minimal nutrient loss. Values >150g/kg indicate a poor fermentation.

The methodology used is adapted from Weatherburn (1967).

Reagents/ Solutions

Solution A

- Phenol (6 B4): 10 g/l
- Sodium nitroprusside with FeIII (4A1): 0,05 g/l (0.05687 if it's dehydrated)

Solution B

- NaOH: 5 g/l
- NaClO: 8.4 ml/l
- If using NaClO (commercial containing 40 g of active Chlorine per liter): 10.5 ml are needed, equivalent to 0.42 g of NaClO)

Preparation of ammonia solution (100 µg N–NH4/ml)

- Dry the $(NH_4)_2SO_4$ at 105 °C in an oven for one hour
- Dissolve 4,714 g of the previous dry reagent in 1000 ml of distilled water
- Using the basic solution of ammonia, prepare a solution containing 10 µg of $N–NH_4$/ml (add 99 ml of water to 1 ml of the basic solution of ammonia)
- Prepare a standard curve using the previous solution (10 µg $(NH_4)_2SO_4$ /ml), as follows : put 0, 1, 2, 4, 6, 8 and 10 ml of the solution in 7 test tubes, adjusting the volume to 10 ml with distilled water. Stir the tubes.

Silage extract conditioning

- Pipette 5 ml of the silage extract in a centrifugation tube, keeping it cold on ice
- Centrifuge at 4000 rpm for 20 min at 4 °C

- Take 0.1 ml from the supernatant and add 4.9 ml of distilled water. Stir and keep cold on ice.

Procedure

- Take 1 ml of each tube from the standard curve and the unknowns
- Add 5 ml of solution A and stir
- Add 5 ml of solution B and stir
- (Up to this step, keep the samples on ice)
- Prepare a blank containing 1 ml water, 5 ml of solution A and 5 ml of solution B
- Incubate at 37 °C for 20 minutes. Optionally, keep at 20 °C or more, for at least 30 minutes
- Read with the spectrophotometer at 625 nm
- Make a standard curve using the concentrations of $N–NH_4$ in µg / ml as abscissas and the optical density as the ordinates
- From the calibration curve, calculate the concentration of the unknown samples, by interpolation.

The ratio ammonia nitrogen/total nitrogen is used as indicator of the fermentation quality. The following scale is recommended:

Table 3. Scale for fermentation quality

Ratio ammonia nitrogen/total nitrogen %	Conservation
0-5	Very good
5-10	Good
10-15	Standard
15-20	Satisfactory
20-30	Poor
>30	Bad

References

- Weatherburn MW. 1967.

2.3.5. Determination of Volatile Fatty Acids

Total fermentation acids (TFA) – the total amount of acid produced during fermentation. It includes lactic, butyric and acetic acids, and may also extend to propionic acid and ethanol.
Volatile fatty acids (VFA) – will be high when fermentation is poor. The undesirable VFAs are butyric and, to a lesser extent, acetic acid. These are associated with high total VFA and give a distinctive and persisting smell to badly made silages.
Acetic acid – is looked upon as a normal constituent of silage, but less desirable than lactic acid. High levels can restrict intake.

Butyric acid – production indicates poorly fermented silage. This can be produced under anaerobic conditions due to a variety of factors including high soil contamination, slow rate of fermentation, low DM content and secondary fermentation. The butyric acid content will often be added to the acetic acid content, termed total volatile fatty acids VFA s, and expressed as a percentage of total fermentation acids (TFA).

Better quality silages with low acetic, ammonia–N, butyric and high lactic and sugars are more prone to aerobic spoilage.

Procedure
The methodology used is based on the one developed by Jouany (1981).

Sample preparation
The silage juice should be stabilized immediately. For this purpose the following steps are recommended:

- Preparation of a conservation reagent: a solution is prepared by adding ortho H_3PO_4 (5 % v/v) and $HgCl_2$ (1 % w/v)
- To 10 ml of silage juice add 1 ml of the conservation reagent.
- Store at –15 °C in a deep freezer
- Prior to analysis, fresh or frozen samples are centrifuged at 2000 rpm for 5 minutes at 4 °C.

Chromatography

- Equipment: Gas chromatograph equipped with flame ionization detector. The column is made of glass, 1.5 m long and with an internal diameter of 2.17 mm. The column is filled with chromosorb W–AW–60–80 mesh, and impregnated with 10 % (w/w) SP 1200 (Supelco) and 1% (w/w) ortho H_3PO_4. It is conditioned at 160 °C overnight with a nitrogen flux of 30 ml/mn
- The analysis is conducted by injecting 1 ml sample (including the conservation reagent) to which is added 0.1 ml of internal standard composed of 1% (w/v) 4–methyl valeric acid
- Working conditions:
  - N_2 (vector gas) : 30 ml/mn.
  - H_2 (detector): 40 ml/mn.
  - Air (detector): 400 ml/mn.
  - Temperature: 125 °C (oven), 155 °C (detector), 165 °C (injector).
  - Volume of the sample to inject: 5 µl.

Calculations
VFA analysis is based on (i) A standard solution (solution A) and an internal standard, which help determine the “response factor” used for calculation.

Determination of the response factor

The following formula is used to calculate the response factor (Ka)

Ka = (Sa/S)/(1/Ca)

Where:
Ka: response factor of component "a".
Sa: pic surface of component "a".
S: pic surface of the internal standard.
Ca: concentration of component "a" in the standard solution "A".

The composition of the standard solution "A" is as follows:

- Acetic acid (C_2) 6.00 g
- Propionic acid (C_3) 1.00 g
- Isobutyric acid (IC_4) 0.125 g
- Butyric acid (C_4) 1.75 g
- Isovaleric acid (IC_5) 0.25 g
- Valeric acid (C_5) 0.25 g

After injecting the silage sample, VFA contents are determined according to the formula:

Ca = (Sa/S)/(1/Ka)

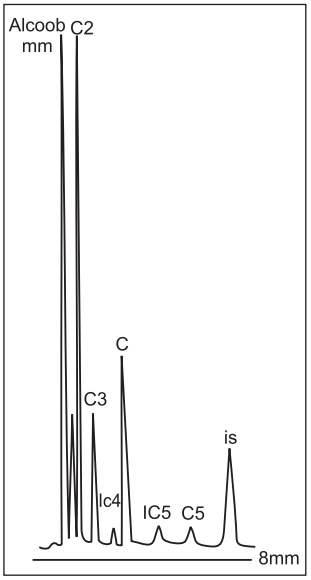


Figure 43. Typical chromatogram of silage juice (C2: acetic acid; C3: propionic acid; IC4: Iso-butyric acid; C4: butyric acid; IC5: Isovaleric acid; C5: Valeric acid; IS: Internal standard

Where
Ca: concentration of component "a" in the silage sample
Sa: Pic surface of component "a" of the silage sample
S: Pic surface of the internal standard injected concomitantly with the silage sample
Ka: Response factor of component "a"

References

- Jouany JP. 1981.

