LABORATORY PROCEDURES

IN

ANIMAL NUTRITION RESEARCH

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PREFACE

This text represents an attempt to summarize and consolidate a considerable amount of information relative to laboratory procedures and experimental techniques that are used commonly in animal nutrition studies. It was originally designed to be a supplement to and reference for Animal Science 507, Laboratory Techniques in Nutrition, at New Mexico State University, and subsequently revised for use in AnSc 5507, Research Techniques in Animal Nutrition, at West Texas A&M University. As such, the text owes much of its development to the course and the people who taught and developed this course over the years. Dr. G. S. Smith deserves special recognition for his many years of work with the course, as do numerous laboratory technicians who compiled and developed procedures over the years.

Basically, the text is an outline of the author's course notes with attempts made to expand and reference where possible. Many of the examples deal with ruminants because the author is most familiar with them; however, this should not detract from its general applicability to other livestock or even to human nutrition. The text can be divided into several general areas, including laboratory safety, proximate analysis, spectrophotometry, liquid scintillation counting, the use of markers in nutrition studies, and microbiology of ruminants. It is not intended to be complete in every detail, and outside reading by students will often be necessary. The author hopes, however, that this text will serve as a useful reference in the years to come for those students who select experimental animal nutrition as a career.

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CHAPTER I

Laboratory Safety

Everyone working in the laboratory should be cognizant of the potential hazards they face while working there. Fires with organic solvents, acid and base burns, and toxic fumes and vapors are common hazards in almost any nutrition laboratory. Generally, lab safety is a matter of common sense, but there are several rules that must be followed. Each student is required to read this chapter, which contains a brief synopsis of safety procedures. In addition, students must read the **Standard Laboratory Procedures** handout that is attached to the end of this chapter.

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

Lab Safety
from
Science Related Materials, Inc.
1980

Laboratory Neatness	Clean and neat work areas avoid risk of damage to clothing and books and injury from spilled chemicals. Neatness also reduces fire hazard.
Laboratory Conduct	Fooling around in the laboratory can be hazardous. Keep the lab in its proper place and fun and games in their place.
Working with Glassware	Remove frozen glass stoppers with proper equipment. Broken or chipped glassware should be discarded. Properly support glassware with ring-stands and clamps when heating and use cork rings with round-bottom flasks.
Working with Glass Tubing	Do not touch heated glass until it has time to cool. Hot glass looks just like cool glass. To remove stoppers from glass tubing or thermometers, grasp tubing close to stopper and push gently with twisting. Use water or glycerin for lubrication.
Laboratory Dress	Pull hair back and wear eye protection when required. Sleeves that are too tight prevent freedom of movement, whereas sleeves that are too loose may cause you to overturn apparatus or glassware. Aprons protect clothing from corrosive or staining chemicals. Gloves protect hands from corrosive chemicals. Handle hot objects with insulated gloves. Do not wear open-toe shoes that allow spilled chemicals or broken glass to come in contact with your feet.

Working with Test Tubes	Gently heat solids or liquids in a test tube near the liquid or solid surface. Be prepared to remove the tube from heat quickly to prevent eruption. Never point a test tube or reaction vessel at another person. For safety and neatness, place test tubes in a rack.
Chemicals in the Eye	**Rapid treatment is vital**. Run large volumes of water over eyeball until medical help is available. Wash with large volumes of water for at least 15 minutes. Alkaline materials in the eye are extremely hazardous. **Know the location of the emergency eyewash station**.
Safety Shower	Use this for chemical spills or a fire victim. Operate by pulling down on ring and keep the area near the shower clear at all times. Remove clothing from area affected by spills.
Fire on Clothing	**Do not run or fan flames**. Smother fire by wrapping victim in fire blanket or lab coat and use the shower or a carbon dioxide fire extinguisher.
Extinguishing a Fire	Using a fire extinguisher: 1. Know its location 2. Remove from mounting 3. Pull pin 4. Squeeze lever 5. Discharge at base of flame 6. Report use and recharge 7. Use dry sand to extinguish burning metals
Unauthorized Experiments	Always work under instructor's or lab technician's supervision in the laboratory.
Eye Protection	Normal eyeglasses are usually not adequate. Do not wear contact lenses in the lab. Eye protection is especially important when working with corrosive materials and vacuum and high pressure apparatus.
Acid/Alkali Spills	For acid spills, use solid sodium bicarbonate followed by water. For alkali spills, wash with water followed by dilute acetic acid.
Handling Flammable Liquids	Flammable liquids should always be stored in an approved storage cabinet. Extinguish all flames in the area where flammable solvents are used, as vapors may travel to ignition source and flash back.

Types of Fire Extinguishers	Rating: A. - For ordinary combustibles; wood, paper, and cloth. B. - For flammable liquids; oil, grease, and gasoline. C. - For use on live electrical equipment. Number on extinguisher (e.g., 10A:5B) denotes square footage the unit is capable of handling.
Handling Mercury	Mercury spills are very hazardous. Droplets should be picked up by suction and a mercury spill kit used to complete cleanup. Notify lab technician immediately when mercury spills occur.
Protection from Toxic Gases	Emergency air masks should be used. However, because our lab is not equipped with such masks, clear the area where gases are, and notify the lab technician.
Waste Disposal	Hot glassware or reactive chemicals should be discarded in a non-metallic container separate from paper and other flammable waste. Test-tube quantities of hazardous liquids can be flushed down the sink with plenty of water. Contact lab technician for disposal of large quantities of hazardous materials or anytime you are not sure of how something should be disposed of.
Labelling Chemicals	All chemicals should be clearly labeled. Do not use materials from unlabeled containers. Avoid contamination. Never return reagents to their container. Clearly label chemicals as you work.
Carrying Chemicals and Equipment	Carry long apparatus such as tubing or burets, in an upright position close to the body. Grasp bottles firmly with both hands and hold them close to the body. Do not carry bottles by the neck. Use a bottle carrier when transporting chemicals any distance.
Transferring Liquids	Remember, **Acid to Water**. Do not pipette by mouth, use a bulb. Use gloves when pouring corrosive liquids. Use a funnel when filling a bottle or flask and prevent an air block by raising the funnel. Pour hazardous liquids over a sink.
Fume Hood	Use a fume hood equipped with a safety glass when working with toxic or flammable materials.

Gas Cylinders	Protect cylinder valve with cap. Fasten cylinders securely. Transport cylinders on a hand truck, don't roll. Do not drop cylinders. Mark cylinders when empty.
Handling Sodium and Potassium	Fire or explosion may result when metallic Na or K are exposed to water. Store them under light oil. Metal can be cut safely with a spatula on a paper towel. Destroy residues with alcohol. Cool if necessary.

THINK SAFETY AT ALL TIMES

No smoking
No food or beverages
No running
Know location of exits
Keep aisles clear - put books and coats in designated areas
Do not leave an experiment unattended
Extinguish burners when you leave the work area

ALWAYS BE PREPARED TO HELP FELLOW STUDENTS IN AN EMERGENCY

ANIMAL SCIENCE NUTRITION LABORATORY

STANDARD PROCEDURES AND SAFETY RULES

I. Personnel using the facilities of the Laboratory are required to:

A. Read, entirely, all assigned laboratory safety material and sign an affirmation that it has been read.

B. Receive, before beginning any activities in the Laboratory, instruction from the Laboratory staff regarding location and proper use of the following safety equipment:

1. EMERGENCY SHOWER

2. FIRE EXTINGUISHERS

3. ELECTRIC POWER PANEL

4. FIRST AID KIT

5. SAFETY GLASSES, GOGGLES, FACE SHIELDS, PROTECTIVE GLOVES, APRONS, AND LAB COATS

6. HOODS AND VENTS

7. TELEPHONE AND EMERGENCY NUMBERS

C. Read and observe the following rules and procedures. **Everyone** using the facilities of the Laboratory is required to abide by these procedures.

II. Personnel using the facilities of the Laboratory area are required to demonstrate an understanding, and proficiency in, the use of any equipment and the conduct of any physio-chemical procedures within the premises before use, unless under direct supervision by the Laboratory staff. Ask for proper instruction if in doubt about procedures.

III. Personnel using the facilities of the Laboratory are required to be aware of the potential hazard involved in any procedure in which they may be engaged (fire, chemical burn, hot liquids, toxic fumes, poisons, electrical shock, etc.). Personnel who initiate the use of any equipment, facilities, or chemical procedures that involve hazard, or that could become hazardous, are required to remain in that particular area until the procedure is properly terminated.

IV. It is not considered good practice to work alone in the Laboratory. Another person should be present or within the range of voice when any potentially hazardous procedure is being conducted.

V. Absolutely **NO SMOKING** in any of the Animal Nutrition Labs or associated rooms.

VI. It is the responsibility of all personnel using flammables to check **first** to ensure that the area is safe from flames and sparking equipment; it is likewise the responsibility of all personnel using flames or sparking equipment to check **first** to ensure that the area is free from flammables.

VII. Foods and drinks are prohibited in any area of the Laboratory where hazardous chemicals are in use, and eating or drinking are prohibited for all persons during whatever period of time they are engaged in usage or handling of toxic or corrosive chemicals.

VIII. Chemicals, equipment, and supplies are to be returned to proper storage **immediately** on completion of use. Desk tops and work areas are to be kept free of "clutter".

IX. Equipment and supplies will not be removed from the premises unless properly checked out. Check out procedure for glassware, equipment, and lab space should be followed. Proper instruction may be obtained from Laboratory personnel.

X. All materials, including samples, should be **properly labeled**. Use proper labeling tape and write legibly. **MATERIALS NOT PROPERLY LABELED WILL BE DISCARDED**.

XI. Work in progress that should not be disturbed must be properly labeled. Every effort must be made to clear ovens, desiccators, and related equipment as soon as possible so that others may use the facilities.

XII. Everyone (students, student aides, and graduate students) is responsible for properly washing his or her own glassware and returning it to storage.

XIII. A glassware breakage list will be posted in the laboratory area. This list must be signed and any breakage recorded. The purposes of this list are:

A. To keep a record of supplies needed in the Laboratory.

B. To instill a greater cautiousness in everyone working in the lab.

XIV. The Laboratory is not an open facility. Permission to use the facility and its equipment must be obtained before use. In the case of proposed extended use of equipment, it is recommended that such use be scheduled, in advance, with the Laboratory supervisor. (NOTE: The use of facilities by scheduled class groups will take priority over other users).

XV. Before any analytical work on samples is allowed:

A. The individual in charge of the samples must make sure each sample has been given a Nutrition Lab code number. Each sample should then be labeled with such code numbers (includes tissue, blood, rumen, as well as feed, feces, etc.)

B. Samples should be adequately described in the code book. Analyses to be performed, project from which samples were derived and time period samples that are to be saved should be indicated.

C. After all analytical work is done, a **copy** of data resulting from the work should be made available to the Nutrition Lab so it can be stored for future reference. Samples will be stored for the time period indicated in section (B) above.

XVI. SCAN BULLETIN BOARDS AND CHALKBOARDS IN THE LABS DAILY FOR NOTICES THAT MAY PERTAIN TO THE USE OF EQUIPMENT OR FACILITIES.

CHAPTER II

Reliability of Laboratory Results

The question of reliability of results is essential to the output of believable, high-quality data from any laboratory. The student who runs a Kjeldahl nitrogen analysis on an alfalfa hay sample should ask the question, "How close is the value I obtained to the true value for this sample?" Moreover, the student should be able to properly evaluate the data to help answer this question.

Some definitions will aid our understanding of this concept of reliability of results. **Accuracy** can be defined as the degree of agreement between a value obtained by a procedure and the actual or true value of the quantity being measured. Because, in most biological settings, the true value is seldom if ever known, the accuracy of any result is seldom known. So, we are still left with the question of how accurate our data really is. Generally, one can increase confidence in the accuracy of a laboratory result by (1) running standards to check for errors in procedures or techniques (e.g., using urea as a standard in the Kjeldahl procedure to check for recovery of nitrogen) and (2) comparing results with others obtained independently. In fact, the use of standards and comparison with other results should be routine procedure in nutrition laboratories. However, these measure still do not ensure accuracy, and for the most part, we must assume the true value is the most probable value from the available data; that is the arithmetic mean of the observations. This is generally a reasonable assumption because the sample mean ($\bar{x}$) is an unbiased estimate of the true mean (population mean, μ).

Precision is a term that many students confuse with accuracy, so a clear distinction must be made between the two terms. Precision can be defined as the closeness of a number of similar measurements to a common value. Although precision in laboratory work is very desirable, the attainment of precision does not necessarily imply the measurements are accurate. This concept is illustrated in Figure 2-1.

The reason for the divergence of precision and accuracy in Figure 2-1 is a common source of error. In this particular example, we might surmise the rifle is not properly sighted in, causing repeated misses of the bulls-eye and poor accuracy. This same situation can occur in the laboratory, as a constant source of error could cause inaccurate results. Precision is usually evaluated as the deviation of individual measurements from a common value; the common value being $\bar{x}$. Precision is the best numerical measure of the repeatability or reliability of a method or instrument, and it is commonly expressed as the standard deviation or coefficient of variation (CV). Although most students are familiar with the method for calculating standard deviation, this information is presented in Table 2-1 for those whom the concept is new.

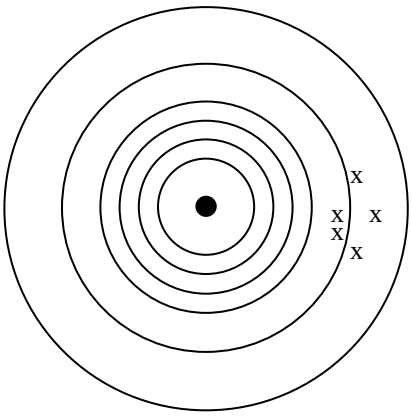

Figure 2-1. Target with x's denoting placement of shots from a rifle. Note that shot placement is very precise (closeness of the x's), but the shooter is not very accurate because of the distance between the x's and the bulls-eye.

As one works in the lab, the value of precision in determining how acceptable or reliable one's results are becomes readily evident. Replication of analyses (i.e., duplicate or triplicate observations on the same sample) allows calculation of $\bar{x} \pm s$ and a quick evaluation of a technique or instrument. When should one run triplicate vs. duplicate analyses? This is somewhat a matter of experience, but the acceptable CV for a number of common nutrition lab analyses listed in Table 2-2 should provide some aid in determining whether more replication is needed.

Table 2-1. Calculation of the sample standard deviation

Standard deviation = $s = \sqrt{\Sigma(x_i-x)^2/(n-1)} = \sqrt{[\Sigma x_i^2-(\Sigma x_i)^2/n]/(n-1)}$

Example: A student ran triplicate analyses for Kjeldahl nitrogen on an alfalfa hay sample and calculated the following crude protein values:

Replicate	% CP	x_i^2
1	17.0	289.0
2	17.5	306.25
3	18.0	324.0

$\Sigma x_i = 17.0 + 17.5 + 18.0 = 52.5$

$\Sigma x_i/n = 52.5/3 = 17.5$

$\Sigma x_i^2 = 289.0 + 306.25 + 324.0 = 919.25$

$(\Sigma x_i)^2 = (52.5)^2 = 2{,}756.25$

$(\Sigma x_i)^2/n = 2756.25/3 = 918.75$

$[\Sigma x_i^2-(\Sigma x_i)^2/n]/n-1 = 0.05/2 = 0.25$

$\sqrt{0.25} = 0.50$ = standard deviation

CV = 0.50/17.5 x 100 = 2.85%

A shortcut method is useful when only two numbers are involved. Calculate as follows:

Take the difference between the two numbers Square the difference and divide it by 2.

Take the square root = standard deviation

Example:

Values of 17.0 and 17.5
Difference is 0.5
$(0.5)^2 = 0.25$
$0.25/2 = 0.125$
$\sqrt{0.125} = 0.354$ = standard deviation

Table 2-2. Some common nutrition lab analyses and acceptable coefficients of variation to use as a guideline in evaluating precision

Analysis	Typical Range in Feedstuffs, %	Acceptable CV, %
DM	80 to 100	0.5
Ash	0 to20	2.0
CP	5 to 50	2.0
CF	5 to50	3.0
ADF	5 to 70	3.0
ADL	0 to 20	4.0
NDF	10 to 80	3.0
Ca	0 to 3	3.0
P	0 to 2	3.0
EE	1 to 20	4.0
IVDMD	20 to 80	4.0

CHAPTER III

Proximate Analysis

General. The proximate analysis is a scheme for routine description of animal feedstuffs devised in 1865 by Henneberg and Stohmann of the Weende Experiment Station in Germany (Lloyd et al., 1978). It is often referred to as the Weende System and was principally devised to separate carbohydrates into two broad classifications: crude fiber and nitrogen free extract (NFE). The system consists of determinations of water (moisture), ash, crude fat (ether extract), crude protein, and crude fiber. As indicated, NFE is a component of the system, but it is measured by difference rather than by analysis. Students should be aware that the proximate analysis system is both comparative and predictive in nature, as this will aid in understanding the broad purposes for development of the system.

First, let us consider the comparative aspects of the system. Most people, even those unfamiliar with livestock feeds and feeding, could look at a sample of first-cut alfalfa hay and a sample of dormant winter range grass and readily surmise that the alfalfa was a higher quality feed than the range grass. Alfalfa simply looks better, or at least from the human vantage point, it has more eye appeal than dormant winter range grass. Fewer people, however, could tell how much better the alfalfa is in terms of any specific nutrient (e.g., protein, fiber) or the production either feed will support. Thus, one important reason for development of the proximate analysis scheme was to allow comparison of feeds on a specific basis. It is often stated that one can not compare apples with oranges, but one can compare the protein as a percentage of dry weight in apples and oranges, and in doing so, make some realistic judgements about the nutritional value of each fruit. By the same token, proximate analysis allows one to make legitimate comparisons of feeds on the basis of specific nutrients, allowing one to judge how much better one feed is than another in terms of specific nutrients.

Now, what about the milk production or growth rate alfalfa hay will support compared with the dormant range grass? This question is addressed by the predictive nature of proximate analysis. If we know all the proximate components of various feeds, and the production the feeds will support, we might be able to develop regression equations to help us predict performance by livestock fed these feeds. In the more typical case, we try to use proximate components to predict factors related to performance, like digestibility and intake and then relate these predicted values to estimates of performance. As will be noted later, the proximate system has some failings that prevent it from being an extremely valuable predictive aid, and considerable research has been conducted in recent years to refine and add to the basic system to make it a better predictive tool.

To summarize, the proximate analysis system is an old scheme of laboratory analyses that allows comparison of feeds on the basis of specific nutrients and, to some extent, prediction of components of animal performance. Next, we will take a look at the specific analyses involved in the proximate analysis scheme.

Dry Matter. Dry matter or, more specifically, moisture determination is probably the most frequently performed analysis in the nutrition laboratory. It is an important analysis, in that the

concentration of other nutrients is usually expressed on a dry matter basis (as a percentage of the dry matter). Because most students seem to have an inordinate amount of difficulty with conversion of nutrients to a dry matter basis, an example showing how this is done, as well as an example of dry matter determination calculations, is given in Table 3-2.

Moisture or dry matter content is extremely important to the livestock industry, particularly those segments that deal with high-moisture feeds. Consider, for example, a feedlot with 20,000 tons of silage inventory. Obviously, as silage is removed from storage, its dry matter content can change, resulting in a change in the feedlot's inventory of silage dry matter. Without routine dry matter determinations, the feedlot could grossly over or underestimate its inventory and perhaps over or undercharge customers for feed. There are numerous examples of the importance of accurate measurements of moisture in the livestock industry that should be readily apparent to most students.

From the author's experience, students typically believe that dry matter analysis is the simplest analysis they perform in the lab. For the most part, this is true because it routinely consists of weighing the sample into a tared (previously weighed) pan, placing the sample in a 100 to 105°C oven for 12 to 24 h and reweighing. Moisture content is simply the loss in weight from evaporation of water. This procedure works well for most feeds, but with some feeds, especially high-moisture, fermented feeds, some problems can be encountered. High-moisture feeds usually contain volatile nutrients that can be lost with 100°C oven drying. Volatile nutrients of greatest importance are short chain fatty acids (acetic, propionic, butyric, etc.), but essential oils (menthol, camphor) also can be important with some feeds. Drying samples at 100°C can volatilize some of these materials, resulting in greater moisture (lower dry matter) values than expected. This concept is considered in some detail along with some other important points in an article by Goss (1980). The errors associated with moisture determination in corn have been the subject of research (Fox and Federson, 1978), and the saponification method of Hood et al. (1971) seems to be a valid method to use with fermented feeds.

Common methods of dry matter determination and suggested occassions for their use are listed in Table 3-1. Students also should check the AOAC (1995) publication for additional methods. . The routine procedure for 100°C oven dry matter determinations is attached to this chapter.

Table 3-1. Common methods of dry matter analysis and suggested occasions for their use

Method	Occasions for use
100°C drying	Most mixed feeds, hays, range grasses with 85 to 99% DM
Freeze drying[a]	High-moisture, fermented feeds
Saponification	High-moisture, fermented feeds
Vacuum drying[b]	Meat or tissue samples

[a]Byers (1980) indicated that freeze drying does not yield significantly different values than saponification. See J. Anim. Sci. 51:158.

[b]Recommended by AOAC for meat samples.

Table 3-2. Dry matter calculations and conversion of nutrients to a dry matter basis (100°C drying oven example)

Replicate	Pan wt.	Pan + sample	Sample wt.	Dry Pan + sample	Dry sample
1	1.0000	3.0000	2.0000	2.9000	1.9000
2	1.0000	3.1000	2.1000	2.9500	1.9500

% DM

Rep 1 = 1.9000 ÷ 2.0000 = .95 x 100 = 95.00%
Rep 2 = 1.9500 ÷ 2.1000 = .9285 x 100 = 92.85%

$\bar{x}$ = 93.93%
s = 1.52
CV = 1.52 ÷ 93.93 = .0161 x 100 = 1.61%

Converting nutrients to a dry matter basis (dmb):

Suppose a feed contains 10% crude protein on a fresh (as-fed) basis (moisture included) and has 10% moisture (90% dry matter).

CP, dmb = 10/0.90 = 11.11%, where 0.90 = dry matter factor or percentage dry matter expressed as a decimal.

To convert from a dry basis to a fresh basis, simply multiply the value on a dry matter basis by the dry matter factor.

Ash. Ash is most commonly performed by burning the sample at 550 to 600°C in a muffle furnace. As with the dry matter analysis, some problems can be encountered in that certain minerals (e.g., Se, Pb, Cd) may be volatilized by high-temperature ashing. In cases when this might be important (usually when analysis for these minerals is going to be performed) a procedure known as wet ashing is used. Wet ashing involves digestion of the sample organic matter with nitric and perchloric acids. This procedure, however, is quite dangerous because of the use of perchloric acid, and special precautions such as a perchloric acid fume hood are required.

Note that total ash content is not a particularly meaningful analysis, in that it reveals nothing about the content of specific minerals. Moreover, ash will contain any contaminating materials like sand or soil that may inflate a sample's ash value. Generally, ash is a preparatory step for further analysis of specific minerals by spectrophotometric or atomic absorption techniques. The general procedure for ash analysis as well as a procedure for putting ash samples in solution for further analyses is attached to this chapter. An example calculation for ash analysis and some comments about organic matter are given in Table 3-3.

Table 3-3. Ash calculations and calculations dealing with organic matter

Example: Most commonly, we use the same pan + sample that was used in the dry matter analysis.

Replicate	Dry pan + sample	Pan + ash sample	Pan wt.	Dry sample wt.	Ash wt.
1	2.9000	1.2500	1.0000	1.9000	0.2500
2	2.9500	1.2700	1.0000	1.9500	0.2700

% Ash, dmb

Rep 1 = 0.2500 ÷ 1.9000 = .1315 x 100 = 13.15%
Rep 2 = 0.2700 ÷ 1.9500 = .1384 x 100 = 13.84%

$\bar{x}$ = 13.50%
s = 0.48
CV% = .48 ÷13.50 = 0.0355 x 100 = 3.55%

Organic matter. Organic matter is that portion of the feed that is not water or ash (i.e., C, H, O, N bonding)

% organic matter, dmb = 100 - Ash,dmb
For the example shown above, OM, % (dmb) = 100 - 13.50 = 86.5%

Animal Science Nutrition Lab

DRY MATTER AND ASH

Materials

Aluminum weighing pans
Forced-air oven
Muffle furnace

Procedure

1) Dry aluminum pans in oven at 100°C for 15 to 30 min
2) Cool pans in desiccator, weigh and record weight
3) Add 1- to 2-g samples and record weight of pan plus sample
4) Dry pan plus sample in oven at 100°C for 12 h or overnight
5) Cool in desiccator, weigh back, and record weight
6) Place pans plus samples in muffle furnace and ash at 500°C for 3 h
7) Cool in muffle for at least 8 h, then in a desiccator, weigh back, and record weight

Calculations:

Pan plus sample weight (before drying) - Pan weight = Sample wet weight

Pan plus sample weight (after drying) - Pan weight = Sample dry weight

% DM = (Dry weight/Wet weight) x 100

Pan plus sample weight (after ashing) - Pan weight = Sample ash weight

%Ash (dmb) = (Ash weight/Dry weight) x 100

Animal Science Nutrition Lab

PREPARATION OF ASH FOR MINERAL ANALYSES

1) Ash 1- to 2-g sample in muffle furnace.

2) Transfer ash residue to 250-mL beaker.

3) Add 50 mL HC1 (1 part HCl + 3 parts water) and add several drops of HNO_3; bring to a boil under a hood.

4) Cool and filter into a 50- or 100-mL volumetric flask that has been rinsed with dilute acid.

5) Dilute to volume with deionized H_20.

Note: Some minerals may not be soluble in 25% HCl. Check AOAC or other reference manuals for information on specific minerals.

Crude Protein Determination by the Kjeldahl Method. Along with dry matter and ash analyses, the Kjeldahl procedure is one of the most common analyses performed in the nutrition laboratory. Although not a measurement of protein *per se*, it does provide an estimate of the content of this important nutrient in feedstuffs. Protein always seems to be in short supply, both in human and animal feeds, and an accurate estimate of protein content is vital in formulating diets for optimum animal performance.

The Kjeldahl method dates back to the 1880's when Johan Kjeldahl used sulfuric and phosphoric acids to decompose organic materials. The method can be conveniently divided into three phases: digestion, distillation, and titration.

The digestion phase is the first phase of the analysis and is designed to oxidize organic matter to CO_2 and H_2O, while reducing nitrogen to ammonia. Sulfuric acid is employed as the principle method of decomposing organic matter. Phosphoric acid also can be used in conjunction with sulfuric acid, but an exact mixture of the two acids is critical, and usually the disadvantages of using two acids outweigh the advantages.

A number of catalysts have been employed in the digestion phase. Potassium sulfate is almost always added to raise the boiling point of H_2SO_4 and thereby increase rate of digestion. A number of other catalysts have been used to assist in reduction of nitrogen including mercuric oxide, copper sulfate, and selenium. Mercuric oxide and copper sulfate seem to be the most popular and along with potassium sulfate, can be purchased in individually packaged catalyst packs. Because of environmental concerns, the use of mercury-based catalysts has decreased in recent years.

At the termination of the digestion phase, organic matter has been decomposed to CO_2 and H_2O, and nitrogen has been changed to ammonium sulfate. Digestion requires a temperature of about 350°C and about 2 to 3 h for most feed samples. A good thumb rule is to boil the vessel until contents become clear and then boil for an additional 30 min to 1 h. We should note at this point that nitrates are not completely recovered in most standard Kjeldahl procedures. Should one wish to recover all the nitrate-nitrogen in a sample, the addition of salicylic acid as a reducing agent in the reaction mixture will accomplish the task.

The distillation phase is the second step of the procedure and involves adding an excess of strong NaOH to the sample from the digestion phase, after adequate dilution of the vessel with distilled water. Specifically, the digestion flask is cooled and 300 to 400 mL of distilled H_2O is added, followed by zinc powder and boiling stones. Then, 100 mL of a saturated NaOH solution is added carefully down the neck of the flask. The base liberates ammonia from the sulfate form, and when heat is applied to the vessel, $NH_4^+OH^-$ is distilled over into a beaker containing a boric acid/indicator solution. The boric acid simply serves to hold the ammonia in solution. Zinc dust and boiling stones are included to provide smooth boiling during the distillation process.

The titration process is simply a matter of neutralizing the collected $NH_4^+OH^-$ with a standard acid. Normally, either HCl or H_2SO_4 is used for this portion of the procedure, and the acid is formulated so that 1 mL of acid will neutralize 1 or 2 mg of nitrogen. Derivation of 0.1428 N HCl, which will titrate 2 mg of nitrogen/mL of acid is attached to this chapter. Rather

than preparing HCl of a known normality, it is often more convenient to purchase standardized acid from commercial sources (e.g., 1 mL of .1N HCl will neutralize 1.4 mg of ammonia-N). Once the milliliters of acid used in the titration process are known, the nitrogen and crude protein content can be calculated. An example of this calculation is shown in Table 3-4.

Generally, samples to be analyzed for Kjeldahl nitrogen are weighed and wrapped in filter paper and then placed in the reaction vessel. Containing the sample in filter paper is simply a method of preventing excessive foaming encountered when loose, ground samples are subjected to H_2SO_4 treatment in the digestion phase. However, because the filter paper contains some nitrogen, it is essential to run a filter paper blank (filter paper + H_2SO_4 + catalysts) to correct for any added nitrogen. This explains the blank subtraction shown in the calculations of Table 3-4.

Standard procedures used in the animal nutrition lab for Kjeldahl analysis are included in this chapter. Before we leave the Kjeldahl analysis, however, it is worthwhile to consider what it really measures. It is called "crude" because it does not necessarily represent a amino nitrogen or true protein but may contain all types of non-protein nitrogen (NPN) like urea, amides, nucleic acids, and free amino acids. Generally, for the ruminant this inclusion of NPN is not a major concern because these compounds as well as true protein would be partially converted to ammonia by ruminal microorganisms and subsequently used for microbial protein synthesis. If knowledge of the true a amino-linked (protein) content of a feed is desired, other methods should be used. Students should consult the AOAC manual for further information on this subject.

In general, the Kjeldahl analysis provides a fairly good estimate of the true protein content of most mixed feeds, hays, grains, and seed meals. Problems are encountered with lush pasture crops and fermented feeds in that a fairly large proportion of the total nitrogen may be present as NPN.

The final question is "where does the 6.25 factor in the calculations come from?" Most original work with proteins showed that they averaged around 16% nitrogen. Thus, if nitrogen content is known, one can simply multiply it by 100/16 = 6.25 to estimate protein content. Unfortunately, all proteins are not 16% nitrogen, and in cases where divergence from the 16% value is known, other factors should be used. For example, the combined proteins of milk contain approximately 15.7% nitrogen, and a factor of 6.38 should be used (Maynard et al., 1979). Students should consult Maynard et al. (1979) for further reading and references on this topic.

Table 3-4. Calculation of crude protein content from the Kjeldahl analysis

Rep	Pan wt	Pan + Sample wt	Sample wt	Sample dmf	mL acid used	mL acid in blank	Corrected mL acid
1	1.0000	2.0000	1.0000	0.900	20.2	0.2	20
2	1.0000	2.1000	1.1000	0.900	20.7	0.2	20.5

Milligrams of N in sample

Rep 1 = 20 x 1.4 mg of N/mL of acid using .1 N HCl = 28 mg
Rep 2 = 20.5 x 1.4 mg of N/mL of acid using .1 N HCl = 28.7 mg

% N in sample

Rep 1 = 28 mg ÷ 1,000 mg = 0.028 x 100 = 2.8%
Rep 2 = 28.7 mg ÷ 1,100 mg = 0.0261 x 100 2.61%

% CP in sample, dmb

Rep 1 = 2.8 x 6.25 = 17.5% ÷ 0.90 = 19.44%
Rep 2 = 2.61 x 6.25 = 16.31% ÷ 0.90 = 18.13%

$\bar{x}$ = 18.79%
s = 0.93
CV = 4.92%

Animal Science Nutrition Lab

PROCEDURE FOR KJELDAHL NITROGEN - MACRO METHOD

CAUTION: FACE PROTECTION AND LAB COATS ARE REQUIRED FOR ALL PORTIONS OF THIS PROCEDURE.

I. Procedures

A. Sample Size

A. Feeds, feces, and dry specimens - weigh a 1-g sample and transfer into #1 ashless filter paper, folded to prevent loss of sample.
B. Urine - measure 5 mL of urine sample into an 800 mL Kjeldahl flask. Be sure that the specimen is at room temperature and uniformly suspended.

B. Catalyst - Use Kel-Pac #2 Gunning, which contains 10 g of K_2SO_4 plus .30 g of $CuSO_4$. For certain procedures it may be preferable to use 15 g of anhydrous K_2SO_4 plus .7 g of HgO.

1. Blanks - Kjeldahl reagents generally contain small amounts of nitrogen, which must be measured and corrected for in calculations. Prepare "blanks" for dry samples by folding one sheet of #1 ashless filter paper and placing it into the Kjeldahl flask. Treat blanks exactly like samples to be analyzed, and subtract the amount of acid titrated for the blanks from the amount of acid titrated for samples.

2. Standards - weigh out two (2) .1-g samples of urea, transfer into #1 ashless filter paper and treat exactly like samples. Calculate percent recovery of nitrogen from urea standards.

C. Acid - add reagent grade, concentrated H_2SO_4 to Kjeldahl flask. For dry samples (feeds and feces) of 1-g size, add 25 mL; for dry samples of a 2-g size, add 35 mL; for urine and aqueous samples, add 25 mL. The ratio of acid to dry matter is important, as the boiling temperature is affected by the ratio of acid to salts in the mixture. Also, if too much acid is used, violent reactions can occur at a later step when alkali is added.

D. Digestion - turn blower on for digestion rack and burners on '5' setting. After adding sample, catalyst, and acid to flask, place on burners of digestion rack. Rotate flasks about every 15 min to speed the oxidation of carbon. If excessive foaming occurs, cool flask and add about .5 g of purified paraffin wax. After all samples are clear, increase heat to 'Hi' setting and digest for 30 more min. Turn heat off and allow flasks to cool. Do not remove flasks from burners and do not turn blower off until flasks are cool to the touch.

E. Dilution - when flasks are cool to touch, add 400 mL of deionized water. Swirl contents to dissolve salts - this is very important!

F. Preparation of receiving flask for steam distillation - add 75 mL of prepared boric acid

solution (2.9% boric acid containing methyl purple indicator) to a clean 500-mL Erlenmeyer flask and place on distillation rack shelf. Place delivery tube from condenser into the flask, making sure that the tube extends well below the surface of the boric acid solution. TURN THE WATER TO THE DISTILLATION SYSTEM ON, and turn on all burners on the '4' setting.

G. Preparation of sample for distillation - Step 1 must be done under the hood as toxic H_2S is given off upon the addition of zinc.

1. Add approximately 0.5 g of powdered zinc to flask, mix thoroughly and allow to settle. Add a scoop of boiling stones.

   FACE SHIELD, GLOVES, AND APRON MUST BE WORN FOR THE NEXT STEPS!!!

2. After digest has settled, measure 100 mL of saturated, aqueous NaOH (50% wt/vol) into a graduated cylinder. Slant Kjeldahl flask containing prepared digest solution about 45° from vertical position. Pour NaOH SLOWLY!! into flask so that a layer forms at the bottom. DO NOT MIX!!!

3. Attach flask to distillation-condenser assembly. DO NOT MIX flask contents until firmy attached. Holding flask firmly, making sure cork is snugly in place, swirl contents to mix completely. Immediately set flask on heater. Withdraw receiving flask from distillation-condenser delivery tube momentarily to allow pressure to equalize and prevent back suction.

H. Distillation

1. Continue distillation until approximately 250 mL of distillate has been collected in receiving flask.

2. Turn heater off. Remove receiving flask partially and rinse delivery tube with deionized water, collecting the rinse water into receiving flask.

3. Replace receiving flask with a beaker containing 400 mL of deionized water. This water will be sucked back into the Kjeldahl flask as it cools, washing out the condenser tube.

I. Titration - titrate green distillate back to original purple using.1 N HC1, and record volume of acid used in titration.

J. Cleaning of flasks - after water has been sucked back into Kjeldahl flasks, allow to cool and then pour liquid into sink, catching the boiling stones in a beaker. Wash flasks, INSIDE AND OUTSIDE!!, with tap water, then rinse with dilute acid, followed by deionized water. Place upside down on Kjeldahl flask racks to dry. Make sure you wash both the inside and outside of flasks!!

K. Cleaning of area - entire work area should be sponged clean and all reagents, supplies, and glassware returned to proper storage. MAKE SURE that all countertops are free of any spilled acid, alkali, or other reagents!!

L. NOTE - ADIN samples are to be digested on '3' setting for the entire digestion - do not turn to 'Hi' setting. Dilute with 500 mL of deionized water.

II. Reagents

A. Mixed Indicator - dissolve 0.3125 g methyl red and 0.2062 g methylene blue in 250 mL of 95% ethanol. Stir for 24 h.

B. Boric Acid Solution - dissolve 522 g U.S.P. boric acid in 18 L of deionized water. Add 50 mL of mixed indicator solution and allow to stir overnight.

C. Zinc -- (metallic) powdered or granular, 10 mesh.

D. Sodium Hydroxide - 50% wt/vol aqueous (saturated) technical grade is adequate.

E. Standardized .1 N Hydrochloric Acid Solution - purchased as such from any major chemical supplier.

III. Summary

DIGESTION

1. Place sample and Kel-pac catalyst into labeled Kjeldahl flask.

2. Add 25 mL of concentrated H_2SO_4 for one gram samples or aqueous samples. Add 35 mL of concentrated H_2SO_4 for two gram samples.

3. Turn blower on, and set burners on '5'.

4. Digest until clear, then set on 'Hi' for 30 more min. Rotate flasks occasionally throughout digestion.

5. Cool digest and add 400 mL deionized water.

6. Swirl flask to dissolve salts.

DISTILLATION

1. Add 75 mL of boric acid solution to a 500 mL Erlenmeyer flask and label appropriately. Place the delivery tube from the condenser into the boric acid.

2. Turn water on to distillation system and set burners on '4'.

3. Add 0.5 g of zinc and a scoop of boiling stones to flask. Do this step UNDER THE HOOD!!

4. Add 100 mL of 50% NaOH slowly to Kjeldahl flask.

5. Place flask on burner, mix thoroughly, and boil until 250 mL of distillate has been collected.

6. Remove receiving flask and replace with beaker containing 400 mL of deionized water.

7. Titrate distillate with 0.1 N HCl, recording amount of acid used.

8. Clean glassware and area thoroughly.

Animal Science Nutrition Lab

OPERATION OF TECATOR RAPID DIGESTOR AND STEAM DISTILLATION UNIT FOR KJELDAHL NITROGEN ANALYSIS

DIGESTION:

1. Turn main power switch located on front of digestion controller to ON position. Set temperature to approximately 400°C using temperature set on controller. Allow time for unit to reach temperature.
2. Carefully insert the digestion tubes into the holes provided in the flask rack. A digestion tube must be set in all positions (even if empty) for the exhaust manifold to work properly.
3. Weigh approximately 0.5 to 1 g of ground homogenous sample in a piece of weighing paper. Fold the paper carefully so that the sample is well contained. Place the folded weigh paper into digestion tube, and add 2 Kjeltabs to each tube. Also, weigh a 0.1 g of urea standard and treat as above. The blank consists of a piece of folded weigh paper and Kjeltabs only. Run one urea standard and one blank with each rack of samples you analyze.
4. When the digestion unit has been on for an adequate warm-up period, add 15 to 20 mL of concentrated sulfuric acid to each tube.
5. After completing sample and reagent addition to the digestion tubes, carefully elevate the rack by grasping the handles provided and place in position on the digestor unit. As the rack is lifted, each tube will rest on its top rim. Carefully lower rack so that each tube enters its respective hole and bottoms in the base of the unit.
6. Place end plates on the flask holder rack, and place the exhaust manifold on top of the tubes. Turn the water supply on for the exhaust manifold to the highest rate of water flow. *Ensure that the water aspiration-fume removal system is functioning properly before use*.
7. Digest samples at 400°C for approximately 1 h, or until the samples have a clear, blue-green appearance. *Turn down the water supply to exhaust manifold to a lower flow rate after the first 5 to 10 min of digestion*.
8. After digestion is complete, remove flask rack containing tubes (with fume removal system still attached) from digestor and place in the rack and tray system next to the digestor. Avoid having the hot tubes come into contact with a cool or wet surface. As a safety precaution, wear gloves and goggles when removing the rack and tubes from digestor.
9. Allow tubes to cool for 15 to 20 min. When tubes have cooled, remove the flask rack containing tubes from the fume hood. Place the fume removal system on the rack provided under the fume hood, and turn off the water supply to the fume removal system. Dilute the digest with 100 mL of deionized water when the tubes are cool enough to handle. Dilution must be made before a cake or gel is formed, but not before the digest is cool enough to contain the exothermic reaction. Make sure digest is fully dissolved.

DISTILLATION:

1. Turn on the cooling water to the Steam Distillation Unit and allow it to flow through the entire unit. The steam generator drain line should be closed for distillation of samples, but water should flow freely from the waste drain line. The system will automatically adjust water flow to the steam generator.
2. Place the distillation vessel with digested sample on the apparatus, twisting the tube to ensure a complete seal.
3. Insert receiver 250-mL Erylenmeyer flask containing 25 mL of boric acid (Kjel-Sorb) indicator, making sure that the delivery tube is fully submerged.
4. After closing the plastic safety shield door, add approximately 50 mL of NaOH by slowly pushing down the alkali dispenser handle, and allow the dispenser handle to return to its closed position (the dispenser handle is spring-loaded and will return without applicationof force to the handle).
5. Open the steam valve to start steam generation. If desired, set the time for approximately 6 min.
6. Collect approximatley 175 mL of distillate in the receiver flask. For the last 15 to 25 mL of collection, carefully lower the receiving flask so that the tube is above the fluid level in the receiving flask.
7. Close the steam valve to halt steam generation. Raise the plastic safety shield door, and remove the receiver flask. Use a small quantity of distilled water to was down the outside of the receiver tube into the receiver flask.
8. Wearing gloves, remove the distillation vessel and place it in the nearby tube rack. After vessel has cooled for 10 or 15 min, carefully pour the contents down the drain, diluting with a large quantity of running tap water.
9. To distill another sample, repeat Steps 2 through 8.
10. Titrate the receiving solution.
11. When you have finished distilling and titrating all samples, place a digestion tube that contains approximately 75 to 100 mL of deionized water in the apparatus, start steam generation, and collect 150 to 175 mL of distillate - DO NOT ADD ALKALI FOR THIS STEP. This serves to wash out the system. If needed, repeat this step until the contents of the digestion flask are clear. Close the steam generation valve, turn off the water supply, and turn off the power on the distillation unit.
12. Use a wet paper towel or Kimwipe to wipe the rubber adapter to which the digestion tubes fit, and wash the removable tray in the bottom of the distillation unit. Check inside the distillation unit for other areas that need to be wiped clean. After power has been off to the distillation unit for 15 min, open the drain line for the steam generator.

ADDITIONAL COMMENTS:

CLEAN UP ANY SPILLED ACID OR BASE when you are finished!! This includes any acid

that has dripped on the top of the digestor, the drip pan under the fume removal system, under the fume hood, or any other counter in the lab!! Also, please rinse the sink with tap water when you are finished.

PLEASE wear protective eyewear (face shield) at all times in the Kjeldahl lab!

Wash all glassware by first rinsing with tap water, then squirting the inside with dilute hydrochloric acid. Finally, rinse well with deionized water.

Derivation of "standard acid" and "standard base" in KJELDAHL METHOD

1 mL of 0.1428 Normal acid = 2 mg of Nitrogen

Derivation: Normality, N = equivalent weights per liter
l N = l g equivalent weight per liter = l milliequivalent weight per mL (milliequivalents = Normality x milliliters)

In the Kjeldahl procedure, nitrogen is titrated as $NH^{+}_{4}OH^{-}$.