2.3.6. Lactic Acid

Lactic acid gives an indication of the quality of forage fermentation, being produced almost exclusively by the lactobacilli responsible for good silage fermentation and effective preservation. Grass silages typically have lactic acid contents of 60–150 g/kg, higher values suggesting more rapid fermentation, better protein preservation and less likelihood of fermentation of by-products that reduces intake. Like butyric acid, lactic acid contents maybe expressed as a proportion of the

total fermentation acids; higher levels indicating better fermentations, and values over 70% TFA being ideal.

Lactic acid should be the primary acid in good silage. This acid is stronger than other acids in silage (acetic, propionic and butyric) and thus usually responsible for most of the drop in silage pH. Secondly, fermentation that produces lactic acid results in the smallest loss of dry matter and energy from the crop during storage.

Some common reasons for low lactic acid content are as follows:

- Restricted fermentation due to high DM content (especially legumes and grasses with >50% DM)
- Restricted fermentation due to cold weather
- Sample taken after considerable aerobic exposure that has degraded lactic acid
- Silages high in butyric acid (Clostridial silages) are usually low in lactic acid.

Determination of lactic acid in silage juice

The method suggested uses a simple colorimetric assay based on the method developed by Barker and Summerson (1941), and modified by Kimberley and Taylor in 1996.

In this method, acetaldehyde is released from lactic acid by hot sulfuric acid. The acetaldehyde is reacted with copper and p–phenylphenol (pPP) to yield a chromagen, which absorbs at 570 nm.

Reagents/Solutions

- Concentrated H_2SO_4 (96%)
- 4% $CuSO_4.5H_2O$ in dd H_2O
- 1.5% p–phenylphenol in 95% ethanol.

Development of the standard curve

To develop a lactic acid standard curve:

- Add 0 to 30 micrograms of lactic acid to 16×150 mm borosilicate tubes. The curve should be in 5–micrograms increments or less
- Add 3 ml of concentrated H_2SO_4 and mix with a vortex mixer. This quantity of acid is defined here as 82% acid
- Incubate at 95–100 °C for 10 minutes in a steam water bath
- Cool at room temperature using a water bath.
- Add 50 µl $CuSO_4$ reagent and then 100 µl of pPP reagent; mix well on vortex mixer keeping the tube at room temperature
- Leave the tubes at room temperature for at least 30 min and then read absorbance at 570 nm
- Blanks will show values of 0.2–0.5 compared to water.

The typical assay involved 1 ml of silage juice sample to which reagents and procedures described above for the standard curve are applied. Absorbance obtained is plotted against the standard curve to calculate the amount of lactic acid.

References

- Jouany JP. 1981.
- Kimberly Taylor. 1996.

2.4. Assessment of Silage Quality Using Fermentation End Products

The following table summarizes normal values for pH, ammonia, volatile fatty acids and lactic acid in different silage types. These values will help assessing silage quality.

Table 4. Recommended values for quality assessing for ensiled forage sample fermentation

Item	Corn silage	Alfalfa silage	Grass silage	(+) or (-) effect	Action(s)
pH	3.7-4.2	4.3-4.5	4.3-4.7	+	Low pH inhibits bacterial activity
Ammonia N% total N	5-7	10-15	8-12	–	High levels indicate excessive protein breakdown
Lactic acid	4-7	7-8	6-10	+	Inhibits bacterial activity by lowering pH
Acetic acid	1-3	2-3	1-3	+/–	Associated with undesirable fermentations. Inhibits yeasts responsible for aerobic spoilage
Butyric acid	0	<0.5	<0.5	–	Associated with protein degradation, toxin formation, and large losses of dry matter and energy

References

- Weatherburn MW. 1967.
- Jouany JP. 1981.
- Kimberly. Taylor. 1996.

3. Milk Analysis

Regular quality control of milk is essential. Routine chemical analyses are carried out in the laboratory to check milk composition for fat and total solids (TS). The laboratory analyzes milk samples from ICARDA's sheep flock, as well as samples from various experiments conducted by ICARDA's scientists and collaborators in the region.

3.1. Sampling

Laboratory samples should kept in closed containers, sealed and labeled indicating the nature of the product, identification number, batch number of the sample and date of sampling. The method of sampling depends on the nature of the product (liquid or solid) and whether chemical or bacteriological analyses are required. The size of the samples is regulated by the size of the product to evaluate.

Technical instructions
Clean and dry all equipment for chemical purposes. Equipment used in sampling for bacteriological purposes should be clean and treated by one of following methods:

- Expose to hot air at 170 °C for 2 h
- Autoclave 15–20 min at 121 °C
- Expose to steam for 1 hour at 100 °C for immediate use
- Immerse in 70% alcohol and flame for immediate use.

Liquid sample
Use clean and dry containers: glass, stainless metal or plastic with a suitable shape and capacity for material to be sampled.
Containers should be closed with a suitable plastic stopper or screw cap.

Note: Containers should be filled well to prevent churning during transportation

Solid and semi-solid samples
Use clean and dry containers of suitable waterproof material like glass, stainless metal or plastic material. Avoid narrow neck containers and close well. Air-tight plastic bags can be used too.

Preservation of laboratory samples
Preservatives have to be added to liquid samples or cheese intended for chemical analysis. The preservative should not affect analysis. Sodium azide or potassium dichromate are the preferred preservatives.

Transport of laboratory samples
Transport samples to the laboratory as fast as possible. Take precautions to prevent exposure to direct sunlight, or to temperatures above10 °C in case of perishable products. For laboratory samples intended for bacteriological examination, maintain temperature at 0–5 °C. Maintain samples of cheese under the same conditions to avoid separation of fat and moisture.

References

- AOAC. 2000.

3.2. Sample Preparation

Principle
Homogenizing of the milk before analysis is essential. Milk fat tends to separate and to flow on the surface.

Procedure
- Put the milk sample in a water bath, at 38 °C
- Mix until homogenization
- Cool samples to approx. 20 °C before analysis.

References

- AOAC. 2000.

3.3. Determination of Fat (Gerber Method)

Principle
Milk is mixed with sulphuric acid in a special glass tube called a Gerber tube. The temperature of the mixture becomes hot due to the dilution of a strong acid. As a consequence milk proteins are digested and fat is set free from the fat globules. It can be measured in the glass scale (Figure 43).

Procedure
- Pipette 10 ml of sulphuric acid into the butyrometer
- Slowly Pipette 10.75 ml milk. Milk should not be mixed with the sulphuric acid, it should appear as a separated layer
- Add 1 ml of Amyl alcohol
- Close with Gerber tube stopper

Caution: Dry neck of butyrometer before
- Shake the butyrometer until all the milk is digested

Caution: Glass becomes very hot
- Centrifuge in Gerber centrifuge for 5 min on 1100±100rpm
- Place the butyrometer for 5 min in water bath, at 65 °C
- Read the percentage of fat.