The molecular wt of NH_4OH is 35.04595; therefore:

l mEq of NH_4OH = 0.03504595 g of NH_4OH, which provides 0.0140067 g of **nitrogen**.

l mL of l N acid would titrate l mL of l N NH_4OH, i.e., l mEq. = l mEq
l milliequivalent of NH_4OH is 35.04595 mg of NH_4OH and it contains 14.0067 mg of **nitrogen**.

If l mL of l N acid is required to neutralize l mL of l N NH_4OH, which contains 14.0067 mg of nitrogen, then what normality of acid is needed for l mL of it to equal 1 mg of N?

ANSWER: "X", the unknown, = 1/14.0067 = 0.071394; thus l mL of 0.0714 normal acid = 1 mg of NH_4OH-nitrogen

and

l mL of 0.1428 normal acid = 2 mg of NH_4OH-nitrogen

If a sample of 2 g size yields 20 mg of NH_4-N, then the sample could be said to contain 1% nitrogen.

NOTE: In practice, Boric Acid (2.9 % solution) is used--*in excess*--to receive the gaseous ammonia during the Kjeldahl distillation, and the boric acid is then "back titrated" with standard acid (either 0.0714 Normal, l mL = l mg N; or 0.1428 Normal, l mL = 2 mg N).

Ether Extract or Crude Fat Determination. Lipids are a group of materials that are insoluble in water but soluble in ether, chloroform, and benzene (Maynard et al., 1979). We often apply the term "fat" to all feed substances extracted by ether; however, as we shall see, this is a somewhat inappropriate use of the term "fat." Several authors have devised classification schemes for lipids, and one example from Maynard et al. (1979) is shown in Table 3-5.

Table 3-5. A classification of lipids[a]

Saponifiable		Nonsaponifiable
Simple	Compound	Terpenes
Fats	Glycolipids	Steroids
Waxes	Phospholipids	Prostaglandins

[a]Reproduced from Maynard et al. (1979).

The broad classification of saponifiable vs. nonsaponifiable distinguishes between lipids that contain alcohol (saponifiable) and those that do not contain alcohol (nonsaponifiable). This separation is convenient because when fats are boiled with alkali, they split into alcohol and salts of the fatty acid. From reactions with alkali, we derive the saponification number, which gives an indication of the average chain length of fats.

Fats are esters of fatty acids with glycerol. Generally, a further division is made in that fats that are liquids at room temperature (20^oC) are referred to as oils, and those that are solids at room temperature are simply called fats.

If three moles of fatty acid combine with one mole of glycerol, the product is referred to as a triacylglycerol, triglyceride, or neutral fat. Most students equate ether extract with neutral fat, which is reasonable in the case of oil seeds and animal products. However, in some feeds, neutral fats may make up only a small percentage of the total ether extract. For further information on characterization of fats, students are referred to Maynard et al. (1979).

Waxes are lipids that result from the combination of fatty acids with higher mono- and dihydroxy alcohols. Waxes are somewhat difficult to digest and have much less nutritive value than neutral fats. They occur as secretions and excretions in animals and insects and as protective coatings in plants (Maynard et al., 1979). A typical example is beeswax, which is a combination of palmitic acid with myricyl alcohol ($C_{30}H_{61}OH$).

Compound lipids, including phospholipids and glycolipids are important in animal and plant tissues because they contain both polar and nonpolar components. Phospholipids consist of glycerol with fatty acids esterified at the 1 and 2 positions and phosphoric acid at position 3 of the glycerol molecule. A nitrogenous base is esterified to the phosphoric acid. A typical example is lecithin, in which the nitrogenous base is choline. Phospholipids are constituents of cell membranes and lipid transport proteins in the plasma (chylomyirons and lipoproteins).

Glycolipids contain glycerol and two polyunsaturated fatty acids at the 2 and 3 positions of glycerol. On position 1 of the glycerol, one or two moles of galactose are attached. Glycolipids are the predominant lipids in the leaves of plants and as such, they represent a major source of lipids for animals consuming high-forage diets (Maynard et al., 1979).

As indicated in Table 3-5, nonsaponifiable lipids include terpenes, steroids, and prostaglandins. Generally, such lipids are only a small fraction of the ether extract of most feeds, but certain of the terpenes can be a notable exception, as we will discuss later.

Prostaglandins are a group of compounds that contain 20 carbon atoms with a cyclic structure between the eighth and twelfth carbon atom (Maynard et al., 1979). They are synthesized by almost all mammalian tissues from arachidonic acid, and their synthesis depends on an intact pituitary gland. Prostaglandins can cure an essential fatty acid deficiency, and they are involved in a number of ways in the reproductive process. Students are referred to Goldberg and Ramwel (1975) for information about the role of prostaglandins in reproduction.

Steroids are a large group of compounds, including cholesterol, ergosterol, bile acids, and adrenal and sex hormones. Steroids generally contribute only a small fraction to the ether extract of feeds, but play a number of important biological roles. Maynard et al. (1979) provide a more complete description of the biological roles of steroids.

Terpenes are materials that yield an isoprene moiety on degredation and include the carotenoids, chlorophyll, and essential oils. Essential oils (menthol, camphor) have no nutritional value and are usually only a small fraction of the ether extract of most feeds. Certain plants, particularly desert shrubs, can contain very high levels of essential oils and thereby grossly inflate the ether extract value and expected energy content of such plants. Cook et al. (1952) discuss the problem of essential oils in such plants.

The ether extraction procedure itself is quite simple and usually involves a reflux apparatus in which the ether is boiled, condensed, and allowed to pass through the feed sample. The Animal Nutrition Laboratory ether extract procedure is attached to this chapter and an example calculation is given in Table 3-6.

Students should be aware that the ether extract procedure is one of the most dangerous they will perform. Ether itself is extremely flammable and great caution should be used in handling it. Moreover, if ether is allowed to boil dry during the extraction process, explosive peroxides can be formed. Generally, a small piece of copper wire is added to the extraction beaker along with ether to prevent peroxide formation. Sparkless switches and adequate ventilation should be standard in areas where ether is in common use.

An alternative lipid classification system and some discussion about the lipid extracting properties of various solvents prepared by Dr. G. S. Smith is attached to this chapter to provide additional reading for students.

Table 3-6. Calculation procedures for the ether extract analysis

Rep	Pan wt	Pan + sample wt	Sample dmf	Beaker wt before	Beaker wt after	Wt of extract
1	3.0000	2.0000	0.90	50.0000	50.0800	0.08
2	3.1000	2.1000	0.90	50.0000	50.0900	0.09

% Ether extract

Rep 1 = 0.08/2.0000 = 0.0400 x 100 = 4.00%
Rep 2 = 0.09/2.1000 = 0.0428 x 100 = 4.28%

% Ether extract, dmb

Rep 1 = 4.00 ÷ 0.9 = 4.44%
Rep 2 = 4.28 ÷ 0.9 = 4.76%

$\bar{x}$ = 4.60%
s = 0.23
CV = 4.91%

Alternative Lipid Classification Scheme

Courtesy of G. S. Smith

CLASSIFICATION OF LIPIDS: Chemically, the lipids are either esters of fatty acids or substances capable of forming such esters. The term "lipid" or "fat" may, however, be encountered in general usage to characterize a wide variety of compounds that are insoluble in water and soluble, to varying extent, in "fat solvents" or "organic solvents" such as: ether (diethyl ether), chloroform, alcohol (methanol, ethanol, etc.), acetone, benzene, and "petroleum ether" (usually a mixture of alkanes, especially hexane, "Skellysolve B", the fraction boiling at about 60-68^{o}C).

I. SIMPLE LIPIDS: Esters of fatty acids and glycerol or other alcohols
   A. Fats and Oils
   B. Waxes

II. COMPOUND LIPIDS: Esters of fatty acids which, on hydroloysis, yield substances in addition to fatty acids and an alcohol.

A. Phospholipids (Phosphatides): Lipids which, on hydrolysis, yield fatty acids, phosphoric acid, an alcohol (usually, but not always, glycerol), and a nitrogenous base:
   1. Lecithins - nitrogenous base is choline.
   2. Cephalins - nitrogenous base is ethanolamine, serine, etc.
   3. Sphingolipids - nitrogenous base is sphingosine.

B. Glycolipids (Cerebrosides): Lipids which, on hydrolysis, yield fatty acids, a complex alcohol, and a carbohydrate. They contain nitrogen but no phosphoric acid.

C. Sulfolipids: complex lipids which contain sulfur, usually in addition to nitrogen and/or phosphorus.

PREPARATION AND ANALYSIS OF LIPIDS – excerpts from Lovern, Chemistry of Lipids of Biochemical Significance.

Extraction "Lipids by definition are soluble in the "fat" solvents, such as ether, alcohol and chloroform. Their extraction from tissues, however, is complicated by at least three factors:

a) much of the lipid may be linked in some form of combination with protein or carbohydrate, and these complexes are usually insoluble in fat solvents.

b) some lipids are only soluble in a limited range of fat solvents; and,
c) some fat solvents are also good solvents for certain non-lipid constituents of tissues. A further, but in practice less troublesome, complication is that the original wet tissue cannot be efficiently extracted with many otherwise good solvents, and must first be dried.

There is no difficulty in extracting triglycerides from fat-rich tissues, e.g. adipose tissue or

oil seeds. In general, triglycerides are not bound into lipoprotein or lipocarbohydrate complexes, and this can certainly be taken for granted with the overwhelmingly greater part of the triglycerides of fat-rich tissues. (The same is probably true for waxes.) Moreover, triglycerides are easily soluble in practically all fat solvents, an exception being cold alcohol. Thus the wet tissue may be extracted with successive batches of a solvent such as acetone, which first extracts the water, and then the triglycerides. Alternatively the tissue may be dried, e.g., by freeze-drying/lyophilizing/, and extracted with any desired solvent, e.g., light petroleum/petroleum ether," heavily contaminated with non-lipids, particularly if a fairly selective solvent such as light petroleum is used, either for the original extraction or for re- extraction of the first extract.

Phospholipids, sphingolipids and sterols are usually present in tissues in 'bound' form /or partly so/. There is a great volume of published work on various methods of ensuring complete extraction of these lipids....

It has long been known that ethanol is able to liberate much of the protein-bound lipid. Although ethanol is not a good solvent for some lipids, e.g. the 'cephalin group', its solvent power is much greater when it also contains such lipids as lecithin. It can also be increased by addition of ether or benzene. Another favorite mixed solvent is methanol and chloroform.

It is a frequent practice to extract the wet tissue solely with such a solvent mixture. The alcohol component removes water and liberates bound lipids. Thus Bloor. ....first used a 3:1 ethanol:ether mixture for the extraction of blood lipids, and this solvent has since found favour for extraction of all kinds of tissues. There is no unanimity as to the relative merits of hot or cold extraction, nor as to the period of extraction. ....the ratio of ethanol to ether could be varied over a wide range without affecting the results..../some/prefer a 1:1 chloroform:methanol mixture....ethanol:ether and chloroform:methanol have been shown to give higher yields. Benzene:ethanol mixtures have found favour in extracting vegetable phosphatides…./ a 2:1 mixture is suitable/.

Acetone, like alcohol, possesses considerable lipid-freeing powers, but since it is a poor solvent (at least when cold) for phosphatides and sphingolipids it is not used in the way just described for alcohol. Acetone is frequently used, however, as a dehydrating solvent, to be followed by some better general lipid solvent such a light petroleum or chloroform. Used in this way, acetone will remove, besides water, the triglycerides, sterols and sterol esters of the tissue, thus ensuring that virtually the only lipids present in the subsequent extracts are phospholipids and sphingolipids....

It should be noted that acetone does extract a certain proportion of phospholipid material, particularly when this is accompanied by large proportions of acetone-soluble lipids such as triglycerides and sterols. In the absence of electrolytes, lecithin and similar substances are appreciably soluble in cold acetone. Some workers add a electrolyte, commonly magnesium chloride to the acetone to reduce this solvent effect, but it is not thereby completely eliminated…. Hot acetone is a good solvent for cerebrosides....

Purification. The problem of non-lipid contaminants has a two-fold origin: a) many of the solvents, e.g., acetone and alcohol, especially with wet tissue, are quite effective extractants for

many of the non-lipid constituents of tissues, e.g., urea, amino acids, various nitrogenous bases, sugars, etc.; and b) substances normally insoluble in fat solvents are readily soluble in the presence of phospholipids.... Removal of contaminants carried into solution by phospholipids is particularly difficult, partly because of the tendency of phospholipids to form complexes with all manner of substances....

Animal Science Nutrition Lab

DETERMINATION OF CRUDE FAT (ETHER EXTRACT)

Materials: Anhydrous ethyl ether

Extraction thimbles

100-mL tall-form beakers

1 to 2 g samples in #1 or #4 filter paper envelopes

Procedure:

1. Label beakers and place a small piece of copper wire (approximately ½" length) in each beaker.
2. Dry beakers at 100°C for approximately 15 min. Cool in dessicator and weigh.
3. Turn on water to condensers. Place sample in extraction thimble and place thimble in clamp on extractor.
4. Pour 50 mL of anhydrous ether into each beaker.
5. Place collar on beaker, attach snuggly to extractor.
6. Turn all burners to "high" setting and turn on main switch. When dripping occurs through the extraction thimble in all samples, turn off the extractor (using main switch) and turn all settings to low. Turn extractor on.
7. Extract at a rate of 2 to 3 drops/second for 16 h.
8. After 16 h, shut-off burners by using main switch, and remove beakers. Replace thimble with glass tube and replace beaker. Bring contents to a boil, collecting ether in glass tube. Boil beaker until almost dry. (Save samples if crude fiber is to be run).
9. Remove beaker and pour collected ether into recycled ether can.
10. Place beakers in 100°C oven for 30 min.
11. Cool beakers in dessicator and weigh.
12. Ash beakers to clean.

Note: The same procedure can be run with a shorter extraction time. The extraction is run for 4 h with a drip rate of 5 to 6 drops/second.

Crude Fiber and Nitrogen Free Extract. The crude fiber analysis is a central part of the Weende system. Originally, the analysis was designed to separate plant carbohydrates into less digestible fractions (crude fiber) and readily digestible fractions (nitrogen free extract; NFE). A tabulation of some common carbohydrates and how they are distributed in the Weende system is shown in Table 3-7, taken from Lloyd et al. (1978). As indicated by this table, and by considerable research, the crude fiber/NFE system fails to make a clear distinction between less digestible and readily digestible materials. In fact, Van Soest (1975) reported that in 20 to 30% of feeds listed in Morrison's *Feeds and Feeding*, the crude fiber fraction is actually more digestible than the NFE fraction.

What are the reasons for this failure? In most plants, the cell contents consist of starches, sugars, soluble proteins, and other water soluble constituents that are highly digestible (95 to 100% digestible). On the other hand, plant cell walls contain much less digestible material like lignin, cellulose, and hemicellulose. A complete description of each of these constituents is beyond the scope of this discussion; however, students are referred to Maynard et al. (1979) for descriptions of the major components of plant cells. In addition, Figure 3-1 provides a graphical representation of the structure of forage cells reproduced from Maynard et al. (1979).

The crude fiber system fails to distinguish between plant cell contents and cell wall material because the analytical procedures result in the solubilization of portions of the cell wall or less digestible constituents. Essentially, the procedure involves boiling a fat-free sample of feed with weak acid followed by weak alkali. The loss in weight on ignition of the residue is crude fiber. Nitrogen free extract is calculated by difference (i.e., 100 - %H_2O - % Ash - %CP - %EE - %CF). The term total carbohydrate (i.e., 100 - %H_2O - % Ash - %CP - %EE) is often associated with human foods because most foods consumed by humans are quite low in crude fiber. Unfortunately, boiling samples in dilute alkali dissolves both hemicellulose and lignin. Moreover, portions of plant cellulose can be solubilized by the procedure (see footnote in Table 3-7). Thus, poorly digestible materials (hemicellulose, cellulose) and indigestible material (lignin) are classified as nitrogen free extract. One can readily see why crude fiber might be more digestible than NFE in certain feeds.

Many scientists have recognized the problems of the crude fiber analysis, and considerable work has been done to revise and improve the system. Probably the most widely accepted new system of fiber analysis was developed by Van Soest and coworkers. We will discuss this particular system in some detail in a later chapter.

The Animal Nutrition Laboratory procedure for crude fiber is attached to this chapter, and Table 3-9 gives an example calculation of a crude fiber analysis.

Table 3-7. Some common carbohydrates of foods and their distribution between the Weende nitrogen free extract and crude fiber fractions (Reproduced from Lloyd et al., 1978, p. 15)

Classification	Carbohydrate	Unit	Reaction to Weende fiber procedure	Weende classification
Monosaccharides	Arabinose Xylose Ribose, etc. Fructose Galactose Glucose Mannose, etc.	$C_5H_{10}O_5$ $C_6H_{12}O_6$	Completely soluble	NFE
Oligosaccharides (2 to 10 units)	Lactose Sucrose Raffinose, etc	$(C_5H_{10}O_5)$ n		
Polysaccharides	Glycogen Starch	$(C_6H_{10}O_5)$ n		
	Hemicellulose Pectin	$(C_5H_8O_4)$n $(C_6H_{10}O_5)$ n	Partially but variably soluble	Crude fiber
	Cellulose	$(C_6H_{10}O_5)$ n		

[a]Van Soest and McQueen [Proc. Nutr. Soc. (1973): 32:123] indicate that acid and alkali extraction removes about 80% of the hemicellulose and 20 to 50% of the cellulose, and only 10 to 50% of the lignin remains in the crude fiber fraction.

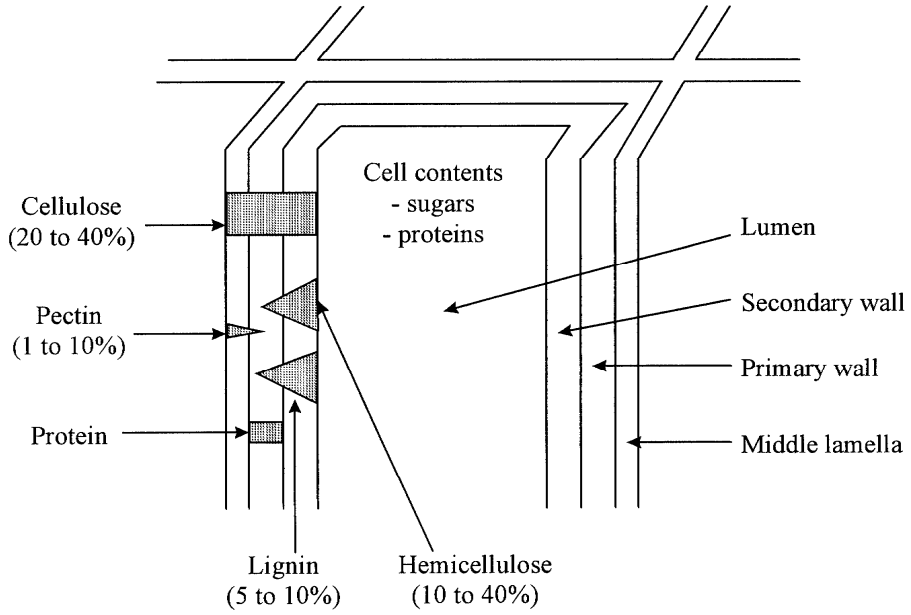

Figure 3-1. Schematic representation of the structure of a forage plant cell showing the component layers. The relative amounts of each of the carbohydrate fractions in the respective layers are depicted by shaded areas, i.e., hemicellulose largely in the secondary wall; pectin largely in the middle lamella. The figures in parentheses are amounts often found in forage dry matter. (Adapted from Maynard et al., 1979).

Table 3-9. Example calculation of crude fiber content

Rep	Sample wt	Sample dmf	Dry crucible wt	Dry crucible + residue wt	Crucible + ash wt	Ignition loss
1	2.0000	0.90	41.0000	41.8500	41.0500	0.8000
2	2.1000	0.90	41.0000	41.8600	41.0500	0.8100

[a]Sample wt from ether extract analysis. A fat free sample should be used for crude fiber analysis.

% Crude fiber

Rep 1 = 0.8000 ÷ 2.0000 = 0.4000 x 100 = 40.00%
Rep 2 = 0.8100 ÷ 2.1000 = 0.3857 x 100 = 38.57%

% Crude fiber, dmb

Rep 1 = 40.00 ÷ 0.9 = 44.44%
Rep 2 = 38.57 ÷ 0.9 = 42.86%

$\bar{x}$ = 43.65%
s = 1.11
CV = 2.56%

Animal Science Nutrition Lab

DETERMINATION OF CRUDE FIBER

REAGENTS

Sulfuric acid solution, 0.255N, 1.25 g of H_2SO_4/100 mL
Sodium hydroxide solution, 0.313N, 1.25 g of NaOH/100 mL, free of Na_2CO_3 (concentrations of these solutions must be checked by titration)
Alcohol - Methanol, isopropyl alcohol, 95% ethanol, reagent ethanol
Bumping chips or granules - antifoam agent (decalin)

APPARATUS

Digestion apparatus
Ashing dishes
Desiccator
Filtering device
Suction filter: To accommodate filtering devices. Attach suction flask to trap in line with aspirator or other source of vacuum with valve to break vacuum.

PROCEDURE

Extract 2 g ground material with ether or petroleum ether. If fat is less than 1%, extraction may be omitted. Transfer to 600 mL beaker, avoiding fiber contamination from paper or brush. Add approximatley l g of prepared asbestos, 200 mL of boiling 1.25% H_2SO_4, and 1 drop of diluted antifoam. *Excess antifoam may give high results; use only if necessary to control foaming*. Bumping chips or granules also may be added. Place beaker on digestion apparatus with preadjusted hot plate and boil exactly 30 min, rotating beaker periodically to keep solids from adhering to sides. Remove beaker and filter as follows:

Using California Buchner -- Filter contents of beaker through Buchner (precoated with asbestos if extremely fine materials are being analyzed), rinse beaker with 50 to 75 mL of boiling water, and wash through Buchner. Repeat with three, 50 mL portions of water, and suck dry. Remove mat and residue by snapping bottom of Buchner against top, while covering stem with thumb or forefinger, and replace in beaker. Add 200 mL of boiling 1.25% NaOH, and boil exactly 30 min. Remove beaker and filter as above. Wash with 25 mL of boiling 1.25% H_2SO_4, three 50 mL portions of H_2O, and 25 mL of alcohol. Remove mat and residue, and transfer to ashing dish.

Dry mat and residue 2 h at 130 $\pm$ 2°C. Cool in desiccator and weigh. Ignite 30 min at 600°C. Cool in desiccator and reweigh.

% CF = (Loss in wt on ignition/wt of sample*) x 100

*Use dry weight for dry matter basis value, or correct as-fed value to dry matter basis.

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CHAPTER IV

Energy in Animal Nutrition

Oxygen Bomb Calorimetry. When a substance is completely burned to its ultimate oxidation products of CO_2 and H_20 or other gases, the heat given off is termed gross energy or heat of combustion. Gross energy is generally measured in an oxygen bomb calorimeter and is the starting point for determining the energy value of feedstuffs (Maynard et al., 1979).

The bomb calorimeter consists of the bomb, an airtight canister in which the substance is burned, and a jacket of H_20 around the bomb that provides a means of measuring the heat produced during burning. In an adiabatic calorimeter, an additional water jacket surrounds the inner jacket. This second water jacket is maintained at the same temperature as the inner jacket to prevent heat loss by conduction. Thus, when a substance is burned in the bomb, the temperature rise in the inner jacket surrounding the bomb reflects the heat produced. A diagram of the Parr oxygen bomb calorimeter reproduced from Maynard et al. (1979) is shown in Figure 4-1.

The method involves placing a small sample (usually not more than l g) in a cup inside the bomb. A fuse wire is attached to terminals in the bomb and placed over the sample. The bomb is then sealed and charged with 20 to 25 atmospheres of oxygen. Then the bomb is placed in the inner water jacket, which contains a known weight of water. When the inner water jacket and outer water jacket have reached the same temperature, a charge is sent to the fuse wire, and the sample burns rapidly in the oxygen-rich environment. Heat from the burning is transferred (in part) to the inner water jacket and the increase in temperature is recorded.

Once the change in water temperature (ΔT) is known, the gross energy of the substance can be calculated by multiplying the thermal equivalent of the calorimeter by ΔT. Every calorimeter has its own thermal equivalent, which is determined by running a standard of known energy content and determining the calories per °C rise in temperature. Once a calorimeter's thermal equivalent has been determined, it should remain the same, provided none of the parts are changed. However, in practice, the thermal equivalent is determined periodically as a check on the instrument and the operator's technique. Normally, benzoic acid (supplied in preformed pellets by Parr Instruments) is used in determining the thermal equivalent.

Some corrections to the gross energy value are necessary because the fuse wire produces some heat when it is burned and nitrogen and sulfur are oxidized to NO^-_3 and SO^-_4 under high 0_2 tension. Because such oxidation would not occur under physiological conditions, the heat generated in the process is subtracted from the determined gross energy value. An example calculation of gross energy content is shown in Table 4-1 and a copy of Animal Nutrition Laboratory bomb calorimetry procedure is attached to this chapter.

If we were to run a number of purified nutrients through gross energy determinations, we would find that on the average, fats contain about 8.9 to 9.6 kcal/g of GE, whereas carbohydrates contain 3.7 to 4.4, and proteins contain about 5.4 to 5.9 kcal/g. For a more extensive tabulation of heats of combustion, students are referred to Lloyd et al. (1978) and Maynard et al. (1979),

but suffice it to say that there are large differences in the gross energy value of classes of nutrients. Why are there such large differences? The answer lies in the elemental composition of the nutrients. Maynard et al. (1979) provide the following answer to the question:

> "These differences are governed by their (nutrients) elemental composition, especially by the relative amount of oxygen contained within the molecule, since heat is produced only by the oxidation resulting from the union of carbon or hydrogen with oxygen from without. The oxygen within has previously liberated heat (or energy) during chemical formation of the compound. In the case of carbohydrates, there is enough oxygen in the molecule to take care of all the hydrogen present, and thus heat arises only from the oxidation of the carbon. In the case of fat, however, there is relatively much less oxygen present and relatively more atoms requiring oxygen from without, and the combustion involves the oxidation of hydrogen as well as carbon. The burning of l g of hydrogen produces over four times as much heat (34.5 kcal/g) as is the case of carbon (8 kcal/g). These facts explain the much greater gross energy values per gram for fats compared with the carbohydrates. The heat produced in the burning of protein comes from the oxidation of both carbon and hydrogen, but the nitrogen present gives rise to no heat at all because it is set free as such in its gaseous form. No oxidation of it has taken place, and thus no heat is produced, contrary to what occurs in the bomb, for which partial corrections are made" - from Maynard et al. (1979; pp 63,64).

Simply stated, nutrients vary in energy content because they vary in degree of internal oxidation, fats being less internally oxidized than carbohydrates. Students can readily see why the inclusion of a small amount of fat in an animal's diet markedly increases the energy content.

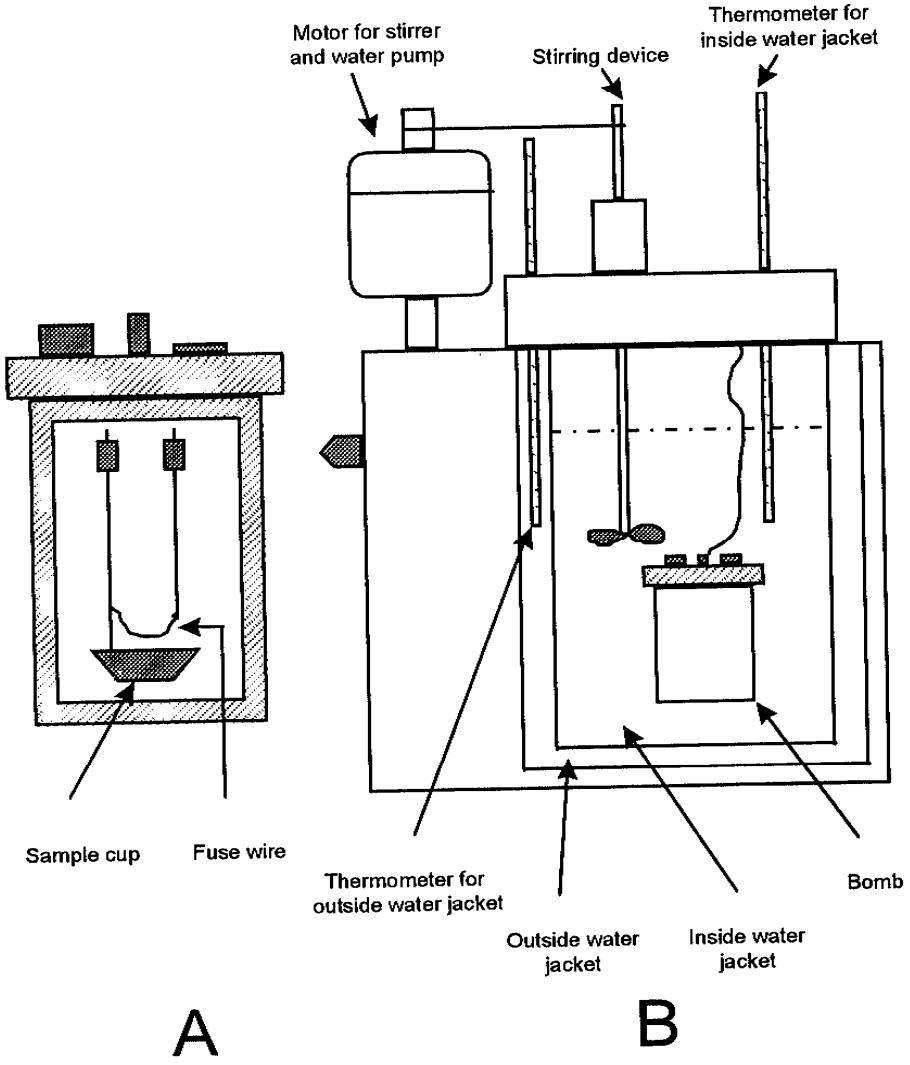

Figure 4-1. The Parr bomb calorimeter: A - Cross section of bomb; B - Cross section of the adiabatic calorimeter with bomb in place. Adapted from Maynard et al. (1979)

Table 4-1. Example oxygen bomb calorimetry data

SAMPLE IDENTIFICATION: Example

Technician: M. Galyean Date: 9/2/96

Energy equivalent of calorimeter: 2,415 cal/°C

Weight of cup and sample 2.0000 Sample dry matter factor:
Sample wt. as rec'd_____
Weight of cup 1.0000 Sample wt. dry _____
Loss on drying _____
Weight of sample 1.0000 Dry matter factor 0.90

Sample weight, dry matter basis: 0.9000

Length of fuse wire 15 cm

Length after ignition 5 cm

Fuse wire ignited 10 cm Correction for wire fused: 23 Cal.
(correction is 2.3 cal/cm of fuse wire burned)

Bucket temperature, initial: 20.5°C Thermometer correction _____
Corrected temperature _____
Bucket temperature, final: 22.5°C Thermometer correction_____
Corrected temperature _____

Temperature change as a result of sample combustion:
2.0^0C

Acid titration: 5 mL: Correction for acids: 5 cal.
(correction is l cal/mL of alkali)

CALCULATION:

Gross heat of combustion = $\frac{\text{(temp. change x energy equiv.)} - (C_{wire} + C_{acid})}{\text{Weight of sample}}$

Dry matter basis:

GE, cal/g = $\frac{(2.0 \times 2415) - (23+5)}{0.9} = 5{,}335.55$

GE, kcal/g = 5.34

Animal Science Nutrition Lab

DETERMINATION OF GROSS ENERGY
(OXYGEN BOMB CALORIMETRY)

Equipment

a. Parr oxygen bomb calorimeter
b. Balance with capacity to 3,000 g

Reagents

a. 0.0725 N Na_2CO_3 Standard sodium carbonate solution,equivalent to 1 cal/mL

3.8425 g Na_2CO_3/liter

b. methyl orange indicator

Procedure

1. Weigh out a 1-g sample and place into a clean combustion crucible. Turn on heater box on instrument.
2. Attach a 10-cm fuse wire between the electrodes of the bomb.
3. Place 1 mL of water into the bomb cylinder and swirl the water to wet the sides.
4. Assemble the bomb. Fill with oxygen to 20 atmospheres gauge pressure. Place the oval bucket in the calorimeter, set the bomb in the bucket, and attach the clip terminal.
5. Weigh out 2,000 g of deionized-water, and pour the water into the calorimeter bucket. Make sure the water temperature is within the range of the calorimeter thermometer.
6. Slide the cover over and lower the thermometers, and turn on the water circulating motor.
7. DO NOT ADJUST CALIBRATION DIALS! The instrument will automatically equilibrate so no adjustment of the water temperature is necessary.
8. Check the calorimeter temperature at 1-min intervals for 3 min.
9. After the entire calorimeter has equilibrated, read and record the initial temperature. Ignite the sample.
10. After ignition has occurred and the temperature of the outer jacket and the inner bucket are equal, read and record the final temperatures at 1-min intervals for 3 min.
11. Raise the thermometers, open up the calorimeter, remove the bomb from the calorimeter bucket, release the remaining pressure on the bomb, and open the bomb. Carefully remove the remaining pieces of fuse wire from the electrodes, and straighten out the wire.
12. Rinse all the inner bomb surfaces with deionized-water, and collect all washings in a clean 100-mL or 150-mL beaker. Titrate the washings using the 0.0725 N Na_2CO_3 and methyl

orange indicator (2 to 3 drops indicator is added to the washings). Titrating with the standard sodium carbonate solution determines the amount of acid formed from the incidental oxidation of nitrogen and sulfur compounds. A correction is made to take care of the heat liberated in the formation of the acid.

Calculations

1. GE (cal/g) on an as fed basis = {(final temp - initial temp) x hydrothermal equiavlent of the bomb - ([length of fuse wire bunred x cal/cm of fuse wire] + mL of Na_2CO_3)}/sample wt
2. Adjusting to a dry matter basis = GE (cal/g) on as fed basis/dry matter factor of sample

NOTE: Determination of the hydrothermal equivalent of the bomb and the procedure for the determination of gross energy in urine may be obtained from the laboratory supervisor.

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Energy Systems in Nutrition. Energy is not a nutrient but a property of nutrients. Energy is a principal requirement for living organisms and as such, most nutrients function to a large extent as sources of energy. As indicated previously, gross energy represents the total energy available in a substance; however, living organisms are not capable of capturing all of the energy in the foods they consume because of digestive and metabolic inefficiencies. Thus, variable amounts of the gross energy in feeds are actually used in productive body functions. Factors affecting energy use by livestock and humans have been extensively studied and a flow chart of energy distribution in body processes taken from Maynard et al. (1979) is shown in Figure 4-2. We will discuss each major loss of energy in the flow chart and also consider other attempts to determine useful food energy for humans and livestock.

The first major energy loss occurs in digestion. If one measures the gross energy of the feed an animal consumes and the gross energy of the feces it excretes, the difference between intake and outgo is the **apparent digestible energy (DE)**. The term apparent is used because some of the fecal energy is of body rather than food origin, arising from digestive fluids, abraded cells, and bacterial cells. Fecal losses represent the largest loss of gross energy, amounting to about 20% of gross energy in pigs fed typical diets, 35 to 40% of gross energy in horses on common diets, 40 to 50% in cattle and sheep fed roughages, and 20 to 30% of gross energy in cattle and sheep fed concentrates (Maynard et al., 1979). Digestible energy is commonly used with swine and horses to describe their energy requirements.

Metabolizable energy (ME) is a step beyond digestible energy, in that it accounts for energy lost in urine and gas. The principal components of urinary energy are incompletely oxidized nitrogenous compounds (primarily urea) and endogenous nitrogen constituents (primarily creatinine). Urine energy amounts to 2 to 3% of gross energy in pigs and 4 to 5% of gross energy in cattle (Maynard et al., 1979). Gaseous losses are quite small in a number of species (man, pig, dog, chicken), but are considerable (7 to 10% of gross energy) in ruminants. Gaseous products arise from fermentation of food in the digestive tract, and, as such, are really a digestive loss; however, it has been convention to group this loss with metabolic losses.

Deduction of **heat increment** from metabolizable energy yields **net energy (NE)**, which represents the portion of the gross energy that is actually useful for body functions. Heat increment is somewhat difficult to measure, usually being determined by direct or indirect calorimetric techniques, but the heat increment loss can amount to 15 to 40% of gross energy. Two components, heat of fermentation and heat of nutrient metabolism, make up what we call heat increment. Most students have some difficulty with the heat increment concept because it is a loss of heat from the body and not a visible substance like feces or urine. Heat of fermentation is really a digestive loss that results from chemical inefficiency of fermentive processes. Technically, it should be subtracted from gross energy in arriving at digestible energy, but because heat of fermentation cannot be separated from heat of nutrient metabolism, it is deducted in arriving at net energy. Heat of nutrient metabolism or the specific dynamic effect (SDE), as it is sometimes called, results from loss of chemical energy in nutrients as a result of variable inefficiency of nutrient metabolism. Heat of nutrient metabolism varies with the type of diet, level of feeding, and body function being supported by the diet; this is why there are differences in the efficiency of utilization of metabolizable energy for various body functions. Thus, NE values of feeds only have meaning in the context of the purpose for which the diet is fed. For

this reason, considerable research has been done to establish partial net energy values, that is NE for maintenance or production. A description of the various net energy systems presently in use for cattle is beyond the scope of this discussion, but students are referred to Blaxter (1965); Lofgreen and Garrett (1968); Moe et al. (1972); Knox and Handley (1973); and Moe and Tyrrell (1973).

Some other approaches to determining the usable energy content of feeds deserve discussion. The **total digestible nutrients (TDN)** system was devised as a means of using digestibility data to account for fecal energy losses in livestock. As such, it should provide values comparable to digestible energy, but as we shall see, it does not do this in all cases. One can define TDN as a measure of the digestible energy content of a feed on a carbohydrate-equivalent basis. The term "carbohydrate-equivalent basis" simply means that all nutrients are scaled to the energy equivalent of carbohydrate. The method of calculation of TDN is shown in Table 4-2.

Table 4-2. Example calculation of total digestible nutrients (TDN)

Proximate component	%	Digestion coefficient	Factor[a]	Value
Crude protein	10	65	-	6.5
Crude fiber	30	50	-	15
NFE	40	90	-	36
Ether extract	5	90	2.25	9
TDN				**66.5**

[a]Factor used to put fat on a carbohydrate-equivalent basis.

The TDN system is appealing, in that as shown in Table 4-2, one can determine the proximate composition of a feed and multiply published or determined average digestion coefficients by each component to arrive at the percentage of total digestible nutrients in the feed. There is some danger in this, however, in that average digestion coefficients may not always apply to the particular feed in question. The 2.25 factor for fat is derived from Atwater's work, which showed that fats have about 2.25 times the digestible energy value of carbohydrates. In addition, based on Atwater's work, protein had the same energy value (i.e., 4 kcal/g) as carbohydrate and, thus, no correction factor was needed.

Unfortunately, the assumption regarding protein was incorrect. The 4 kcal/g figure developed by Atwater already adjusted for incomplete tissue oxidation and urinary excretion of nitrogen. Thus, TDN as normally calculated adjusts for urinary losses of nitrogen to some extent. In fact, in species with minimal gas losses, TDN is very similar to metabolizable energy. Actually, TDN should be calculated by multiplying protein by 1.3 to put it on a carbohydrate-equivalent basis (5.2/4 = 1.3).

The most significant problem with TDN, however, is that it does not account for additional energy losses, particularly heat increment, and to some extent, gaseous losses. For this reason, TDN does not accurately estimate the energy value of feeds, particularly for ruminants, for which there is wide variation in the heat increment between roughage and concentrates. This same criticism applies to digestible and metabolizable energy, which is why so much effort has gone into developing net energy systems for ruminants.

Physiological fuel values (PFV) are caloric values for nutrients developed by W. D. Atwater for use in human nutrition. Atwater evaluated a number of typical human diets and found that on the average, carbohydrates, fats, and proteins contained 4.15, 9.40, and 5.65 kcal/g, respectively. He also studied the digestibility of the nutrients and the energy lost in urine with metabolism of protein; from these, he calculated average caloric values for the three classes of nutrients. Because urinary excretion is considered, PFV values are quite similar to metabolizable energy. An example calculation of PFV is shown in Table 4-3, taken from Lloyd et al., (1978). Atwater's values have been refined and modified over the years and still form the basis for estimating the energy value of human diets. Students are referred to Watt and Merrill (1963) for revised energy values based on Atwater's work.

Before we leave energy, students should be aware that gross energy can be calculated from the proximate composition of a feedstuff. This is accomplished by multiplying the percentage of each proximate component times its appropriate energy value followed by summation of these products. Garrett and Johnson (1983) report the following formula for computation of GE from proximate components: GE = 5.72 x Crude protein + 9.5 x Ether extract + 4.79 x Crude fiber + 4.03 x NFE. This formula is based on the work of Nehring and collegues in Germany and generally provides reasonable estimates of GE content (kcal/g).

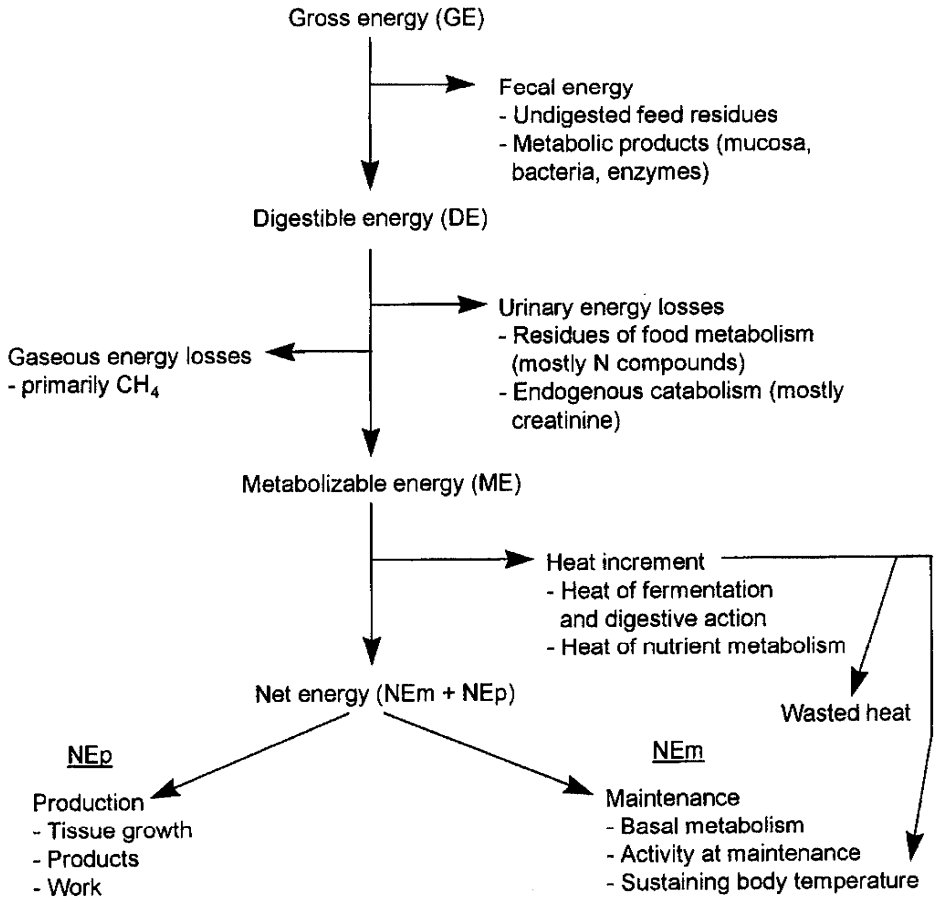

Figure 4-2 - Energy distribution in body processes. Adapted from Maynard et al. (1979).

Table 4-3. Calculation of Atwater's physiological fuel values. From Lloyd et al. (1978)

Source	GE, kcal/g	Apparent digestibility	Urinary energy loss, kcal/g	PFV, kcal/g	Rounded PFV, kcal/g
Carbohydrate	4.15	97	0	4.03	4
Fat	9.40	95	0	8.93	9
Protein	5.65	92	1.25	4.05	4

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CHAPTER V

Detergent Fiber Analyses

As discussed in Chapter III, the crude fiber/NFE system does not provide an accurate picture of the carbohydrate fraction of feedstuffs, primarily because of solubilization of variable amounts of hemicellulose and lignin in the crude fiber analysis. Many researchers recognized this problem; however, it was not until recently that an alternative analytical scheme was developed.