Calculations
The reading represents the percentage of fat content in milk

Reagents / Solutions
- Sulphuric acid density 1.81
- Amyl alcohol

Equipment
- Water bath (Fig. 44)
- Gerber milk butyrometer (Fig. 46)
- Standard rubber stopper
- Volumetric pipette (H_2SO_4) with safety bulb, 10 ml (Fig. 47)
- Volumetric pipette (milk), 10.75 ml (Fig. 47)
- Volumetric pipette (Amyl alcohol), 1 ml (Fig. 47)
- Gerber centrifuge (1100±100 rpm, diameter 45–50 cm), (Funke Gerber, Germany) (Fig. 45).

References

- Marth EH. 1978.

3.4. Total Solids

Principle
Milk is dried under constant temperature in the oven until weight is stabilized.

Procedure
- Weigh a clean pre-heated crucible
- Add 3–4 g of milk and record weight
- Dry the sample in an air forced oven at 105 °C overnight to stabilize weight
- Weigh the crucible with the dried sample.

Calculations
%TS = [(Weight of dried milk)/(weight of milk)]×100

Equipment
- Air forced oven (Fig. 6)
- Crucibles (Fig. 9)
- Desiccator (Fig. 7)
- Pipette (Fig. 28).

References

- Hui YH 1992.

Figure 44. Water bath

Figure 45. Gerber centrifuge

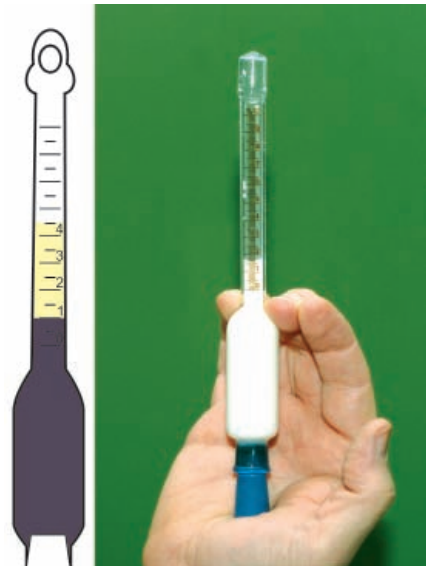


Figure 46. Gerber milk butyrometer

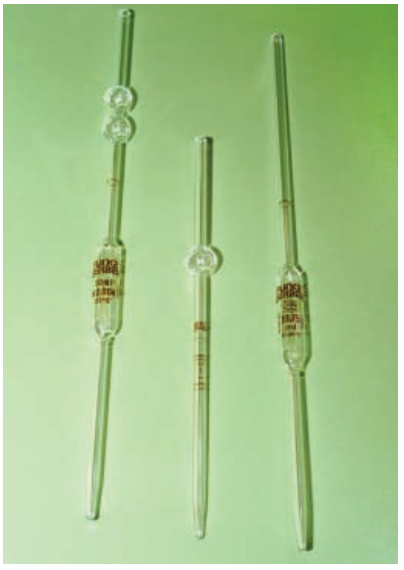


Figure 47. Volumetric pipettes

3.5. Fat, Protein Total Solids and Lactose Analysis

Principle

The analysis of milk with the Milkoscan device is based on absorption of Near Infrared energy at specific wavelength:

- by CH groups (3.48 µm) and carbonyl groups of fat (5.723 µm)
- by peptide bonds of protein molecules (6.465 µm), and
- by OH groups in lactose molecules (9.610 µm).

The Total Solids (TS) are set by the factory based on other solid milk components (e.g. protein, fat, lactose).

Procedure

Cleaning and calibration of the Milkoscan

- Warm Triton 1% solution and Stella 5% solution to 40 °C
- Purge the device with Stella
- Purge the device with Triton
- Calibrate the device with Triton 1%.

Measurement

- Warm milk samples to 40 °C in water bath
- Insert sample in aspirator device of Milkoscan.

Equipment

- Milkoscan 133B device (Foss Electric, Hillerød, Denmark)(Fig. 48)
- Water bath (Fig. 48).

References

- Manual for Milkoscan 133B, 1993.
- AOAC. 2000.

Figure 48. Milkoscan with water bath

3.6. Milk Density

Principle

Based on specific gravity a special device is used to determine the milk density.

Procedure

- Inject approximately 5 ml of milk to ensure that the milk line is well flushed with the sample
- Read the density on the device.

Note: Ensure that there are no bubbles in the milk line, bubbles cause an error.

Equipment

- Density meter DMA 35, PAAR, Austria (Fig. 49).

References

- Milchanalytik 755.302. 2008

3.7. Milk Conductivity

Principle

Mastitis causes an increase of salt concentration. Conductivity is measured with an electronic conductivity meter by immersing a probe directly into the milk sample.

Procedure

- Immerse the conductivity probe into the milk sample
- Read the result on the device.

Equipment

- Electric conductivity meter Hanna HI 98360 Microprocessor, Hanna Instruments, Italy (Fig. 50).

References

- Hui YH. 1992.

3.8. Milk pH

Principle

pH is the negative logarithm of the hydrogen ion concentration. Measurement of pH is particularly important in the dairy industry since it provides, in many cases, a more meaningful measurement than titratable acidity. pH values from 0–7 are acidic, while those from 7–14 are alkaline. pH measures the level of the acidity.

Procedure

- Pour the sample into a clean, dry beaker
- Carefully press electrodes into the milk-containing beaker and determine the pH.

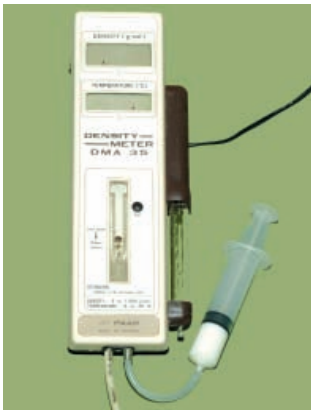


Figure 49. Density meter

Figure 50. Electric conductivity meter

Figure 51. pH meter

Equipment

- pH meter HANNA HI 113, Hanna Instruments, Italy (Fig. 51)

References

- FAO. 1986.
- Nielsen EW and Ullum JA. 1989.

3.9. Milk Acidity

Principle

The pH value of fresh milk is normally about 6.6 at 25 °C, which indicates a natural acidity. The acidity is determined by titration with alkali NaOH, in the presence of phenolphthalein as indicator.

Procedure

- Pipette 10 ml of milk
- Add 5 drops of 1% phenolphthalein
- Titrate with 0.1 *N* NaOH until a slight pink color appears.

Figure 52. Round bottomed white porcelain dish, 100 ml

Solids non-fat (SNF) define the natural acidity of fresh milk. The higher the solids, that are not fat content, in milk, the higher the natural acidity and vice versa. Developed or real acidity is due to lactic acid, formed as a result of bacterial action on lactose in milk. Hence the titratable acidity of stored milk is equal to the sum of natural acidity and developed acidity. The titratable acidity is expressed as percentage of lactic acid. Colostrum has a high "natural acidity" in part because of its very high protein content.

Reagents / Solutions

Sodium hydroxide 0.1 N solution

- Dissolve equal parts of sodium hydroxide (sticks or pellets) in equal parts of water in a flask
- Tightly close the flask with a rubber bung and allow insoluble sodium carbonate to settle down for 3 to 4 days
- Use the clear supernatant liquid for preparing the standard 0.1 *N* solution
- Use 8 ml of stock solution per liter of distilled water
- Standardize solution accurately against acid potassium phthalate or oxalic acid.

Phenolphthalein indicator 1%

- Weigh 1g of phenolphthalein in volumetric flask 100 ml
- Dissolve in ethanol and make up to volume.

Calculations

%acidity of milk = $[(V_a \times 0.009)/V_m] \times 100$

where:

V_a : volume of 0.1*N* NaOH

V_m : volume of milk sample

0.009 : conversion factor from milliliter of 0.1*N* NaOH to gram of lactic acid

Equipment

- Glass burette 50 ml capacity with 0.1 ml graduation (Fig.10)
- Round bottomed white porcelain dish, 100 ml (Fig. 52)
- Pipette 10 ml
- Pipette 1 ml
- Stirrer
- Stirring rods.

References

- AOAC. 2000.

3.10. Volatile Fatty Acids

Principle

Fat samples are esterified and evaporated in the GC system; fatty acids are separated in a stationary phase of the installed column depending on the chemical characteristics.

Sample preparation

Extraction (Folch et al. 1957)

- 1 ml milk
- 2 ml MeOH
- 1 ml $CHCl_3$
- Vortex 1min
- 1 ml $CHCl_3$
- 1 ml H_2O
- Extract the lower part
- Evaporate with N_2.

Methylation (Joachim Molkentin, Dietz Precht 2000)

- 100±20 mg of fat
- 5 ml methyl valerate standard (n–heptane, containing 0.4 mg/ml methyl valerate).

1 ml of this solution was mixed with

- 20 µl sodium methylate solution (2 *N* in methanol) in a sample vial

- Vortex for 3 min
- Centrifuge for 1min at 2000 rpm
- 100 mg sodium hydrogen sulphatemonohydrate
- Cover vial with new cap
- Vortex for 2 min mix
- Centrifuge for 1 min at 2000 rpm
- Use supernatant for GC analysis.

Procedure

Switch on procedures

- Switch on device
- Switch on attached PC
- Run GC solution program
- Click on the GC–Device
- Load the cool–heat method and click on 'Download'
- Click on 'System on' and let the method work for 5 min
- Load the heat method and click on 'Download'
- Run method for 20 min while observing GC parameters on monitor When FID temperature is above 100 °C, switch on the detector and ignite the flame clicking the left button on system monitor screen
- Load the analytical method
- Click on 'Download'.

Injection procedure

Before injecting a sample there are some steps to follow:

- Click on 'Sample log on'. Fill in the requested data. This is important for post–acquisition data work
- Fill the GC analysis lab–book with the requested data
- Do not inject the sample before 'Ready' appears on the PC monitor in blue; LED's on the GC should appear in green
- Inject the sample and press the 'Start' buttom on the GC simultaneously.

Thermal program

- 50 °C for 3 min
- 5 °C/min to 140 °C for 2 min
- 2 °C/min to 170 °C for 5 min
- 10 °C/min to 220 °C for 2 min
- 5 °C/min to 225 °C for 21 min.

Injection

Thermal method:

- 1 µl solvent
- 1 µl air
- 1 µl sample
- Inject sample manually after a dwell–time of 5 seconds
- Remove the syringe after 5 seconds.

Switch off procedure

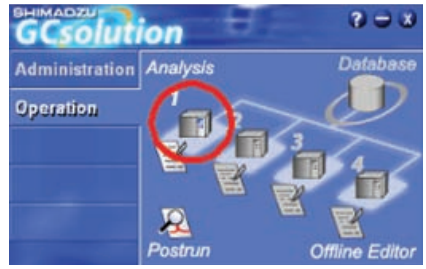
- Load the cool–heat method and click on 'Download'
- Switch off the flame and then switch off the detector by clicking the left buttons on system monitor screen
- Wait till the FID and injector temperature are less than 100 °C, then press the 'System off' button
- Close the program
- Switch off the GC
- Close running programs and shut down the PC.

Note: Do not open the oven at any time. This may damage the installed column.

If any error message appears, inform the person responsible immediately. Any delay in conveying this information could damage the whole system.
Do not inject wet samples.
Do not inject more than 1 µl of air.

Calculations

The qualitative and quantitative assessment of fatty acids is calculated by the GC solution software.



Reagents / Solutions

- He gas (99.999%)
- H_2 gas (99.999%)
- N_2 gas (99.999%)
- Sodium methylate 2*N*
- Methanol
- Chloroform.

Equipment

- Gas chromatograph (Shimadzu 2010) with FID system, Japan (Fig. 53)
- Vortex mixer
- Syringe 1 µl
- Pasteur pipettes.

References

- Folch J, Lees M, and Stanley GHS. 1957.
- Molkentin J and Precht D. 2000.

3.11. Organic Acids

Principle

A filtered sample is carried by the mobile phase through the stationary

Figure 53. Gas chromatograph

phase. The separated organic acid is detected by an electric conductivity detector after mixing with the post-column pH buffered electrolyte to enhance detection (Fig. 54).

Sample preparation

- Dilute 5 g yogurt with H_2O to 25 ml
- Shake, 5 min
- Centrifuge at 2000 rpm, 10 min
- Filter through 45 µm disc filter
- Inject 20 µl into injector.

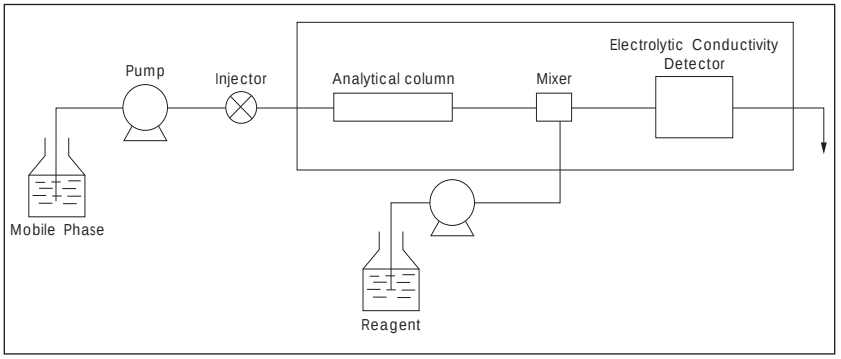


Figure 54. Flow diagram of organic acids analysis system

Procedure

Separation

- Column: Shim–pack SCR–102H (300 mm×8.0 mm i.d.) two columns in series
- Mobile Phase: 5 m*M* p–toluenesulfonic acid

- Flow Rate: 0.8 ml/min
- Temperature: 45 °C.