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

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

A general scheme of the detergent analysis is shown in Figure 5-1 and a comparison with the Weende analysis is shown in Figure 5-2. These figures are reproduced from Maynard et al. (1979) and Maynard and Loosli (1969). In addition, a brief description of some of the nutritionally important constituents of forages is given in Table 5-1.

An excellent review of the chemistry of forages has been compiled by Van Soest (1982). Procedures for ADF, ADL, and NDF are attached. Example calculations for these analyses are shown in Tables 5-2 and 5-3, respectively, and a brief synopsis of what each analysis measures and its nutritional value is in Table 5-4.

Before we leave this subject, however, students should consider once again, the reasons for the development of this new system. As indicated in our discussion of the Weende system, two important reasons for development of the system were so that comparisons of nutrient content and prediction of animal performance could be made. The same reasons apply to the detergent scheme; fortunately, the detergent scheme does a better job than the Weende system in both respects.

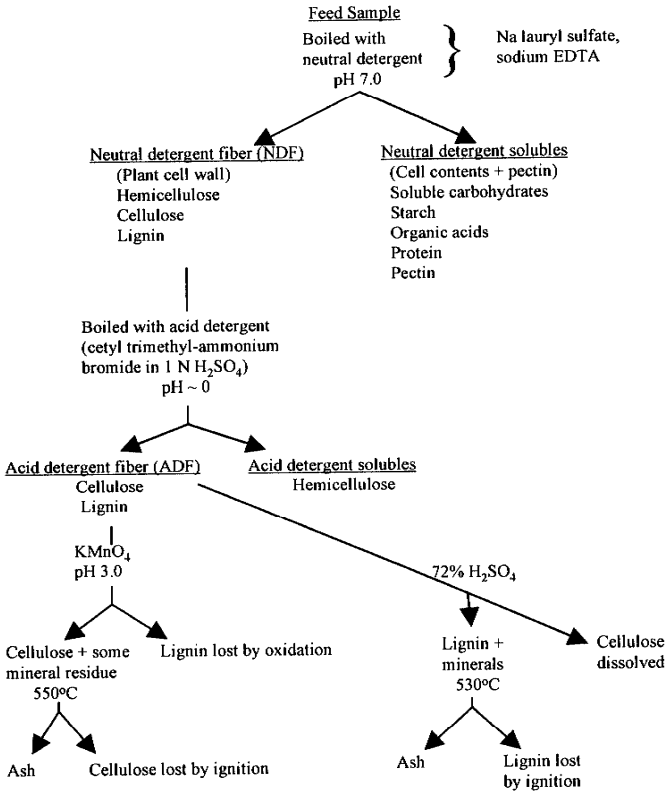

Figure 5-1. The Van Soest method of partitioning fiber in feeds. (Source: H. K. Goering and P. J. Van Soest, Forage Fiber Analyses. Agric. Handbook No. 379., ARS, USDA, 1970.). Adapted from Maynard et al. (1979).

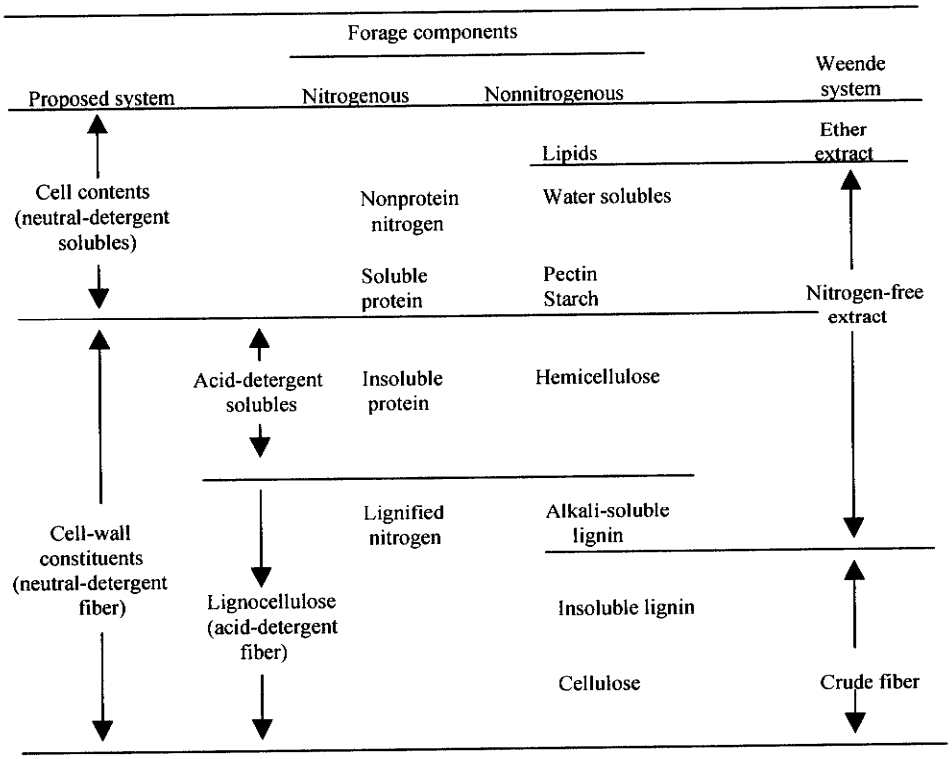

Figure 5-2. Relationship of two systems of dividing forage organic matter. Adapted from Maynard and Loosli (1969).

Table 5-1. Characteristics of some important forage cell wall constituents

Constituent	Characteristic
Lignin	Major non-carbohydrate portion of cell wall. Three dimensional polymer of phenylpropanes. Lowers availability of hemicellulose and cellulose it is associated with. Provides structural support for plant.
Cellulose	Major skeletal carbohydrate in plants. Polymer of glucose in ± (1→4) linkages. Cellulase enzyme not secreted by mammals. Digestibility varies with amount of lignin, silica, cutin. Provides structural support for plant.
Hemicellulose	Polymer of xylose and other five carbon sugars (arabinose side chains). Digestibility depends on lignin, etc. Provides structural support for plant.
Pectin	Polymer of methyl D-galacturonic acid. Highly digestible, and availability not greatly influenced by lignin, etc.
Cutin	Composed of waxes and waxy polymers. May be integrated with lignin and is measured as lignin in ADL. Lowers availability of cellulose and hemicellulose.
Silica	Taken up by grasses more so than by legumes. Content in plants may vary from l to approximately 22% of dry matter. Plants in sandy soils have higher levels. Has similar effect to lignin on digestibility of cellulose and hemicellulose. May be direct effect on cellulose or hemicellulose or may tie up some trace minerals needed by ruminal microorganisms. Composed of SiO_2 polymers.

Table 5-2. Example calculation of ADF and ADL

Rep	Pan	Pan + sample	Sample wt	Sample dmf	Crucible wt	Crucible +ADF	Dry crucible after 72% H_2SO_4	Dry crucible after ash
1	1.0000	2.0000	1.0000	0.90	40.0000	40.5000	40.1000	40.0200
2	1.0000	2.1000	1.1000	0.90	40.0000	40.5100	40.1050	40.0205

ADF %, dmb

Rep 1 40.5000 - 40.0000 = (0.5000 ÷ 1.0000) x 100 = 50.00% ÷ 0.9 = 55.56%
Rep 2 40.5100 - 40.0000 = (0.5100 ÷ 1.1000) x 100 = 46.36% ÷ 0.9 = 51.51%

$\bar{x}$ = 53.54%
s = 2.86%
CV = 5.35%

ADL %, dmb

Rep 1 40.1000 - 40.0200 = (0.0800 ÷ 1.0000) x 100 = 8.00% ÷ 0.9 = 8.89%
Rep 2 40.1050 - 40.0205 = (0.0845 ÷ 1.1000) x 100 = 7.68% ÷ 0.9 = 8.53%

$\bar{x}$ = 8.71%
s = 0.255
CV = 2.92%

Cellulose, dmb

Rep 1 40.5000 - 40.1000 = (0.4000 ÷ 1.0000) x 100 = 40.00% ÷ 0.9 = 44.44%
Rep 2 40.5100 - 40.1050 = (0.4050 ÷ 1.1000) x 100 = 36.81% ÷ 0.9 = 40.90%

$\bar{x}$ = 42.67%
s = 2.50
CV = 5.87%

AIA %, dmb

Rep 1 40.0200 - 40.0000 = (0.0200 ÷ 1.0000) x 100 = 2.00% ÷ 0.9 = 2.22%
Rep 2 40.0205 - 40.0000 = (0.0205 ÷ 1.1000) x 100 = 1.86% ÷ 0.9 = 2.07%

$\bar{x}$ = 2.15%
s = 0.11
CV = 4.93%

Table 5-3. Example calculation of NDF

Rep	Pan	Pan + sample	Sample	Sample dmf	Dry crucible	Dry crucible + NDF
1	1.0000	2.0000	1.0000	0.90	40.0000	40.7000
2	1.0000	2.0000	1.0000	0.90	40.0000	40.7100

NDF %, dmb

Rep 1 = 40.7000 - 40.0000 = (0.7000 ÷ 1.000) x 100 = 70.00% ÷ 0.9 = 77.78%
Rep 2 = 40.7100 - 40.0000 = (0.7100 ÷ 1.000) x 100 = 71.00% ÷ 0.9 = 78.89%

$\bar{x}$ = 78.34%
s = 0.785
CV = 1.00%

Table 5-4. Components of the detergent forage fiber analysis system and what they measure.

Component	What it measures
NDF[a]	Primarily cellulose, hemicellulose and lignin. Availability depends on lignification.
ADF[a]	Primarily cellulose and lignin. Availability depends on lignification.
ADL	Primarily lignin, but some cutin and bound nitrogen (Maillard product). Unavailable.
AIA	Fairly good estimate of silica content (acid insoluble ash).

[a]Note that NDF - ADF will provide an estimate of hemicellulose content.

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

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Animal Science Nutrition Lab

ACID DETERGENT FIBER (ADF) PROCEDURE

MATERIALS: 600 mL Berzelius beakers
Fiber digestion apparatus
Sintered glass crucibles - 40 to 50 mL - coarse porosity
Filtering flasks

REAGENTS: Acid Detergent Solution:

Add 27.84 mL of H_2SO_4* to a volumetric flask and bring to 1 L volume with H_2O. Then and 20 g of $CH_3(CH_2)_{15}N(CH_3)_3Br$ to the 1 L of acid solution.

*Add some H_2O first (before acid).

Acetone

Digestion of Sample

A. Transfer 1 g air-dried sample to Berzelius beaker.

B. Add 100 mL acid detergent solution.

C. Heat to boil (5 to 10 min), and boil exactly 60 min.

D. Filter with light suction into previously tared crucibles. Refer to procedure for matting crucibles. #541 filter paper may be used instead of crucible if ADL is not being done. Must preweigh filter paper first.

E. Wash with hot water 2 to 3 times.

F. Wash thoroughly with acetone until no further color is removed. Suction dry.

G. Dry in drying oven at 100°C for 24 h.

H. Cool in dessicator. Weigh and record weight.

ADL PROCEDURE

REAGENTS: 72% H_2SO_4 standardized to specific gravity of 1.634 at 20°C.

A. Place ADF crucible in a 50 mL beaker on a tray.

B. Cover contents of crucible with 72% H_2SO_4. (Fill approximately half way with acid.)

C. Stir contents with a glass rod to a smooth paste.

D. Leave rod in crucible, refill hourly for 3 h, stirring the contents of the crucible every hour.

E. After 3 h, filter contents of crucible using low vacuum at first, increasing it only as more force is needed.

F. Wash contents with hot water until free of acid (minimum of five times).

G. Rinse rod and remove.

H. Dry crucible in oven at 100°C for 24 h.

I. Cool in dessicator. Weigh and record weight.

J. Ash in muffle at 500°C for 4 h.

K. Cool in dessicator. Weigh and record.

ADIN PROCEDURE

A. Run ADF on sample just as described in procedure. Filter through #541 filter paper.

B. Place contents of filter paper and residue from ADF procedure in Kjeldahl flask. A blank for this procedure would consist of a #541 filter paper taken through ADF procedure.

C. Follow procedure for determining Kjeldahl nitrogen, and do calculations on original sample weight.

NEUTRAL DETERGENT FIBER (NDF) PROCEDURE

REAGENTS AND MATERIALS

NDF solution: To 1 L of H_2O add:
30 g of sodium lauryl sulfate
18.61 g of disodium dihydrogen ethylene diamine tetraacetic dihydrate
6.81 g of sodium borate decahydrate
4.56 g of disodium hydrogen phosphate
10 mL of triethylene glycol

Amylase solution - Heat-stable α-amylase (Sigma No. A3306 – from the Dietary Fiber Kit).
Acetone
Refluxing apparatus
Whatman #541 filter paper
Aluminum pans

Procedure

1. Place 0.5 to 1.0 g sample in 600-mL Berzelius beaker.
2. Add 100 mL of neutral detergent fiber solution.
3. Heat to boiling (5 to 10 min). Decrease heat as boiling begins. Boil for 60 min.
4. After 60 min, filter contents onto preweighed Whatman #541 filter paper under vacuum. Use low vacuum at first, increasing only as more force is needed.
5. Rinse contents with hot water, filter, and repeat twice.
6. Wash twice with acetone.
7. Fold and place in preweighed aluminum pan.
8. Dry overnight in 100°C oven.
9. Cool in dessicator. Weigh - yield is CWC.
10. For samples with a high starch content: Add 50 μL of heat-stable amylase to the beaker along with NDF solution as in Step 2, and follow remaining steps. For the most difficult samples, a 1-g sample can first be treated with 30 mL of 8 M urea solution plus 50 μL of heat-stable amylase solution. The mixture can be heated on a steam bath at 80 to 90^0C for 5 min, then incubated at room temperature for 4 h or overnight. After incubation, add 100 mL of NDF solution and treat as in Step 3 and following. An additional 50 μL of heat-stable amylase can be added at this point if desired.

Reference: Goering and Van Soest (1970), as modified by Van Soest et al. (1991; J. Dairy Sci. 74:3583).

PROCEDURE FOR MATTING CRUCIBLES

1. Sonicate crucibles for 2 min.
2. Reverse flush with 50 mL of dilute acid.
3. Rinse with hot water.
4. Add a teaspoon of Hiflo supercell.
5. Add 30 mL of hot water, swirl and place on sidearm flask.
6. Pour 100 mL of dilute acid through crucible.
7. Pour 100 mL of hot water through crucible.
8. Place in muffle (500°C) for 4 h.
9. Cool in dessicator, record tare weight.
10. After analyses, discard mat.
11. Wash, then sonicate for 2 min.
12. Backflush with 50 mL of dilute acid and 50 mL of hot water.

CHAPTER VI

Nitrogenous Constituents of Feeds

For many years, the Kjeldahl analysis has been the standard method of evaluating the nitrogen content of feedstuffs. However, in recent years, more attention has been given to the availability of feed nitrogen. It has long been recognized that the total nitrogen content of feeds could be divided into non-protein nitrogen and protein nitrogen fractions. Moreover, a portion of the true protein may be bound or unavailable as a result of excessive heating of the feed or other treatments and, in the case of ruminants, nitrogen is either insoluble or soluble in ruminal contents. With all these variations, the need for a clearer description of feed nitrogen is evident.

Such a description of the nitrogen or protein content of feedstuffs is given in Figure 6-1, which has special applicability to ruminants, but is useful for nonruminants. We will attempt to discuss each fraction, and consider some laboratory methods for evaluating these fractions.

Soluble protein is the first of the three major classifications in Figure 6-1. It consists of true protein and various types of non-protein nitrogen (NPN), including amino acids, nucleic acids, nitrates, amines, urea, and so on. Soluble protein is considered to be rapidly degraded to ammonia by ruminal microorganisms. In fermented feeds, a large portion of the soluble protein may be in the form of non-protein nitrogen and, hence, it is desirable to divide the soluble protein fraction into soluble NPN and soluble true protein. A procedure for separating soluble protein into these two fractions has been published by Prigge et al. (1976). Essentially, this involves placing the sample in a buffer solution and agitating for 1 h at 39^0C. The sample is then filtered and the filtrate divided into two portions. One portion is analyzed by Kjeldahl and the total N x 6.25 is an estimate of soluble crude protein. The second portion is treated with tungstic acid to precipitate proteins, filtered and the filtrate analyzed for N by Kjeldahl, yielding an estimate of soluble NPN. Total soluble nitrogen minus soluble NPN is an estimate of soluble true protein nitrogen. Waldo and Goering (1979) evaluated several potential methods of fractionating feedstuff N. The Animal Nutrition Laboratory procedure for soluble and insoluble N, based on the .15 M NaCl procedure of Waldo and Goering (1979), is attached to this chapter.

Insoluble available protein has been the subject of considerable research in ruminant nutrition. This fraction consists largely of true protein with small amounts of NPN and is essentially insoluble in ruminal fluid and degraded to a variable extent by ruminal microorganisms, depending on the nature of the protein. Because it is only partially degraded in the rumen, insoluble protein is often considered as the portion of total protein with escape potential. Escape of feed protein could be important in ruminants, particularly when the escape protein is of greater quality (biological value) than microbial protein. A number of methods of treating protein to increase ruminal escape have been studied. Formaldehyde treatment, heating, and coating the protein with oil have all met with some success. Students are referred to Ferguson (1975) for more reading on treatment of proteins to increase ruminal escape.

Methods for estimating the insoluble available protein fraction of feeds are usually related to those for measuring the soluble fraction. The procedure discussed previously (Prigge et al., 1976) can be applied to determination of insoluble protein, simply by subtracting the soluble

nitrogen content from the feed's total nitrogen content to arrive at an estimate of insoluble nitrogen. Sniffin et al. (1980) provide additional references and discussion on this topic. These authors recommend determination of soluble protein and bound protein, with insoluble available protein determined by difference from total protein.

It should be noted that knowledge of the solubility and insolubility of protein in buffers or ruminal fluid is of limited value in describing feeds for nonruminants. A more common approach for nonruminants would be to evaluate digestibility in pepsin. Pepsin indigestible protein provides a fairly good index of the portion of the total protein that would be unavailable to nonruminants. It also seems, however, that pepsin indigestible protein is not digested in ruminants (F. N. Owens, personal communication, DuPont Specialty Grains, Des Moines, IA). A procedure for determination of pepsin indigestible protein is in the AOAC (1980) manual.

Finally, let us consider the bound protein fraction. This fraction is essentially the undigestible portion of the feed nitrogen. Components of the bound fraction include naturally occurring nitrogen in lignin, protein-tannin complexes, and proteins or amino acids bound to carbohydrates by the Maillard reaction. Perhaps the most important of these three from the standpoint of feed preparation is the Maillard, or browning reaction. In this reaction, free amino groups of the protein (usually the ε-amino of lysine) combine with the aldehyde group of a reducing sugar to form an indigestible complex. The reaction is maximized at moisture levels of 30% and temperatures greater than 60^0C. Moreover, increased time of exposure to these conditions will enhance the reaction (Maynard et al., 1979). The complex formed by this reaction is measured as lignin in the ADL analysis and is sometimes referred to as artifact lignin. Van Soest (1975) provided an excellent discussion of the Maillard reaction.

The Maillard reaction is of considerable interest to the protein processing industry, particularly with regard to the effects of heat on protein quality. Proteins that have undergone browning have considerably decreased value, both for ruminants and nonruminants. Several methods have been developed to measure bound protein. One method involves mixing fluorodinitrobenzene (FDNB) with the protein. The FDNB complexes with free amino groups and upon hydrolysis of the protein, the FDNB-amino acid complex produces a yellow color. Thus, the more intense the yellow color, the less the extent of amino acid-sugar condensation (Maynard et al., 1979; see also Carpenter and Booth, 1973).

Pepsin indigestible protein also would provide an index of bound protein; however, the most common procedure with forages is the measurement of acid detergent insoluble nitrogen (ADIN). The ADIN procedure was included with Chapter V. In forages, the principal carbohydrate involved in the Maillard reaction is hemicellulose, and the reaction may be a considerable problem in ensiled or artificially dried hays where moisture and temperature conditions are conducive to development of the reaction.

Values for soluble and insoluble protein as well as ADIN for a number of feedstuffs are shown in Table 6-1. Measurement of the various nitrogen fractions will aid nutritionists in the development of feeding programs and proper use of protein supplements.

Figure 6-1. Protein composition of feedstuffs in terms of ruminant protein nutrition. Adapted from Sniffen et al. (1980)

Soluble nitrogen	Insoluble Available nitrogen	Bound nitrogen
Non-protein nitrogen (nitrates, amino acids, nucleic acids, amines, urea if added) True protein	Some non-protein nitrogen True protein	Protein-lignin complex
Rapidly degraded in rumen	Partially degraded in rumen	Not degraded in rumen or digested

Table 6-1. Protein partition of various feedstuffs, DM basis

Ingredient	IRN[a]	Crude protein[b]	Soluble	ADF bound	Insoluble available
Apple pomace	4-00-423	4.0	2.3	60.3	36.9
Beet pulp	4-00-669	8.5	3.4	10.9	85.7
Brewers dried grains	5-02-141	28.9	6.0	13.2	80.3
Citrus pulp	4-01-237	6.0	25.9	10.6	63.5
Corn	4-02-931	9.6	14.6	5.0	80.4
Corn fermented solubles	5-02-890	24.5	67.3	4.6	27.5
Corn gluten feed	5-02-903	22.2	54.5	2.6	42.3
Corn gluten feed, heat treated	5-02-903	23.8	31.6	3.3	65.1
Corn gluten meal	5-02-900	68.2	4.4	10.6	85.0
Corn solubles with germ meal and bran	5-09-333	29.4	62.5	2.9	34.6
Cotton seed meal	5-01-617	44.3	11.9	3.1	85.0
Cotton seed meal	5-01-872	50.6	17.1	1.7	31.2
Cotton waste product	5-01-608	22.3	24.4	1.5	74.0
Distillers dried grains	5-02-842	25.7	5.5	28.8	75.7
Distillers dried grains w/solubles	5-02-843	29.1	19.4	15.3	55.3
Distillers dried solubles	5-02-844	34.9	43.9	12.3	13.3
Hominy	4-02-337	11.0	24.1	2.3	73.1
Oats	4-03-309	12.3	31.0	4.3	64.2
Oat hulls	1-03-281	2.7	29.6	11.7	58.7
Peanut skins	4-03-631	16.1	4.4	15.5	79.1
Potato meal	4-07-850	8.5	43.5	5.6	50.9
Potato pulp	4-03-775	7.1	35.8	8.2	56.0
Rice mill feed	1-03-941	5.2	15.2	20.2	64.5
Rye middlings	4-01-032	18.3	48.2	2.0	49.3
Soybean meal feed	5-05-594	15.2	22.1	20.2	57.7
Soybean oil meal	5-04-512	52.3	23.5	1.3	74.3
Wheat bran	4-04-190	17.3	41.3	3.3	55.4
Wheat flour	4-08-112	15.2	35.3	0.2	54.2
Wheat middlings	4-05-205	18.4	37.0	2.3	60.7

[a]International reference number.
[b]Crude protein = N x 6.25.

Animal Science Nutrition Laboratory

Soluble Nitrogen Content of Feedstuffs

1. Determine total N content of feed sample by Kjeldahl.
2. *Weigh out samples of 0.5 g into a 125-mL Erlenmeyer flask.
3. Add 50 mL of .15 M NaCl (preheat NaCl to 40°C). The pH of the NaCl should be adjusted to 6.5 with orthophosphoric acid.
4. Place flask in H_2O bath (40°C) for 6 h. Stir occasionally.
5. Filter contents of flask through Whatman #4 filter paper held in a Buchner funnel.
6. *Determine by Kjeldahl amount of N in residue and calculate soluble N as difference between total N and insoluble N.

Reference - Waldo and Goering. J. Anim. Sci. 49:1560 (1979).

*Sample size and volume of NaCl could be increased proportionally for samples low in crude protein content.

**By determining ADIN on the same sample, the insoluble fraction can be subdivided into insoluble available and unavailable fractions.

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CHAPTER VII

Measures of Digestibility

General. Nutritive value of feeds is determined by a number of factors, including composition, odor, texture, and taste. These factors are generally measurable in the case of the animal as digestibility and intake. Digestibility is simply a measure of the availability of food nutrients. When digestibility is combined with intake data, one can make a rather accurate prediction of overall nutritive value and, hence, the potential production a given feed can support. Of the two factors, intake is relatively more important than digestibility in determining overall nutritive value because highly digestible feeds are of little value unless consumed by the animal in question. However, digestibility usually provides a fairly reliable index of nutritive value because more digestible feeds are normally consumed to a greater extent than less digestible feeds. In addition, measures of digestibility are somewhat easier to obtain than measures of intake, and, thus, considerable effort has been made by animal nutritionists to develop effective means of determining digestibility. The first of these means we will discuss is the conventional digestion trial.

Conventional Digestion Trials. The conventional digestion trial is the most reliable method of measuring the digestibility of a feedstuff. Unfortunately, however, it is somewhat time consuming, tedious, and costly. Basically, the feed in question is fed in known quantities to an animal. Usually, the animal is restrained in an individual cage so that a quantitative collection of feces can be made. Accurate records of feed intake, refusals and fecal output are kept, and a subsample of each (usually 10% of daily output in the case of feces) is retained for analysis. When estimates of nitrogen balance are desired, urine output also is measured.

Three animals per feed are required as a minimum, with more animals preferred. The animals are usually allowed from 7 to 14 d to adjust to the feed and their cages, followed by a 5- to 7-d period in which feces is quantitatively collected and feed intake is recorded. Samples can then be dried, ground, and analyzed for the nutrients of interest. Digestibility of any given nutrient can be calculated as follows:

$$\text{Nutrient digestibility} = [(\text{nutrient intake} - \text{nutrient in feces})/\text{nutrient intake}] \times 100$$

Even though conventional digestion trials are the standard with which all other measures of digestibility are compared, the values obtained still vary ± 1 to 3 % as a result of animal-to-animal variation and sampling and analytical errors. Thus, it is clear why nutritionists would seek alternative measures of digestibility because of the time and expense of conventional trials. A typical protocol for nutrient balance trials is detailed in a short paper attached to this chapter.

Prediction of Digestibility from Chemical Composition. One such alternative measure of digestibility is the prediction of digestibility from chemical composition of the feed in question. This process involves development of multiple regression equations relating various chemical components to in vivo digestibility. Students are referred to Johnson and Dehority (1968) and Oh et al. (1966) for examples of this method. Generally, the digestibility estimates obtained

from prediction equations are not as precise as one might desire (±3 to 4 % of values obtained from conventional trials), and at the present time, in vitro digestibility measurements are more extensively used to estimate digestibility than are prediction equations based on chemical composition.

In Vitro Fermentation Methods. In vitro digestibility techniques provide a quick, inexpensive, and precise prediction of in vivo or conventionally determined digestibility in ruminants. Essentially, the method simulates the processes that occur in the rumen, and probably the most commonly used in vitro technique is the one devised by Tilley and Terry (1963). Although the original Tilley and Terry procedure has been modified by many researchers, the basic procedure is the same as that used in the Animal Nutrition Laboratory. A copy of the procedure is provided with this chapter.

Estimates of digestibility by the Tilley and Terry procedure are within 1 to 3% of conventionally determined values. The in vitro procedure does a better job of prediction than chemical composition because it accounts for all factors affecting digestibility, whether known or unknown, which is not possible with current chemical methods. This accounting for additional factors is primarily a function of the use of ruminal fluid from a donor steer as the digestive agent, thereby including unknown factors that simple chemical analyses of the feed do not reveal.

As indicated previously, the in vitro procedure is quite simple, but nonetheless subject to a number of variables that may influence the results obtained. Basically, a small sample of feed (~.5 g) is weighed into a 50-mL centrifuge tube. McDougall's buffer (based on the composition of sheep saliva) and ruminal fluid from a donor animal are added, and the tube is allowed to incubate for 48 h at 39^0C. The fermentation is then stopped, tubes are centrifuged, and supernatant fluid discarded. Acidified pepsin is added, and the tube is allowed to incubate for another 48 h at 39^0C. Finally, the contents are filtered, and the residue is dried and weighed. In vitro dry matter disappearance is determined by the following formula:

IVDMD % = 100 x [(initial dry sample wt - (residue - blank))/initial dry sample wt]

The blank value is determined by incubating a tube containing ruminal fluid and buffer, without any feed sample. This accounts for indigestible materials introduced into the vessel by the ruminal fluid inoculum that should not be "counted against" the feed.

The procedure is useful in that estimates of digestibility can be obtained in a few days on a large number of samples. However, we should consider some of the variables that influence the procedure. Four major ones are listed below from Johnson (1969).

A. Variations in the microbial population
    1. Diet of donor animal
    2. Animal to animal differences
    3. Inoculum processing

B. Variations resulting from different storage, grinding, and processing techniques in sample preparation

C. Differences attributable to the fermentation medium

1. Sample:inoculum ratio

2. Buffer

3. Nutrients in medium

D. Procedural variations such as length of fermentation and laboratory errors.

Given these variations, one can develop methods to standardize the in vitro procedure. The largest source of variation among the four major sources listed above is the variation in the microbial population. Difficulties associated with this source of variation can be partially overcome by using more than one animal and by feeding donor animals the same or a similar diet to that being evaluated in the in vitro system. In addition, fluid should be removed at a standard time after feeding. The author's personal preference is to remove fluid ~4 h after feeding when microbial numbers are maximal; however, several researchers prefer removing fluid after the donor has been withheld from feed and water for 12 to 14 h. Generally, the standard method of processing the inoculum is simply to strain whole ruminal contents through at least four layers of cheesecloth.

With regard to variations in the sample, it has been observed that finely ground samples are more highly digested than are coarsely ground samples. Hence, all samples should be ground in the same manner, and grinding to pass a 1- to 2-mm screen usually is adequate. A commonly used sample:inoculum:buffer ratio is 0.5 g:8 mL of ruminal fluid:32 mL McDougall's buffer. Some laboratories add nutrients to the buffer, the most common being urea, to prevent nutrient deficiencies during fermentation. Fermentation should be maintained at a pH of 6.9 to 7.1 for optimum results.

Procedural variations can be minimized by standardizing temperature, time of fermentation, centrifugation speeds, and so on. In fact, the key to successful in vitro analysis is to standardize as much as possible. In this regard, it is a good practice to include a standard forage with each in vitro run as a means of determining the validity of individual runs.

All things considered, the in vitro digestibility technique is the best means of laboratory evaluation of digestibility available today. The procedure will continue to be used extensively for some time to come. Students are referred to Johnson (1969) for further reading on the in vitro technique.

Nylon Bag Digestibility Techniques. Another method of estimating digestibility of feeds is the nylon bag technique. In this procedure, nylon bags (~5 cm x 15 cm) are filled with 2 to 3 g of the feed in question and incubated in the rumen of a cannulated animal. Generally, bags are secured to a weighted cord to prevent floating in the rumen and to ensure adequate exposure to microbial digestion. Bags are then removed, washed under tap water (until wash water is clear), dried (50^0C for approximately 24 h, followed by 1 h at 100^0C), and the weight of residue

determined. An empty bag should be incubated and serve a similar purpose to the blank tube in the Tilley and Terry procedure. A 72-h period in the rumen often provides estimates of digestibility similar to those obtained by the Tilley and Terry method.

Many of the same variables (i.e., diet of animal, time of fermentation) that affect the Tilley and Terry method also affect the nylon bag procedure. In addition, one should consider the pore size of the nylon material, which should be small enough to prevent passage of feed from the bag, but large enough to permit microbial entry. A pore size of 50 μm or less is desirable. Furthermore, the sample:bag size ratio is quite important, and a ratio of ~10 mg/cm^2 of bag surface is probably adequate.

One disadvantage of the nylon bag technique is that fewer samples can be run at one time than with the Tilley and Terry method, and a donor animal with a large diameter cannula is desirable. Nonetheless, nylon bag (or in situ) techniques, are quite useful for evaluating kinetic aspects of digestion in ruminants. Through the use of multiple incubation times and computer models, rates of nutrient digestion can be estimated. Digestion rate models proposed by Ørskov and McDonald (1979) and D. R. Mertens (personal communication, Dairy Forage Research Lab, Madison, WI) are attached to this chapter. Students also are referred to Lowrey (1969) for further reading on the nylon bag procedure. Van Soest (1982) also provides some information and references on the subject.

In a later chapter, we will consider other methods of estimating digestibility by the use of indigestible markers. These methods have special application to conditions where conventional methods are difficult to apply or when representative samples for in vitro methods are difficult to obtain (i.e., grazing animals).

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Table 7-1. Example calculations for in vitro dry matter and organic matter disappearance

Rep	Sample wt	Sample dmf	Sample omf[a]	Dry wt	OM wt	Pan wt	Filter paper wt	Dry pan and residue wt	Ashed pan and residue wt	Dry residue wt	OM residue wt[b]
1	0.5000	0.90	0.95	0.4500	0.4275	1.5000	0.4500	2.1500	1.5200	0.2000	0.1800
2	0.5000	0.90	0.95	0.4500	0.4275	1.5200	0.4700	2.1950	1.5400	0.2050	0.1850
Blank	-	-	-	-	-	1.5000	0.4500	1.9600	1.5050	0.0100	0.0050

[a]Sample omf = organic matter factor, dmb = 100 - ash%, dmb
[b]OM residue wt = Dry residue wt - (Ashed pan + residue wt - pan wt)

Rep 1 - IVDMD = 100 x [(0.4500 - (0.2000 - 0.0100))/0.4500] = 57.78%

Rep 1 - IVOMD = 100 x [(0.4275 - (0.1800 - 0.0050))/0.4275] = 59.06%

Rep 2 - IVDMD = 100 x [(0.4500 - (0.2050 - 0.0100))/0.4500] = 56.67%

Rep 2 - IVOMD = 100 x [(0.4275 - (0.1850 - 0.0050))/0.4275] = 57.89%

IVDMD: $\bar{x}$ = 57.23%; s = 0.78; CV = 1.37%
IVOMD: $\bar{x}$ = 58.48%; s = 0.83; CV = 1.41%

Animal Nutrition Laboratory

IN VITRO PROCEDURE

REAGENTS

McDougall's artificial saliva (mix four parts McDougall's to one part ruminal fluid.)

9.8 g $NaHCO_3$/L

7.0 g $Na_2HPO{\cdot}7H_2O$/L or use 3.71 g anhydrous/liter

0.57 g KCl/L

0.47 g NaCl/L

0.12 g $MgSO_4{\cdot}7H_2O$/L

4% (wt/vol) $CaCl_2$ solution: 4 g $CaCl_2$/100 mL

Mix the first five chemicals in 500 mL of water and stir until dissolved. Add remainder of water. Before using, add in the 4% $CaCl_2$ solution (use 1 mL of the 4% $CaCl_2$ solution per 1 L).

Place the McDougall's solution, after the addition of the 4% $CaCl_2$ solution, into the 39^0C water bath and bubble in CO_2 gas until the pH of the McDougall's solution reads 6.8 to 7.0.

When using the CO_2 tank, open the top release valve, and then open the smaller valve to release CO_2 into the plastic line. After you have finished, close the top release valve and close the smaller line release valve. Failure to close **BOTH** valves results in emptying the tank. When the tank reads at 50 lb of pressure, **please** tell the technician so a new CO_2 tank can be ordered.

Pepsin solution

6.6 g of 1:3,000 pepsin

100 mL of 1 N HCl (to prepare 1 N HCl add 80.4 mL of HCl/L of H_2O)

Add deionized H_2O to 1 L.

PROCEDURE

1. Weigh out a 0.5-g sample and place into a labeled 50-mL centrifuge tube.

2. To this tube, add 28 mL of the McDougall's solution. Prewarm McDougall's in 39^0C H_2O bath. Add 7 mL of ruminal fluid (can alter quantity, but use 4:1 ratio of buffer to ruminal fluid). Place ruminal fluid on stir plate to avoid settling. Ruminal fluid is strained through four layers cheesecloth before use. If possible, ruminal fluid should be obtained from at least two animals.

3. Flush tube with CO_2 (gently so sample is not blown out). Place cap on tube, invert several times to suspend the sample, then place tubes into a rack, and place the rack into a 39^0C water bath.

4. Also include at least four blanks (tubes containing **no** sample and 35 mL of the McDougall's to ruminal fluid mixture). Include two blanks per time interval if rates of digestion are to be determined. Include 0.5-g samples of lab standards.

5. Incubate the tubes for 48 h.

6. Invert the tubes at 2, 4, 20, and 28 h after initiation of incubation to suspend the sample.

7. After 48 h of incubation, remove the tubes from the water bath. Centrifuge for 15 min at 2,000 x *g* and suction off the liquid by vacuum. At this point, one may freeze samples until they can be filtered or until the pepsin digestion can be completed.

8. If you are doing the acid pepsin digestion, mix the pepsin solution, and add 35 mL of pepsin solution to each tube. Incubate for 48 h in a 39^0C water bath, shaking at 2, 4, and 6 h after pepsin addition.

9. After the completion of the digestion (either McDougall's and ruminal fluid or the pepsin solution digestion), filter your samples using the modified Buchner funnel and ashless filter paper.

10. Dry the filter paper containing the sample in an aluminum pan for 12 to 24 h. Record weights.

11. Ash each sample and record the weights. Ash at 500°C for 4 h.

12. Complete calculations.

Procedures and General Consideration for Nutrient Balance Trials with Ruminants (Courtesy of G. S. Smith)

I. Selection and care of animals

A. Select animals of similar size, sex, age, weight, and breeding. They should be free of parasites, healthy, and vigorous; but not unduly excitable.

B. Place animals in preliminary feeding pens, and allow them to become adjusted to environment and to the routines of handling and feeding. Gradually change the diets to those that will be used in the trial. During this adjustment period, replace animals that are "poor eaters," and those that are overly excitable or otherwise unsuitable. Weigh the animals at regular intervals, and keep a record of these "preliminary weights."

C. Allow the animals a few days for adjustment to metabolism cages after their transfer from the preliminary holding pens. Protect them from flies and pests, keep them clean, and provide for their comfort within the limits of the trial objectives.

D. Keep a daily record of temperature, weather conditions, unusual events in the animal laboratory, and any observations of pertinence to animal behavior or performance.

II. Experimental Feeds

A. During the preliminary period, calculate the amount of diets (roughage, concentrates, and supplements) necessary for the entire period of the study. Trials with ruminants should consist of at least a 10-d "adjustment period" followed by a "collection period" of 10 d. It is wise to provide a surplus of all dietary ingredients.

B. Ensure that all feed ingredients are uniformly mixed, so that ingredients fed initially are comparable to those fed finally. In the case of concentrate mixtures and supplements, mix amounts sufficient to provide at least 1¼ to 1½ times the amount calculated to be fed during the actual trial. This allows for "repeat" days, spillage, and so on.

C. Store all feed ingredients in covered containers **conspicuously labeled** and **COMPLETELY LABELED**:

1. Project number
2. Trial number
3. Animal number (to receive diets)
4. Dates of trial
5. Experimenter

D. Secure the labels on containers! (**Never label lids only**). Situate feed cans conveniently, and protect against inadvertent interchange of locations. For

roughages, select a quantity of feed that is in excess of needs, uniform in quality, and properly identified. Store conveniently, and protect from contamination-especially dusts from feedstuffs and chemicals.

E. Preliminary sampling and analyses. Take a representative sample of all feedstuffs and analyze **before** the onset of the actual trial to ensure that composition of diets is as prescribed for the trial.

III. Procedures

A. Check the accuracy and precision of all scales, balances, and volumetric equipment to be used in measurement of feedstuffs and excreta.

B. Plan **in detail** a definite routine to be used in the trial - both for the preparation and provision of diets and for the collection of excreta - and follow this routine throughout. Generally, this involves weighing dietary ingredients directly into individual feed pans, and, if necessary, mixing each animal's diet by hand to ensure uniform distribution of ingredients and to prevent selective consumption by the animal.

C. During the preliminary period, while animals are in metabolism cages, collect daily a "running grab sample" from each of the dietary ingredients. Composite these into single, 10-d samples for each of the diets. Label thoroughly! Use the same procedure to collect additional 10-d, composite samples for each of the ingredients fed during the subsequent 10-d collection period. **LABEL COMPLETELY**.

D. Feed consumed during the adjustment period will be excreted during the subsequent collection period. Therefore, the daily feed must be kept constant throughout the 20-d feeding period - constant in amount, and unvarying in composition.

E. Maintain a "Barn Record" showing the daily feeding performance of each animal, the daily excreta, and any notes of consequence.

F. At the onset of the "collection period", start the collection of urine and fecal samples at a specified time, and strive to meet the same schedule throughout the trial. Be sure to end the trial at the same time on the final day of collection.

G. If there should be any feed uneaten from feeding time to feeding time, remove the entire refused portion, weigh and record the weight, and collect a **representative** sample for analysis. **LABEL COMPLETELY**.

H. Collection of urine

1. Equipment used must afford quantitative collection of entire 24-h excreta. Collect urine into clean containers that are safe-guarded against contamination from feedstuffs, fecal material, and extraneous material (e.g., flies, bugs, dust, and so on). Protect also from spillage and breakage (plastic jugs are preferred in most cases).

2. Add a few drops of toluene into the collection jugs at the onset of each 24-

h period to retard microbial activity (this will have insignificant effects on most analyses that are conducted in routine trials).

3. When making the daily collections, provide a temporary container to collect any excretion made during the process (a "stitch in time…").

4. During the collection, suspend sediment by shaking the collection jug, and pour quickly into a volumetric cylinder (graduated), and record the daily urine volume to ± l mL. Test the reaction of the urine to litmus (note if basic) and determine specific gravity and/or total solids by refractometry, if applicable.

5. Dilute the urine to a prescribed, constant volume (generally 1 L) with distilled water, mix thoroughly by pouring back and forth between two containers, and collect a prescribed portion (generally 100 mL per day from a l-L total volume) into the container for the 10-d composite sample. Make sure that each of the 10 daily collections is represented equally in the 10-d composite sample. (It is wise to make **duplicate**, 10-d collections for each animal. Use of plastic bottles for these composite samples will both allow freezing, if desired, and also prevent breakage.)

6. Add to the collected portion of urine an amount of concentrated HCl to ensure that the sample is slightly acid to litmus (avoid excess). Check reaction to litmus each day, and add acid only when necessary. RECORD the amounts of acid added. Add a few drops of toluene to the composite sample, (as well as to the collection jugs), stopper the composite samples, and store under refrigeration (approx. 4^0C).

7. LABEL COMPLETELY:

a) Project number

b) Trial number

c) Animal number

d) Dates of collection

e) Experimenter

8. Inspect the apparatus and assembly for urine collection daily (or more often) to ensure that they are clean and free-flowing.

I. <u>Collection of feces</u>

1. Feces must be collected daily. Follow a prescribed routine.

2. Weigh the entire excretion. Mix thoroughly, and collect a **representative** sample amounting to one-tenth of the daily excretion. Record the weights of daily excretions for each animal.

3. Into the container for the composite sample of feces add a few thymol crystals (approx. 0.1 g per 100 g of feces collected into the vessel); mix, seal, and store under refrigeration.

4. **LABEL COMPLETELY** (see above).

IV. Analysis of samples

A. All analysis should be conducted according to recognized, accepted procedures. It is wise for initiates to become familiar with procedures using "check" samples before beginning work on experimental samples.

B. Urine. In sub-sampling urine samples for analysis, it is desirable to pour the sample into a clean vessel - check for sediment in collection bottle and make a **quantitative** transfer - and then add a (clean) magnetic stirring bar for magnetic stirring while sub-sampling, thereby assuring suspension of any sedimentary material. **LABEL** all sub-samples.