Detection
- Flow Rate: 0.8 ml/min
- Temperature: 48 °C
- Detector: Electrolytic conductivity.

Calculations
The qualitative and quantitative assessment of organic acids are calculated by the CLASS–VP software.

Reagents / Solutions
Mobile and buffer solution
- 5 m*M* p–toluenesulfonic acid
- 20 m*M* Bis–Tris
- 100 µ*M* EDTA.2Na .

Standard
- Acetic acid
- Formic acid
- Citric acid
- Ortho phosphoric acid
- Succinic acid
- Lithium lactate

Equipment
- HPLC assembly (Fig. 55):
- Conductivity detector (Shimadzu CDD10A vp)
- Oven (Shimadzu CTO 10 AC vp)
- Control Unit (Shimadzu SCC 10Asp)
- Pump (Shimadzu LC10 ADvp)
- Degasser (Shimadzu DGU 14A).

Figure 55. HPLC

References

- Zhang H, Zhou F, Ji B, Nout R, Fang Q, and Yang Z. 2008.

3.12. Tests for Abnormal Milk

3.12.1. Somatic Cell Count (SCC)

Principle
Cell counts are used to determine levels of mastitis infection in indi-

vidual ruminants, or in bulk milk samples. SCC over 500,000 indicates subclinical mastitis (Al–Majali and Jawabreh, 2003; Kiossis et al, 2007).

Procedure

- Immerse the cassette nipple in the milk sample
- Press the white knob on the cassette to load the milk sample
- Put the cassette in the cell counter device and press run
- Read the results.

Equipment

- Cell counter DCC (DeLaval, Sweden) (Fig. 56)
- Cell counter cassettes (DeLaval) (Fig. 57).

Figure 56. Somatic cell counter

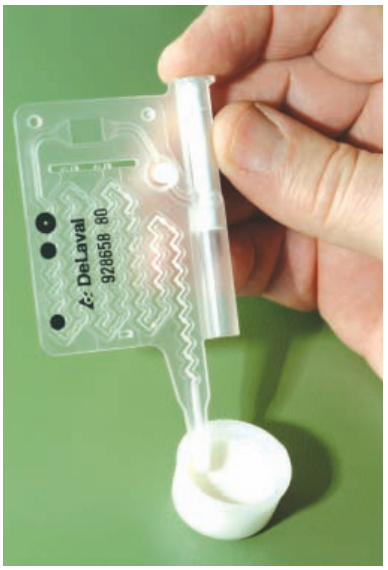


Figure 57. Somatic cell counter cassettes

References

- Al–Majali AM and Jawabreh S. 2003.
- Kiossis E, Brozos CN, Petridou E and Boscos C. 2007.

3.12.2. California Mastitis Test (CMT)

Principle

CMT reagent consists of a detergent and acid base indicator. Somatic cells are ruptured by the detergent, releasing the DNA. Reagent will precipitate the DNA resulting in a gel formation that is proportional to the quantity of DNA present.

Procedure

- Take 1 teaspoon (2 ml) milk from each part of the udder. Hold paddle in vertical position
- Add an equal amount of CMT solution to each cup of the paddle
- Rotate the CMT paddle in a circular motion to mix the contents not longer than 10 seconds.
- Read the test quickly. Visible reaction disintegrates after about 20 seconds. The reaction is visually scored according to Table 5.

Note: CMT paddle should be rinsed after each test.

Table 5. Score for CMT reading

Score	Description
0 = Negative(Not Infected)	No thickening of the mixture
1 = Trace (Possible Infection)	Slight thickening of the mixture
2 = Weak Positive (Infected)	Distinct thickening of the mixture
3 = Distinct Positive (Infected)	Immediate thickening of the mixture with a slight gel formation
4 = Strong Positive (Infected)	Gel is formed and surface of the mixture becomes elevated

Equipment

- CMT paddle (Fig. 58)

Reagents/ Solution

- CMT Solution reconstituted according to package instructions.

References

- González–Rodríguez MC and Cármenes P. 1996.
- Hui YH. 1992.

Figure 58. CMT paddle

4. Blood Analysis

4.1. Determination of Progesterone

Principle

Blood samples are collected in vacutainer tubes. Blood serum or EDTA plasma is used for progesterone determination. Progesterone is bounded with a constant amount of rabbit anti–progesterone. The determination is carried out using progesterone enzyme immuno-assay test kit.

Procedure

Sample preparation

- Centrifuge blood samples at 6000 rpm for 20 minutes
- Collect 2 ml of serum/ plasma
- Freeze samples till analysis.

Assessing the test

- Melt the frozen sample to room temperature
- Pipette 25 µl of the sample and standard
- Pipette 100 µl of progesterone–HRP conjugate reagent
- Pipette 50 µl of rabbit anti–progesterone reagent
- Shake for 30 sec on plate shaker
- Incubate for 90 min at room temperature
- Wash wells 5 times with distilled water
- Pipette 100 µl of TMB reagents
- Shake for 5 sec on plate shaker
- Incubate for 20 min at room temperature
- Pipette 100 µl of stop solution
- Shake for 30 sec on plate shaker
- Read absorbance at 450 nm within 15 minutes.

Calculations

Plot the standard curve and calculate concentration based on absorbance using Microsoft excel equations.

Figure 59. ELISA reader and plate shaker

Equipment

- ELISA reader (Multiskan Ascent, Thermo Labsystems, Finland) (Fig. 59)
- ELISA plate shaker (Cole Parmer 51400.05, USA) (Fig. 59)

References

- Linear Chemicals.1994.

APPENDICES

Appendix 1. Request form for Analyses in Animal Nutrition Laboratory

ICARDA Animal Nutrition Laboratory: Request for Analysis
BATCH NUMBER :
Date received:
Date dispatched: Approval lab manager:
Requisitioner:
Program/Department:
Budget Code: Total costs:
Approval project manager:

Type of Analysis sample	Number of samples	Description of samples	Cost per sample US$	Total costs US$
N/CP				
DM & Ash				
ADF				
NDF				
ADL				
IVDMD				
IVOMD				
Fat				
Milk analysis				
Minerals				
Gas Production Test				
Tannins				
TP& Tannins				
Condensed tannins				
			Total	

Appendix 2. Safety in the Laboratory

Hazards in laboratory

- Flammable liquids
  - Do not let vapors concentrate to flammable levels in work area.
  - Try to eliminate all chance of sparks from static electricity even if electric equipment is grounded.
  - Use fume hood.
  - Use minimum quantity.
  - Store in special storage cabinet.
- Toxic liquids
  - Avoid contact with skin.
  - Do not inhale.
  - Use fume hood.
  - Do not pipette any liquids by mouth. Use pipette fillers or rubber balls.
- Breakage of glassware
  - Use correct techniques for the insertion of tubing onto glassware.
  - Never use glassware under pressure or vacuum unless it is designed for the job and suitably shielded.
  - Dispose broken glassware.
- Spillages
  - In case of chemical spills on skin or clothes rinse with plenty of water. If eyes are affected rinse immediately with water for at least 10 minutes.
  - Clear up spillage promptly.
- Pressure equipment and gas cylinders
  - Never use without prior training.
  - Minimise the number of gas cylinders in a laboratory and store externally whenever possible.
  - Chain cylinders when in use.
  - Use regulators, tubing and control equipment suitable for the gas concerned.

For Atomic Absorption Spectrophotometer use effective fume hood to remove gaseous effluents from burner. Use only C_2H_2 that is dissolved in solvent recommended by manufacturer. Open C_2H_2 tank stem valve only a quarter turn. Change tank when C_2H_2 pressure shows 75–100 lb. For compressed gas cylinders let contents of C_2H_2 cylinders settle, and let all cylinders come to room temperature prior to opening. Use only correct pressure gauges, pressure regulator, flow regulator, and tubing for each size of gas cylinder and type of gas as specified by supplier. Close gas tank valve and diaphragm on regulator when gas is not in use. Service regulator yearly.

Note: Check for gas leakage.

Safe handling of concentrated corrosive acids

– Use acid–resistant fume hood when heating acids or performing reactions that emit acid fumes. In diluting, always add acid to H_2O 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 H_2O.

Sulfuric acid. Always add H_2SO_4 to H_2O. Wear face shield and heavy rubber gloves to protect against splashes. Do not mix with HCl.
Nitric acid. Nitric acid reacts vigorously or explosively with aniline, H_2S, flammable solvents, hydrazine and metal powders. Gaseous nitrogen oxides from HNO_3 can cause severe lung damage. Use fume hood. Handle with disposable polyvinylchloride, not rubber, gloves.

Safe handling of alkalis

Alkalis can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face–shield to protect against concentrated alkali liquids. Use fume hood or gas mask.
Sodium and Potassium hydroxides are extremely caustic and can cause severe burns. Protect skin and eyes when working with these alkalis, whether as solids or concentrated solutions. Add pellets to H_2O, not vice versa.

Safe handling of special chemical hazards

Acetone. Highly flammable, forms explosive peroxides with oxidizing agents. Use fume hood.

Chloroform. Do not inhale. Use fume hood. Can react explosively with Al, Li, Mg, Na, K, N_2O_4, and NaOH plus methanol. Tumor producing agent.

Di– and Trichloroacetic and Trifluoroacetic Acids.
Can cause severe burns to skin and respiratory tract. Use rubber gloves, eye protection and fume hood.

Ethanol. Flammable. Use fume hood when heating or evaporating.

Methanol. Flammable, toxic. Avoid contact with eyes. Avoid inhaling vapors. Use fume hood. Can react vigorously with NaOH plus $CHCl_3$, and KOH plus $CHCl_3$ or KOH plus $HClO_4$.

Petroleum ether. Extremely flammable. Use fume hood. Avoid static electricity.

Hexane. Highly flammable. Use fume hood.

General precautions in laboratory

- Wear laboratory coats when working in the laboratory. Wear protective clothes and face shield or goggle when handling hazardous chemicals
- All laboratory workers should undergo simple first aid training
- First aid kits should be available in the laboratory
- Class B and C dry chemical fire extinguishers (for flammable, liquid and electric fires) should be placed in the laboratory. Carbon dioxide fire extinguishers should be used on fires in electronic equipment. Staff need to be instructed on use of fire extinguishers.

References

- AOAC.1995.

Appendix 3. Preparing Standard Solutions

A. Molar (M) solutions

1 Molar solution (1*M*) contains 1 mole of solute dissolved in a solution totaling 1 l. Molecular weight is MW.
1 *M* = 1 g MW of solute per liter of solution

Example: NaCl

- MW of sodium chloride (NaCl) is:
  Na (23)+Cl (35.5) = 58.5 grams/mole
- 1 *M* aqueous solution of NaCl, dissolve 58.5 grams of NaCl in 1 l. of distilled water.
- 0.1 *M* solution of sodium chloride contains 5.85 grams per 1 l.

Example: H_2SO_4

- Molar (1 *M*) aqueous solution of H_2SO_4
- Check on bottle label for Molar concentration (e.g. 18.0)
  x ml = 1 ml×1 mole/ 0.018 moles
  x ml = 55.6 ml of H_2SO_4

Note: Add H_2SO_4 slowly to 500 ml of distilled water and then complete to 1 l. Never add water to sulfuric acid.

Typical concentrations of concentrated acids and bases

Acid/Base Name	Wt%	Density (g/ml)	Molarity (*M*)
Acetic acid	99.7	1.05	17.4
Ammonium hydroxide	28	0.89	14.6
Hydrochloric acid	37	1.18	12.0
Nitric acid (HNO_3)	37	1.40	15.6
Phosphoric acid	85	1.69	14.7
Sulfuric acid	96	1.84	18.0

B. Normal (N) solutions

Equivalent weight (EW) = molecular weight g/n
mEW = millieauivqlent weight
n = valence of solute
N = Weight of solute/ mEW of solute×Volume (ml) of dilution

Example: Normal solution with salt

2.9216 grams of NaCl in 500 ml distilled water
MW of NaCl = 58.44
EW = 58.44/1
mEW = 0.05844
N = 2.9216 g/ (0.05844×500 ml)
N = 0.099

Example: Normal solution with acids

EW = MW/n

n = number of replaceable hydrogen atoms in the reaction

HCl	n = 1
HNO_3	n = 1
H_2SO_4	n = 2
HF	n = 1

For HCl MW = 36.46, EW = 36.46

1 *N* = 36.46 g/l

For H_2SO_4 MW = 98.08, EW = 98.08/ 2 = 49.04 g/l

N = 49.04

Example: Normal solution with alkalis

EW = MW/n

n = number of hydrogen ions that are required to neutralize the base

NaOH	n = 1
$Ca(OH)_2$	n = 2

For NaOH MW = 40, EW = 40

1 *N* = 40 g/l

For $Ca(OH)_2$ MW = 74, EW = 74/2 = 37

1 *N* = 37 g/l

C. Preparation of normal solutions from concentrated reagents

Example: H_2SO_4

Prepare 2 l. of 0.2N 96% H_2SO_4

Density = 1.84 g/ml

MW = 98.08

EW = MW/2 = 49.04 g/l

V (ml) = Volume of H_2SO_4 needed

V = N needed × Volume of solution×EW/density x concentration reagent
= 0.2×2.0 l × 49.04/1.84 × 0.96
= 11.105 ml

Therefore, slowly add 11.105 ml of 96% H_2SO_4 to 1 l of distilled water and then add up to 2 l to get a 0.2 *N* solution.