C. Feces. Composite fecal collections should be mixed thoroughly, and a **representative** sub-sample collected into a weighed container suitable for drying at 100^0C. Usually 200-g portions are selected for this determination. Dry the samples to a constant weight in a forced-draft oven at 100^0C, and record loss in weight as "moisture". Such samples will lose appreciable amounts of nitrogen and energy. These losses may be reduced by drying of the samples at 65^0C, and generally such samples are suitable for routine determinations of both nitrogen and "gross energy" of feces. For precise measurements, samples of fresh feces should be chosen for determinations of nitrogen and "total energy". Alternatively, samples of fresh feces may be dried while frozen. Dried samples should be ground through a Wiley Mill, using at least a 2-mm screen.

D. It is a wise practice to preserve sub-samples of all feeds, feces, and urine in the fresh state, refrigerated, until after all analyses are completed **and until after the data have been evaluated and interpreted**.

Ørskov and McDonald - Digestion Model

SAS Proc NonLin Statements

Note: Data are entered as fraction of nutrient (designed for protein) that has disappeared at various incubation times.

```
data dmd;
input t 1-2 p 4-7;
y=p;
cards; run;
12 .375
24 .622
48 .731
72 .766
proc nlin iter=50 method=marquardt;
parms d=.90 b=.70 c=.05;
* fraction a equals d-b;
bounds d <=1.0;
temp=exp(-c*t);
model y=d - b + b*(1-temp);
der.d=1;
der.b=-1+(1-temp);
der.c=b*t*temp;
output out=points predicted=yhat residual=yres parms=d b c;
proc print data=points;
proc plot;
plot yhat*t='*'y*t='y'/overlay;
plot yres*t;
run;
quit;
```

See Ørskov and McDonald (1979) - J. Agric. Sci. (Camb.). 92:499

Mertens - Digestion Rate Model

SAS Proc NonLin Statements

Note: Data are entered as fraction of nutrient (designed for NDF) remaining vs. time of incubation.

```
data dmd;
input tp 1-2 p 4-7;
t=tp;
y=p;
if t=0 then ndf0=y;
retain ndf0;
cards; run;
00 1.00
06  .95
12  .75
18  .64
24  .52
48  .31
72  .29
96  .27
proc nlin data=dmd iter=100 method=marquardt;

*Model statements provided by D. R. Mertens - August, 1988;

parms k=.05 lag=5 i=.20;
td=abs(t-lag);
at=(t-lag+td)/2;
tr=at/td;
e=exp(-k*at);
do=ndf0-i;
model y=do*e+i;
der.k=-at*do*e;
der.i=1-e;
der.lag=tr*k*do*e;
output out=points predicted=yhat residual=yres parms=k lag i;
proc print data=points;
proc plot;
plot yhat*tp='*' y*tp='y'/overlay;
plot yres*tp;
run;
quit;
```

CHAPTER VIII

Spectrophotometry

A clear understanding of the principles of spectrophotometry is important to students of nutrition. Spectrophotometric procedures are used for the analysis of many minerals, vitamins, blood constituents, and other biologically important compounds. At some point in time, each of us has probably applied a basic spectrophotometric principle in everyday life. For example, one often can determine whether a solution is more concentrated than another simply by its color. The deeper the color, the more concentrated we assume the solution to be. This is essentially the same process used in any spectrophotometric analysis; except that solutions need not be colored to measure concentration.

We can define **spectrophotometry** as **the measurement of the light transmitting power of a solution to determine the concentration of light absorbing material present in the solution**. There are essentially two units of measurement in spectrophotometry: transmittance and absorbance or optical density. Transmittance is defined as the ratio of the intensity of light emerging from a solution and the light entering the solution. Mathematically, transmittance (T) can be expressed as follows:

$T = I_2/I_1$,
where I_2 = intensity of emerging light and I_1 = intensity of incident light.

In practice, we do not really measure the light intensities, but rather we measure the ratio of a solution of light-absorbing material to the solvent. Essentially, the instrument is set at 100 % T with the solvent, and light-absorbing materials in a solution will result in a decrease in % T. Although values of transmittance range from 0 to 1.0, transmittance is usually expressed as a percent (e.g., 0.45T = 45% T).

Absorbance is the most common unit of measurement in spectrophotometry. It is the negative logarithm of T and is of great value because under certain conditions it is linearly proportional to the concentration of light-absorbing materials in solution (Fritz and Schenk, 1979).

Two major approaches are used commonly in spectrophotometric analyses. One approach is to measure the light absorbed by an ion or molecule itself (Fritz and Schenk, 1979). Colored compounds obviously absorb light, and many colorless compounds absorb light in the ultraviolet or infrared regions. The second approach is used with compounds that **do not** absorb appreciable amounts of light. In this case, a reagent is added to the compound to produce a complex that will readily absorb light. This principle is applied to many analyses (e.g., phosphorous, glucose) commonly performed in nutrition laboratories.

Before we continue our discussion of spectrophotometric measurements, we should first consider the absorption of light by various substances. Radiant or light energy is characterized by its wavelength. The following table provides a guide to measurements in common use.

Table 8-1. Units of measurement in radiant energy. From Fritz and Shenk (1979).

nm = nanometer = 10^{-9} meter (millimicron, mμ)
Å = Angstrom = 10^{-10} meter
μm = micrometer = 10^{-6} meter (micron, μ)

Infrared (750 + nm), visible (380 to 750 nm) and ultraviolet (10 to 380 nm) regions of the electromagnetic spectrum are those most commonly used in spectrophotometric analyses. Light of these wavelengths is absorbed by a chemical species only when its wavelength corresponds to the energy needed to cause a change in the electronic configuration of the species (Fritz and Schenk, 1979). Absorption of light by molecules causes electronic, vibrational, and rotational changes in the molecule. Electronic changes result when the energy of the electrons in the molecule is changed. Vibrational changes occur when there is a change in the internuclear distance of two or more atoms in the molecule, and rotational changes occur as a molecule rotates around its center of gravity (Fritz and Schenk, 1979). Because of the variety of changes that can occur, absorption of light by a molecule may occur at a number of wavelengths, and it is important in spectrophotometric analyses to select the wavelength at which absorption is maximized. For this reason, it is common to evaluate the absorption spectrum of a given molecule in order to select the optimum wavelength for analysis.

Once the absorption spectrum has been determined, we can apply a principle of spectrophotometry to determine the concentration of the compound in question. The fundamental principle or law of spectrophotometry is **Beer's Law**, which states that the transmittance of a solution containing light-absorbing material depends on the nature of the substance, the wavelength of light, the length of the light path, and the concentration of the substance. **At a given wavelength**, Beer's Law can be expressed as follows:

$$T = 10^{-klc}$$

where k = constant for substance, l = length of light path, and c = concentration of light-absorbing material.

By taking the log of both sides, the equation can be rewritten as:

$$-\log T = klc$$

As we have seen earlier, -log T is termed absorbance or optical density. Thus, if Beer's Law holds true for a given situation, absorbance is directly proportional to concentration. This concept is illustrated in Figure 8-1.

Now, let us consider how we would perform a spectrophotometric analysis and apply the

principles discussed above. First, we set the spectrophotometer to provide light of the proper wavelength. Recall that we can determine the proper wavelength by determining the absorption spectrum for the substance to be measured. We then make up a series of standard solutions (i.e., known concentrations of the substance to be measured), and measure the absorbance of each standard in the spectrophotometer. This is done by placing each standard solution in a cuvette, which is basically a test tube through which light is passed in the spectrophotometer. Cuvettes are designed to have the same dimensions, so the length of the light path is the same for all measurements. The next step would be to determine the absorbance of the solution of unknown concentration. Concentration of the solution can then be calculated by application of Beer's Law. We will now consider three ways of determining the concentration of an unknown solution.

The first method is what we will call the "closest standard method". In this case, we have determined absorbance of a number of standards and the unknown. We then select the standard that has an absorbance value nearest the unknown. Recall from Beer's Law we have the following:

Equation 1 $A_{unknown} = k\ l\ c_{unk}$
Equation 2 $A_{standard} = k\ l\ c_{std}$

Because both standard and unknown are the same substance, k should be equal in both equations. In addition, 1 would be equal in both equations because our cuvettes have the same dimensions. Thus, if we divide Equation 1 by Equation 2, we obtain:

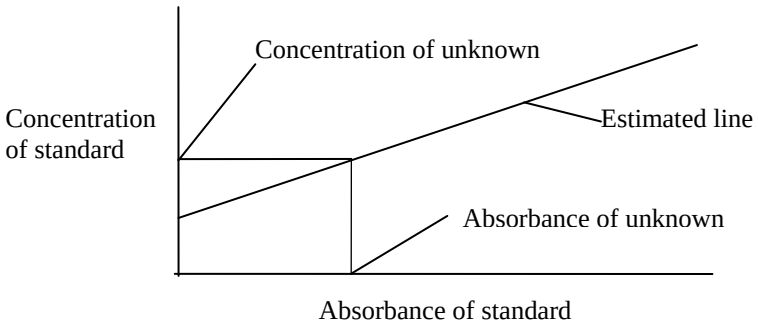
$A_{unknown}/A_{std} = C_{unk}/C_{std}$

Solving for C unk we have:

$C_{unk} = C_{std} \times (A_{unknown}/A_{std})$.

Now we can determine the concentration of the unknown by simply "plugging in" the appropriate values.

We will call the second method the "standard curve method". In this case we simply plot absorbance vs. concentration of standards and estimate a line that fits the points. This method is depicted below:



For this second method, concentration of the unknown is estimated by extrapolation from absorbance of the unknown.

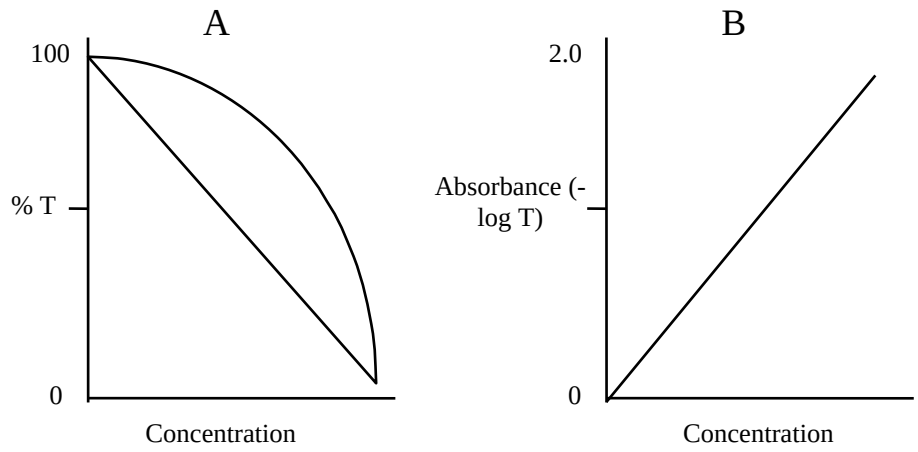
The third method also involves generation of a standard curve, except that linear regression is used to estimate the line that “best fits” the data. This method is highly desirable because regression techniques allow one to put confidence limits on the predicted unknown concentration. An example calculation of all three methods in the determination of phosphorus is hown in Table 8-2.

We have spent considerable time discussing situations in which Beer's Law is applied to calculate unknown concentrations. But how do we know whether Beer's law applies to a particular analysis? This can be determined by plotting absorbance vs. concentration (or vice versa) throughout a range of concentrations. The linear region of the curve indicates those concentrations for which Beer's law applies. Generally, most reported spectrophotometric procedures have considered regions of linearity, absorption spectra, and so on, and we need not worry about such matters in routine analyses. Knowledge of such procedures, however, will assist the student in recognizing the steps needed to develop spectrophotometric procedures.

The Animal Nutrition Laboratory procedures for phosphorus and ammonia are attached to this chapter. In the case of phosphorus, molydovanadate reagent is added to form a light-absorbing complex. In the ammonia procedure, the reagents result in the formation of a blue color complex with ammonia.

Several variables can influence the results of spectrophotometric analyses. These factors should be considered in every spectrophotometric analysis and are listed and discussed in Table 8-4. In addition, it is important to recognize that analysis of a particular constituent may be subject to a number of interferences. For example, other anions or cations besides the one of interest might bind with reagent and prevent formation of light-absorbing complexes. In the case of carotene, the sample must be chromatographed before analysis and losses of carotene can occur during the process. In such instances, it is common to use an internal standard to adjust for interferences. A known quantity of the desired constituent is added to the sample and its recovery is calculated. Based on internal standard recovery, sample values can be adjusted for interferences. This process is illustrated for carotene analysis in Table 8-3.

Figure 8-1. Relationship between transmittance, absorbance, and concentration.



Graph A shows that T does not decrease linearly with concentration. Graph B shows that conversion of T to absorbance produces a linear relationship. This situation holds true when Beer's Law is applicable to the data.

Basic Components of a Spectrophotometer. We will now briefly discuss the components of a spectrophotometer. Although there is considerable variation in what is available "on the market", Figure 8-2 depicts the essential components of a spectrophotometer.

The radiation or light source is usually of the continuum type that emits light over a large range of wavelengths. More than one source is needed to emit light in both the UV and visible ranges (200 to 750 nm). The tungsten filament lamp is commonly used in the visible region, and a deuterium discharge lamp is used in the UV region (Fritz and Schenk, 1979).

The monochromator is used to select particular wavelengths of light from the light source. Actually, it selects a narrow range of wavelengths, this range being called the spectral bandwidth or bandpass (Fritz and Schenk, 1979). Usually, the wavelength selected is expressed as the mean wavelength, although students should be aware that a narrow band is emitted rather than one particular wavelength. The essential elements of a monochromator are (1) entrance slit, (2) lens or mirror that causes light to travel in parallel rays, (3) dispersion device to select light of different wavelengths, (4) focusing lens or mirror, and (5) exit slit (Fritz and Schenk, 1979). The dispersion device is usually a diffraction grating or prism. Diffraction gratings are surfaces that have been grooved with parallel grooves approximately one wavelength wide. Light hitting the grating is diffracted so that different wavelengths come off at different angles, and by moving the grating, light of the desired wavelength can be selected. Slits adjust the spectral bandpass of the monochromator.

The sample cell or cuvette may vary considerably in shape and size, depending on the brand of instrument. Glass cells can be used effectively in the visible range, but quartz or silica cells are required in the UV range because borosilicate glass will absorb radiation at lower wavelengths. In any case, dimensions of the cuvette must be constant. Many spectrophotometers use flow-through cells, in which the sample is automatically pumped into or out of the cell.

A typical detector would be the vacuum phototube. In this device, a silver/silver oxide-plated nickel cathode coated with cesium and a wire anode are sealed in an evacuated glass tube. The anode is maintained at a positive voltage relative to the cathode. As photons of light strike the cathode, electrons are ionized away from cesium and strike the anode, producing a current. This current can then be amplified and converted to a useful form of readout (Fritz & Schenk, 1979). An example of vacuum phototube is shown in Figure 8-3.

Figure 8-2. Basic components of a spectrophotometer. Adapted from Fritz and Schenk (1979).

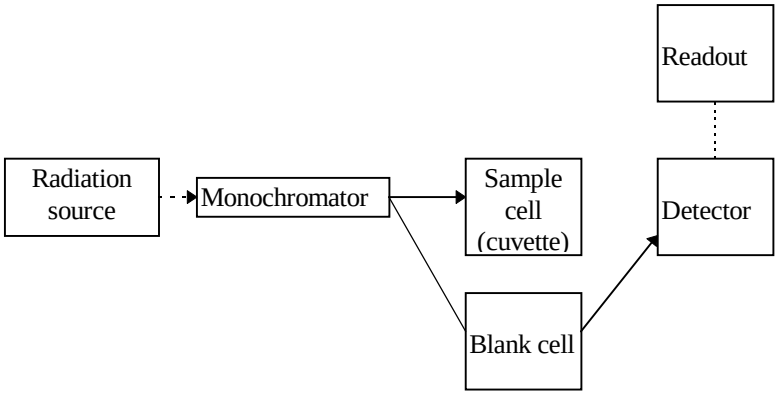


Figure 8-3. Diagram of a typical vacuum phototube. Adapted from Fritz and Schenk (1979).

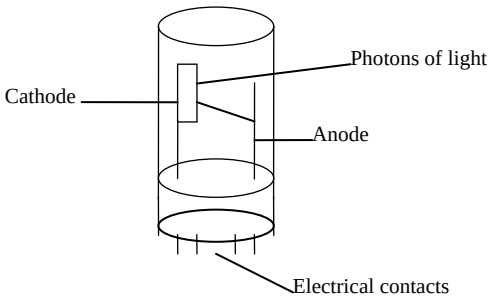


Table 8-2. Calculation of Phosphorus Content of Feeds by Spectrophotometric Methods

A l-g sample of feed was ashed, put into solution with 25% (vol/vol) HCl and brought up to 100 mL in a volumetric flask. A 10-mL aliquot was drawn, mixed with molybdovanadate reagent, and diluted to 50 mL in a volumetric flask. Standard phosphorus solutions also were mixed with reagent, and absorbance of unknown and standard solutions was determined at 400 nm in a spectrophotometer. The standard and unknown data were as follows:

Y, conc, mg/100 mL	X, Absorbance @ 400 nm
Blank (0.2)	0
0.5	0.185
0.8	0.330
1.0	0.440
1.5	0.595

Unknown

Rep 1 - Absorbance = 0.20

Rep 2 - Absorbance = 0.185

Now, by using data from the standards, we can calculate the P concentration in the unknown sample in one of three ways.

1. Closest standard method

   Recall that if Beer's Law holds for our data, the following relationship is also true:

   $Conc_{unk} = Conc_{std} \times (Abs_{unk}/Abs_{std})$

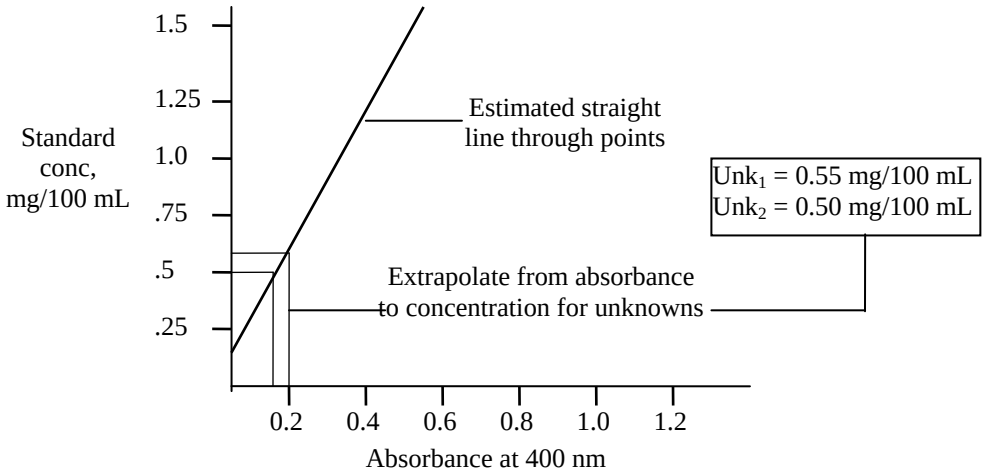
   For our data:

   $Conc_{unk1} = 0.5 \times (0.20/0.185) = 0.54$ mg/100 mL

   $Conc_{unk2} = 0.5 \times (0.185/0.185) = 0.50$ mg/100 mL

2. Standard curve method

We can plot concentration vs. absorbance and extrapolate concentrations for unknown samples.



3. Linear regression

We can calculate an equation for the line that we have "guessed at" in method 2 above by using simple linear regression. The form of the equation will be:

$Y = b_0 + b_1X$

Where: Y = predicted concentration
b_o = Y intercept
b_1 = slope of the line
X = Absorbance of standards

Using the data from the standards listed at the beginning of this table (except the blank):

$\Sigma Y = 3.800$
$\Sigma Y/n = 0.95$
$\Sigma Y^2 = 4.140$
$(\Sigma Y)^2/n = 3.610$
$\Sigma y^2 = 0.530$

$\Sigma X = 1.550$
$\Sigma X/n = 0.3875$
$\Sigma X^2 = 0.69075$
$(\Sigma X)^2/n = 0.600625$
$\Sigma x^2 = 0.090125$

$\Sigma XY = 1.6890$
$[(\Sigma X)(\Sigma Y)]/n = 1.4725$
$\Sigma xy = 0.2165$

$b_1 = \Sigma xy/\Sigma x^2 = 2.4022$

$$b_0 = (\Sigma Y/n) - [b_1 \cdot (\Sigma X/n)] = 0.01915$$

Hence, the equation describing the line is:

$$Y = 2.4022X + 0.01915$$

Plugging the unknown absorbance values into this equation yields:

Rep 1 Absorbance = 0.20 = 0.50 mg/100 mL
Rep 2 Absorbance = 0.185 = 0.46 mg/100 mL

The advantage of the linear regression method is that it allows us to put confidence limits on the predicted concentration. This can be done by calculating the standard error of the estimate, $S_{y.x}$, as follows:

$$\Sigma dy.x^2 = \Sigma y^2 - [(\Sigma xy)^2/\Sigma x^2]$$

$$Sy.x^2 = \Sigma dy.x^2/n-2$$

$$Sy.x = \sqrt{Sy.x^2}$$

For the preceding data, the value of Sy.x = 0.07. The number is a measure of the error with which any observed value of Y can be predicted from X, using the regression equation.

It is usually beneficial to calculate the correlation coefficient between X and Y. This value measures the strength of the linear relationship between the two variables and can vary from -1.0 to + 1.0.

$$r_{xy} = \Sigma xy/[\sqrt{\Sigma x^2} \cdot \sqrt{\Sigma y^2}]$$

For our data, the value is +.99. In spectrophotometric data, a correlation of +0.95 or greater should be achieved.

<u>Calculation of the phosphorus content of the sample</u>

The three methods above allow us to calculate the concentration (mg/100 mL) of P in the unknown samples. Now, how can one calculate the % P in the original feed sample?

1. Remember our aliquot was 10 mL, from an original volume of 100 mL. This aliquot was made to volume in 50 mL volumetric flask. The concentration of P in this 50-mL flask expressed in mg/100 mL was 0.54 and 0.50 mg/100 mL for Reps 1 and 2 as calculated by the closest standard method.

2. If there was 0.54 mg/100 mL, and the actual volume was 50 mL, then there would be half as much or 0.27 mg of P in the 50-mL flask. The value for Rep 2 would be 0.25 mg P.