Example: HCl

Prepare 2 l. of a 0.2 *N* 38% HCl

Density = 1.188 g/ml

MW = 36.461

EW = MW/1 = 36.461

V (ml) = Volume of HCl needed

V = *N* needed × Volume of solution × EW/density x concentration reagent
= 0.2 × 2 × 36.461/(1×1.188 × 0.38)
= 32.604 ml

Therefore, slowly add 32.604 ml of 38% HCl to 500 ml of distilled water and then add up to 2 l to get a 0.2 *N* solution.

Appendix 4. Abbreviations

°C	Degree Celsius
ADF	Acid Detergent Fiber
ADIN	Acid Detergent Insoluble Nitrogen
ADL	Acid Detergent Lignin
AOAC	Association of Official Analytical Chemists
cm	centimeter
CMT	California Mastitis Test
CP	Crude Protein
CT	Condensed Tannins
CTAB	Cetyl Trimethyl Ammonium Bromide
DM	Dry Matter
FID	Flame Ionization Detector
g	gram
GC	Gas Chromatograph
GPT	Gas Production Test
IVDMD	In vitro Dry Matter Digestibility
IVOMD	In vitro Organic Matter Digestibility
l	liter
m	meter
M	Molarity
ME	Metabolizable energy
MeOH	Methanol
EtOH	Ethanol
mg	milligram
ml	milliliter
mm	millimeter
µg	microgram
µl	microliter
µm	micrometer
N	Nitrogen
N	Normality
NDF	Neutral Detergent Fiber
NEL	Net Lactation Energy
NPN	Non protein nitrogen
ODM	Organic Dry Matter
PEG	polyethylene glycol
PVPP	Polyvinylpolypyrrolidone
SCC	Somatic Cell Count
SCFA	Small Chain Fatty Acids
TCA	Trichloroacetic Acid
TP	Total Phenolics

Appendix 5. Units and Conversion Tables

Unit	Description	Conversion	Remarks
Pressure			
Pa	Pascal	Standard unit	Pascal = Newton/m²
atm	Atmosphere (physical)	101,325 Pa	Pressure in gases
atm	Atmosphere (technical)	98,066.5 Pa	One atmosphere =1 kp/cm²
bar	Bar	100,000 Pa	Pressure in gas and liquid
psi	Pounds per Square Inch	6,894.757293168 Pa	1 psi equivalent 14.5 bar
mbar	Millibar	100 Pa	Equivalent one hecto-pascal (hPa)
mmHg	Millimeter-mercury column	133.32 Pa	
Energy			
J	Joule	Standard unit	
BTU	British Thermal Unit	1055.058138 J	
cal	calorie	4.186794846 J	
HPh	Horsepower*hour	2684517.413 J	
Wh	Watt*hour	3599.9982 J	
Area			
m²	Square meter	Standard unit	
ha	Hectare	10,000 m²	
ft²	Square foot	0.0929 m²	
km²	Square kilometer	1,000,000 m²	
mi²	Square mile	2,589,988.11 m²	
Volume			
l	Liter	Standard unit	
bl, bbl	Barrel	ca. 159 l	
gal	Gallon (UK)	4.55 l	
gal	Gallon (US)	3.7862 l	
pt	Pint	0.4733 l	
qt	Quart	0.9466 l	

Appendix 5. Continued

Unit	Description	Conversion
Speed		
m/s	meters per second	Standard unit
km/h	kilometers per hour	0.278 m/s
mi/h	miles per hour	0.447 m/s
Power		
N	Newton	Standard unit
p	Pond	approx. 9.81 N
W	Watt	Standard unit
hp	horsepower	745.701 W
Length		
m	meter	Standard unit
ft	foot	0.3048 m
in	inch	0.0254 m
mi	mile	1609.344 m
yd	yard	0.9144 m
Mass		
kg	kilogram	Standard unit
lbm	pound	0.4536 kg
t	ton	1000 kg
oz	ounce	0.02834 kg
Temperature		
K	Kelvin	Standard unit
°C	Degree Celsius	T/°C+273.15 K
°F	Degree Fahrenheit	(T/°F+459.67 K)×1.8
		T/°C = (T/°F−32)/1.8
		T/°F = T/°C×1.8+32
Time		
s	second	Standard unit
m, min	minute	60 s
h	hour	3600 s
d	day	86400 s

Appendix 6. Frequently used Standards of Concentration

Measurement	Notation	Generic formula	Typical units
Mass percentage	wt% or w/w%	g solute x 100/ g solution	%
Mass-volume percentage	m/v%	g solute x100/ml solution	% though strictly % g/ml
Volume-volume percentage	vol% or v/v%	ml solute x 100/ ml solution	%
Molarity	*M*	moles solute/ liter solution	mol/l (or *M* or mol/ dm^3)
Molinity	-	moles solute/kg solution	mol/kg
Molality	m	moles solute/kg solvent	mol/kg (or m)
Molar fraction	X (chi)	moles solute/ moles solution	(decimal)
Formal	F	moles undissolved solute/liter solution	mol/l (or F)
Normality	*N*	gram equivalents/ liters solution	*N*
Parts per hundred	%(or pph)	dekagrams solute/kilograms solution	dg/kg
Parts per thousand	%(or ppt)	grams solute/kilograms solution	g/kg
Parts per million	ppm	mg solute/kg solution	mg/kg
Parts per billion	ppb	µg solute/kg solution	µg/kg
Parts per trillion	ppt	ng solute/kg solution	ng/kg

Appendix 7. Table of Elements

Sym-bol	Name	Atomic Number	Mass	Electro-negativity	Density g/m³	Melting Point °C	Boiling Point °C
Ac	Actinium	89	227	1.10	10.07	1050	3200
Ag	Silver	47	108	1.93	10.50	962	2212
Al	Alumi-num	13	27	1.61	2.70	660	2467
Am	Ameri-cium	95	243	1.28	13.67	640	2607
Ar	Argon	18	40	0.82	1.74	64	-186
As	Arsenic	33	75	2.18	5.72	81	613
At	Astatine	85	210	2.20		302	337
Au	Gold	79	197	2.54	19.32	1064	2807
B	Boron	5	11	2.04	2.34	2300	2550
Ba	Barium	56	137	0.89	3.59	725	1140
Be	Beryllium	4	9	1.57	1.85	1278	2970
Bh	Bohrium	107	264				
Bi	Bismuth	83	209	2.02	9.75	271	1560
Bk	Berkelion	97	247	1.30	14.78	986	
Br	Bromine	35	80	2.96	3.12	-7	59
C	Carbon	6	12	2.55	2.26	3500	4827
Ca	Calcium	20	40	1.00	1.70	839	1484
Cd	Cad-mium	48	112	1.69	8.65	321	765
Ce	Cerium	58	140	1.12	6.77	795	3257
Cf	Califor-nium	98	251	1.30	15.10	900	
Cl	Chlorine	17	35	3.16	3.21	-101	-35
Cm	Curium	96	247	1.30	13.50	1340	
Co	Cobalt	27	59	1.91	8.90	1453	2870
Cr	Chro-mium	24	52	1.66	7.19	1857	2672
Cs	Caesium	55	133	0.79	1.87	29	678
Cu	Copper	29	64	1.90	8.96	1083	2567

Appendix 7. Continued

Symbol	Name	Atomic Number	Mass	Electronegativity	Density g/m³	Melting Point °C	Boiling Point °C
Db	Dubnium	105	262				
Ds	Darmstadtium	110					
Dy	Dysprosium	66	162	1.22	8.55	1412	2562
Er	Erbium	68	167	1.24	9.07	1522	2510
Es	Einsteinium	99	252	1.30		860	
Eu	Europium	63	152		5.24	822	1597
F	Fluorine	9	19	3.98	0.09	-220	-188
Fe	Iron	26	56	1.83	7.87	1535	2750
Fm	Fermium	100	257	1.30		1527	
Fr	Francium	87	223	0.70		27	677
Ga	Gallium	31	70	1.81	5.91	30	2403
Gd	Gadolinium	64	157	1.20	7.90	1311	3233
Ge	Germanium	32	73	2.01	5.32	937	2830
H	Hydrogen	1	1	2.20	1.55	-259	-253
He	Helium	2	4		0.18	-272	-269
Hf	Hafnium	72	178	1.30	13.31	2150	5400
Hg	Mercury	80	201	2.00	13.55	-39	357
Ho	Holmium	67	165	1.23	8.80	1470	2720
Hs	Hassium	108	277				
I	Iodine	53	127	2.10	4.93	114	184
In	Indium	49	115	1.78	7.31	157	2000
Ir	Iridium	77	192	2.20	22.40	2410	4527

Appendix 7. Continued

Symbol	Name	Atomic Number	Mass	Electronegativity	Density g/m³	Melting Point °C	Boiling Point °C
K	Potassium	19	39	0.82	0.86	-189	774
Kr	Krypton	36	84		3.75	-157	-153
La	Lanthanum	57	139	1.10	6.15	920	3469
Li	Lithium	3	7	0.98	0.53	180	1347
Lr	Lawrencium	103	262				
Lu	Lutetium	71	175	1.27	9.84	1656	3315
Md	Mendelevium	101	258	1.30			
Mg	Magnesium	12	24	1.31	1.78	639	1090
Mn	Manganese	25	55	1.55	7.43	1245	1962
Mo	Molybdenum	42	96	2.16	10.22	2617	4612
Mt	Meitnerium	109	268				
N	Nitrogen	7	14	3.04	1.25	-210	-196
Na	Sodium	11	23	0.93	0.97	98	883
Nb	Nobium	41	93	1.60	8.57	2468	4927
Nd	Neodym	60	144	1.14	7.01	1010	3127
Ne	Neon	10	20		0.90	-249	-246
Ni	Nickel	28	59	1.88	8.90	1495	2732
No	Nobelium	102	259	1.30		827	
Np	Neptunium	93	237	1.38	20.20	1132	3902
O	Oxygen	8	16	3.44	1.43	-218	-183
Os	Osmium	76	190	2.20	22.60	3045	5027
P	Phosphorus	15	31	2.19	1.82	44	280
Pa	Protactinium	91	231	1.30	15.40	1750	

Appendix 7. Continued

Symbol	Name	Atomic Number	Mass	Electronegativity	Density g/m³	Melting Point °C	Boiling Point °C
Pb	Lead	82	207	2.33	11.35	327	1740
Pd	Palladium	46	106	2.20	12.02	1552	2927
Pm	Promethium	61	145		7.30	1100	3000
Po	Polonium	84	209	2.00	9.30	254	962
Pr	Praseodymium	59	141	1.13	6.77	935	3127
Pt	Platinum	78	195	2.28	21.45	1772	3827
Pu	Plutonium	94	244	1.30	19.84	994	3235
Ra	Radium	88	226	0.89	5.50	700	1737
Rb	Rubidium	37	85	0.82	1.63	39	688
Re	Rhenium	75	186	1.90	21.04	3180	5627
Rf	Rutherfordium	104	261	1.30		1627	
Rg	Roentgenium	111	272				
Rh	Rhodium	45	103	2.28	12.41	1966	3727
Rn	Radon	86	222		9.73	-71	-62
Ru	Ruthenium	44	101	2.20	12.37	2250	3900
S	Sulphur	16	32	2.58	2.07	113	445
Sb	Antimony	51	122	2.05	6.68	630	1750
Sc	Scandium	21	45	1.36	2.99	1539	2832
Se	Selenium	34	79	2.55	4.79	217	685
Sg	Seaborgium	106	266				
Si	Silicium	14	28	1.90	2.33	1410	2355
Sm	Samarium	62	150	1.17	7.52	1072	1900

Appendix 7. Continued

Symbol	Name	Atomic Number	Mass	Electronegativity	Density g/m³	Melting Point °C	Boiling Point °C
Sn	Tin	50	119	1.96	7.31	232	2270
Sr	Strontium	38	88	0.95	2.54	769	1384
Ta	Tantalum	73	181	1.50	16.65	2996	5425
Tb	Terbium	65	159		8.23	1360	3041
Tc	Technetium	43	98	1.90	11.50	2200	4877
Te	Tellurium	52	128	2.66	6.24	449	990
Th	Thorium	90	232	1.50	11.72	1568	4790
Ti	Titanium	22	48	1.54	4.54	1660	3287
Tl	Thallium	81	204	1.62	11.85	303	1457
Tm	Thulium	69	169	1.25	9.32	1545	1727
U	Uranium	92	238	1.36	18.95	640	3818
V	Vanadium	23	51	1.63	6.11	1890	3380
W	Wolfram	74	184	2.36	19.35	3410	5660
Xe	Xenon	54	131	2.60	5.90	-112	-108
Y	Yttrium	39	89	1.22	4.47	1523	3337
Yb	Ytterbium	70	173		6.90	824	1466
Zn	Zinc	30	65	1.65	7.13	420	907
Zr	Zirconium	40	91	1.33	6.51	1852	4377

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