3. Note, however, that all this P came from the 10-mL aliquot drawn from the original sample. Thus, if there were 0.27 mg in 10 mL of the original solution, there would be 10 times as much in the original 100-mL volume. So, for Rep 1, the original sample of feed had 2.7 mg of P. For Rep 2, the value would be 2.5 mg of P.

4. If the dry weight of the sample was 1.000 g or 1,000 mg, the % P in the sample would be:

Unknown
Rep 1 = (2.7/1,000) x 100 = 0.27 % P
Rep 2 = (2.5/1,000) x 100 = 0.25 % P

$\bar{x}$ = 0.26 % P
s = 0.01414
CV = 5.4 %

Table 8-3. Carotene Analysis and Internal Standard Recovery Calculations[a]

Purified β-carotene (100 mg/mL) was diluted in hexane to provide a series of standard concentrations. Absorbance was measured at 440 nm and the values are shown below.

Y, mg/mL	X, Absorbance @ 440 nm
5	0.15
10	0.30
15	0.45
20	0.59
40	1.20

The regression equation calculated from the data was: $Y = 3.336X + 0.0053$

Six samples of 2.0 g of forage were extracted using 30 mL acetone + hexane (3 + 7). Aliquots of .5 mL of a carotene standard containing 100 mg carotene/mL were added into three of the samples. Samples were chromatographed, brought to 100-mL volume, and absorbance determined on a spectrophotometer at 440 nm. The standard curve shown above was used to calculate the following values:

Replicate	Internal standard amount, μg	Absorbance	Y	Carotene in sample, μg
1	0	0.025	0.0887	8.87
2	0	0.020	0.0720	7.20
3	0	0.025	0.0887	8.87
4	50	0.108	0.3656	36.56
5	50	0.109	0.3687	36.89
6	50	0.091	0.3089	30.89

The average carotene concentration in the three samples was 8.31 μg. The IS recovery can be calculated as follows:

Rep 4: 36.56 - 8.31 = (28.25/50) x 100 = 56.50%
Rep 5: 36.89 - 8.31 = (18.58/50) x 100 = 57.16%
Rep 6: 30.89 - 8.31 = (22.58/50) x 100 = 45.16%

Hence, the average IS recovery for the three replicates was 52.94%.

Carotene content of the sample would be adjusted for incomplete recovery by:
Sample adjusted carotene = 8.31/.5294 = 15.70 μg/2g = 7.85 μg/g.

[a]This example provided courtesy of G. S. Smith, ANSC 507 notes (1969).

Table 8-4. Factors to consider in spectrophotometric analyses.

Concentration of constituent	Should have optimum concentration range for application of Beer's law
Concentration of reagent	Use in excess of amount needed for full reaction. Add in carefully repeated amounts
pH	Important when organic dyes or chelating agents are used as reagents
Time	Color stability should be checked against time. Read at standard time after reagent addition if possible
Sequence of operations	Standardize dilutions, order and method of reagent addition
Temperature	Heating may alter color development or reaction
Ionic interferences	Cations or anions may consume reagent so that not enough is left to react with constituent
Nature of solvent	Always check background absorbance of solvent at wavelength where test is to be conducted

References

Fritz, J. S., and G. H. Schenk. 1979. Quantitative Analytical Chemistry (4th Ed.). Allyn and Bacon, Inc., Boston, MA.

Animal Nutrition Laboratory

Determination of Phosphorus

Method: Colorimetric Method – Modified from AOAC 965.17

Reagents

1) Molybdovanadate reagent - Dissolve 40 g NH_4-molybdate $\cdot 4H_2O$ in 400 mL hot H_2O and cool. Dissolve 2 g NH_4-metavanadate in 250 mL hot H_2O, cool, and add 250 mL 70 % $HClO_4$. Gradually add molybdate soln. to vanadate soln with stirring and dilute to 2 L.

2) Phosphorus standard solns. - (1) stock soln. - 2 mg P/mL. Dissolve 8.788 g KH_2PO_4 in H_2O and dilute to 1 L. (2) working soln. - 0.1 mg P/mL. Dilute 50 mL stock soln. to 1L.

Preparation of Standard Curve

Transfer aliquots of working std. soln. containing 0.2, 0.5, 0.8, 1.0, and 1.5 mg P to 100 mL volumetric flasks (this corresponds to 2, 5, 8, 10, and 15 mL of working soln). Treat as below. Use water as the blank (i.e., molydovanadate solution and water only as indicated below).

Determination

Ensure that all glassware has been rinsed with dilute acid before use. Place aliquot of sample solution containing 0.2 to 1.5 mg P in 100-mL volumetric flask. Add 20 mL of molybdovanadate reagent, dilute to volume with H_2O, and mix well. Let stand 10 min, then read at 400 nm using H_2O as the blank. Determine milligrams of P from standard curve.

Alternative Small-Volume Procedure

Transfer aliquots of 0.1, 0.2, 0.4, and 0.6 mL the working std. soln. prepared above to 10-mL disposable test tubes (this yields 2, 4, 8, and 12 µg/mL standards). Add 1 mL of molybdovanadate reagent and sufficient H_2O to bring the total volume to 5 mL (e.g., for the 2 µg/mL standard, add 3.9 mL of H_2O). For samples, place an aliquot of sample solution containing between 10 and 60 µg into a 10-mL test tube, add 1 mL of molybdovanadate reagent, and bring to 5 mL volume with H_2O. Mix well, let stand 10 min, and read at 400 nm using H_2O as the blank (i.e., blank would contain 4 mL of H_2O plus 1 mL of molybdovanadate reagent).

Animal Nutrition Laboratory

Phenol-Hypochlorite Assay for Ammonia

Adapted from Broderick and Kang JDS 63:64 (1980)

Phenol reagent

Dissolve .15 g of sodium nitroferricyanide (sodium nitroprusside) in 1.5 L of distilled H_2O. Then add 33 mL (90% wt/vol) phenol (measured by a graduated cylinder) and mix thoroughly. Make solution up to 3 L with distilled H_2O, and store in brown glass bottle.

Hypochlorite reagent

Dissolve 15 g of sodium hydroxide in about 2 L of distilled H_2O. Then dissolve 113.6 g of disodium phosphate (heptahydrate - $Na_2HPO_4 \cdot 7H_2O$) in this solution with mild heating and mixing. After cooling, add 150 mL of commercial bleach (5.25% sodium hypochlorite) and mix. Then make solution up to 3 L with distilled H_2O. Filter solution through Whatman #1 filter paper, and store in polyethylene bottle protected from light.

Ammonia standard solution

A stock solution of 100 mM ammonia was prepared by diluting 0.6607 g of ammonium sulfate (dry ammonium sulfate overnight in 100°C oven before use) to 100 mL with .1 N HCl.

Working standards of 1, 2, 4, 6, and 8 mM are made by diluting aliquots of the stock solution.

Procedure

1. Add 0.05 mL (50μL) of sample or standard into test tube (blank is 50 μL of H_2O).
2. Mix with 2.5 mL of phenol reagent.
3. Add 2.0 mL of hypochlorite reagent and mix.
4. Place in 95°C H_2O bath for 5 min.
5. After cooling, read on spectrophotometer at 630 nm.

CAUTION: Wear gloves and protective clothing when running this analysis.

CHAPTER IX

Atomic Absorption and Emission Spectroscopy

Atomic absorption and flame emission spectrometry are analytical techniques developed in recent years for the determination of metallic element concentrations. Flame emission involves measurement of the intensity of radiation of an excited atom at a given spectral line, whereas atomic absorption involves measurement of light absorption by free atoms. We will briefly discuss each method and consider their application to mineral analysis.

Flame Emission Spectrometry. When a sample is sprayed into a flame, the following events occur (Fritz and Schenk, 1979):

1. The solvent is evaporated, leaving airborne particles of solids that were dissolved in the solvent.

2. Solid compounds are vaporized and partially converted into gaseous atoms.

   $Mg\ Cl_2\ (solid) \rightarrow MgCl_2\ (gas) \rightarrow Mg\ (gas) + Cl\ (gas)$

3. A small fraction of the gaseous atoms are excited by the thermal energy. When electrons in these atoms return to the ground state, they emit a photon (hv) of UV or visible light:

   $Mg\ (gas) \xrightarrow{heat} Mg^*\ (gas) \rightarrow Mg\ (gas) + hv$ (* = excited state)

4. If a constant set of conditions is maintained, the light emitted by the atoms will be proportional to the concentration of the element in the sample.

In most atoms, a number of electron transitions to higher energy states are possible, each giving rise to light of a specific wavelength or spectral line. The most prominent line, however, is that resulting from an electron returning from the lowest excited state to the ground state, and this line is usually chosen for analysis (Fritz and Schenk, 1979).

We will discuss instrumentation for both flame emission and atomic absorption later in this chapter. Now, let us consider the analytical procedure for determining the concentration of elements by flame emission. Inorganic samples are dissolved in acids as with most spectrophotometric procedures. Standard solutions containing elements of interest are prepared and aspirated into a hot flame, producing some free atoms of the element. These atoms become excited and give off light. A monochromator is set to select light of the spectral line of the element in question and the intensity of emitted light, as indicated by the current from a photomultiplier tube, is plotted against standard concentration. The unknown samples are aspirated into the flame, and their concentration is determined by reference to the standard curve. It should be noted at this point that intensity of emitted light is usually not linear with concentration in flame emission procedures, and polynomial or curvilinear regression techniques are often used to find the equation that best fits the standard data. This problem of non-linearity

is the result of self-absorption in the flame, (i.e., energy emitted by an atom is absorbed by another atom of the same element, thereby decreasing the intensity of emitted light). Self-absorption can be decreased by using dilute solutions.

There are several interferences that can influence the results of flame emission procedures. Most of these affect atomic absorption measurements also, but to different extents. Spectral interferences result from the emission of light by atoms of other elements in the sample that have spectral lines close to the element of interest. Although monochromators remove most of the problems with this type of interference, it is not uncommon to correct flame measurements for background or solvent emissions.

Ionization interference results when metal atoms become ionized and, thus, emission of light at the desired wavelength is decreased. This problem is reduced by adding the salt of an easily ionized element to the samples. An excess of electrons from the easily ionized element reverses the equilibrium of the element of interest, so very few of its atoms ionize (Fritz and Schenk, 1979).

Exitation interferences can be a considerable problem in flame emission measurements. These result from changes in the flame or other conditions that alter the number of atoms in the excited state. It is possible to use an internal standard to adjust for such variations. This involves adding (to all samples) a known amount of another element with a spectral line close to the element of interest. If the emission intensity of the internal standard changes, adjustments can be made to intensity values of the element of interest.

The final type of interference is chemical interference. This results from the formation of salts or oxides in the flame that do not form free atoms. A classic example is the effect of phosphate on calcium, in which a stable calcium phosphate complex is formed that does not emit light. This problem can be overcome by adding lanthanum oxide to samples and standards, which binds phosphate, freeing calcium for exitation in the flame. The Animal Nutrition Laboratory procedure for analysis of Ca by atomic absorption is attached to this chapter. Another chemical interference is the formation of stable metal oxides in the flame. However, oxide formation can be decreased by fuel-rich flames.

Inductively Coupled Plasma Emission Spectroscopy. A recent development in emission spectrometry has been the use of electrically generated plasmas, rather than fuel-fired flames to produce atomic emission. A plasma is a gas in which a significant number of the atoms or molecules are ionized. Because it is an electrical conductor, it can be heated rapidly by inductively coupling it to a time-varying magnetic field (Fritz and Schenk, 1979). The temperature reached by the process is two to three times that obtained in routine flame emission. The high temperature seems to overcome many of the interferences in flame emission, and spectral interferences are about the only problem with the procedure. Use of ICP allows for excellent detection limits, and when computer-coupled, 20 or more elements can be analyzed in a matter of minutes. For more information, students are referred to Fassel (1978).

Atomic Absorption Spectrometry. Atomic absorption is essentially the opposite of flame emission. As in flame emission, the sample is converted into free atoms by aspirating it into a

flame. In this case, however, a light source emitting the spectral line of the element of interest is used and free atoms in the flame absorb the light. Generally, the great majority of free atoms remain in the ground state in the flame, and are thereby capable of absorbing the light. The amount of absorption is proportional to the concentration of the element in question (i.e., Beer's Law may be applied).

As should be evident, the atomic absorption spectra of free atoms is very narrow, compared with the broad spectra of molecules in solution (Fritz and Schenk, 1979). In comparison with flame emission, atomic absorption has the advantage of fewer interferences. In fact, chemical interferences are the only significant concern in atomic absorption procedures. A comparison of the theoretical aspects of flame emission and atomic absorption spectrometry is shown in Figure 9-1.

Unknown concentrations can be calculated for atomic absorption procedures in the same manner as previously discussed with spectrophotometric measurements in Chapter VIII. A list of elements that can be analyzed by atomic absorption and flame emission, including detection limits and sensitivity to flame or atomic absorption is shown in Table 9-1.

Instrumentation. Figure 9-2 depicts the basic components of an atomic absorption/flame emission spectrometer. The primary difference between flame and atomic absorption units is the presence or absence of the light source.

The **hollow-cathode lamp** is an evacuated tube containing a noble gas, usually neon or argon. The hollow- or tube-shaped cathode is made of the element to be determined. When voltage is applied, the gas is ionized, and ions bombard the cathode (Fritz and Schenk, 1979), causing release of metal atoms that collide with gas ions. This elevates the metal atoms to an excited state. Upon fall to the ground state, the metal atoms give off light of the desired wavelength.

Generally, the light emitted by a hollow cathode tube is modulated or chopped so that the detector can distinguish it from light emitted by atoms in the flame. If this is not done, absorbance values will seem to be lower than they actually are. Modulation is accomplished by either manually chopping the light beam or alternating the current to the light source and tuning the detector to the same frequency. In either case, the constant emission of light by excited atoms in the flame will not influence the detector.

The flame is equivalent to the defined space or cuvette in general spectrophotometry. Generally, there are two types of burners: total consumption or pre-mix. Total-consumption burners do not provide a very steady flame because gas and sample enter at a single opening. This type of burner is not extensively used in newer instruments. Pre-mix burners mix fuel and sample before entering the flame, resulting in a much steadier flame than a total-consumption burner. All newer instruments use pre-mix burners. The nebulizer is simply an aspirating device to draw up the sample and convert it to a fine mist before it enters the flame.

Monochromators in atomic absorption units are essentially the same as described in Chapter VIII for spectrophotometers. The same is true for detectors, except that most units employ

photomultiplier tubes rather than vacuum phototubes. The photomultiplier tube is similar to a vacuum phototube, except that it contains a series of anodes, called dynodes, that successively amplify the current produced by incoming light. This allows very minute amounts of light to be amplified to levels appropriate for the electronics and readout system.

References

Fassel, V. A. 1978. Quantitative elemental analyses by plasma emission spectroscopy. Science 202:183.

Fritz, J. S., and G. H. Schenk. 1979. Quantitative Analytical Chemistry.

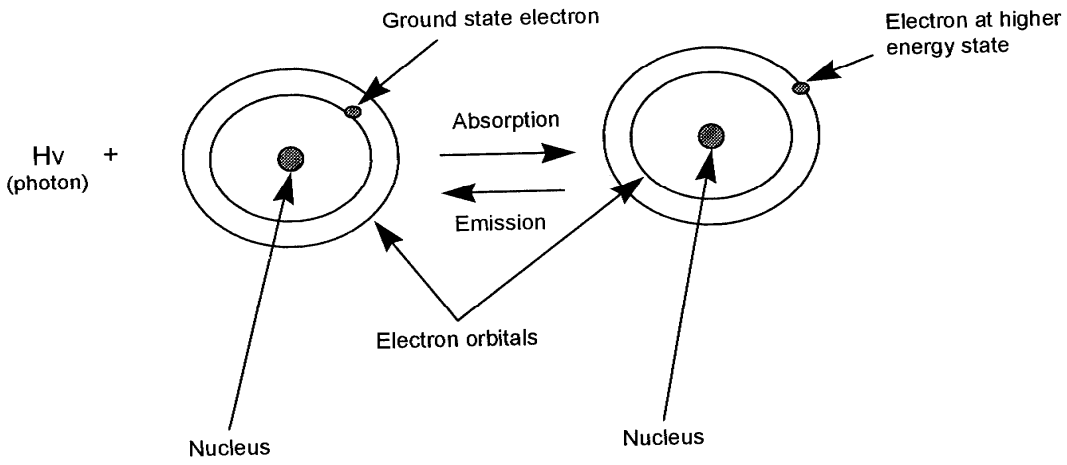


Figure 9-1. Relationship between atomic absorption and atomic emission.

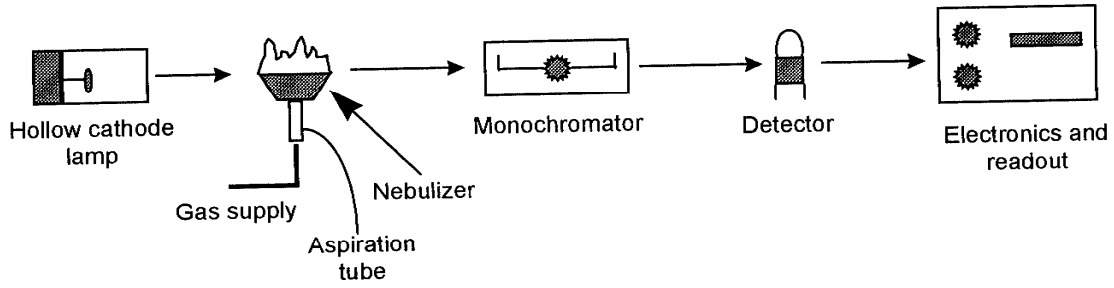


Figure 9-2. Basic components of an atomic absorption/atomic emission spectrophotometer.

Table 9-1. Detection limits of elements analyzed by atomic absorption and emission spectroscopy

Element**	Emission	Absorbance	Oxidant + Acetylene
Ag - 2	0.008	0.005	Air
Al - 3	0.05	0.1	N_2O
As - 2	12	0.2	Argon-Hydrogen
Au-2	2.6	0.02	N_2O
B - 1	0.05	6	N_2O
Ba - 1	0.006	0.05	N_2O
Be - 2	1.1	0.002	N_2O
Bi - 2	23	0.05	Air
Ca - 1	0.0005	0.002	Air
Cd - 2	9.3	0.005	Air
Ce - 1	0.76	-	-
Co - 2	0.09	0.005	Air
Cr - 3	0.004	0.005	Air
Cs - 2	0.57	0.05	Air
Cu - 2	0.028	0.005	Air
Dy - 1	0.046	0.4	N_2O
Er - 3	0.068	0.1	N_2O
Eu - 1	0.06	0.2	N_2O
Fe - 2	0.026	0.005	Air
Ga - 2	0.31	0.1	Air
Gd - 3	4.6	4	N_2O
Ge - 3	0.7	1	N_2O
Hf - 3	18.9	15	N_2O
Ha - 2	9.6	0.5	Air
Ho - 1	0.009	0.3	N_2O
In - 2	0.14	0.05	Air
Ir - 1	0.38	2	N_2O
K - 1	0.00005	0.005	Air
La - 1	0.08	2	N_2O
Li - 1	0.0002	0.005	Air
Lu - 3	1.3	3	N_2O
Mg - 2	0.07	0.005	Air
Mn - 2	0.008	0.003	Air
Mo - 2	0.5	0.1	N_2O
Na - 1	0.0006	0.005	Air
Nb - 1	1.2	5	N_2O
Nd - 1	0.74	2	N_2O
Ni - 2	0.02	0.005	Air
Os - 3	1.7	1	N_2O
Pb - 2	0.21	0.01	Air

Table 9-1 (cont.). Detection limits of elements analyzed by atomic absorption and emission spectroscopy

Element**	Emission	Absorbance	Oxidant + Acetylene
Pd - 2	0.07	0.02	Air
Pr - 1	0.069	10	N_2O
Pt - 2	7.2	0.1	Air
Rb - 2	3.4	0.005	Air
Re - 1	0.1	1.5	N_2O
Rh - 1	0.0006	0.03	Air
Ru - 3	0.3	0.3	Air
Sb - 2	-	0.2	Air
Se - 2	-	0.1	Argon-Hydrogen
Si - 2	3.1	0.1	N_2O
Sm - 1	0.23	5	N_2O
Sn - 2	0.2	0.06	Air
Sr - 1	0.0005	0.01	Air
Ta - 3	5	6	N_2O
Tb - 1	0.03	2	N_2O
Te - 2	2.4	0.3	Air
Ti - 3	0.22	0.3	Air
Th - 2	11.1	0.15	N_2O
Tl - 1	0.014	0.2	N_2O
U - 1	5.5	30	N_2O
V - 2	0.13	0.04	N_2O
W - 1	0.6	3	N_2O
Y - 1	0.6	3	N_2O
Yb - 1	0.009	0.04	N_2O
Zn - 2	16.2	0.002	Air
Zr - 2	48.2	5	N_2O

*All emission detection limits in N_2O-acetylene flame.

**Numbers beside element means: 1 = more sensitive by emission (25); 2 = more sensitive by absorption (30); 3 = equally sensitive (12).

Table courtesy of Mr. Andy Bristol, NMSU, Soil and Water Testing Laboratory.

Animal Nutrition Laboratory

Calcium Determination

Reagents: Lanthium oxide solution

Add 58.65 g of La_2O_3 to 1-L volumetric flask, **slowly** add 250 mL of HC1 under hood, dissolve completely, bring to volume with reagent grade H_20, and cap tightly.

Sample preparation: (see previous procedure)

Ash -1 to 2-g sample in muffle in duplicate
Place residue in 250-mL beaker
Add 50 mL of 1:3 HC1 (1 part HC1 to 3 parts H_20) and several drops of HNO_3; bring to boil under hood
Cool and filter into 50- or 100-mL volumetric flask that has been rinsed with dilute acid
Dilute to volume with reagent grade H_20

Standard preparation:

Place 1 mL of stock Ca solution (1,000 μg/mL) into 100-mL volumetric with Hamilton syringe, and bring to volume with reagent grade H_20. This is working solution (10 μg/mL). Place 0, 2.5, 5, 10, 20, and 30 mL of working solution into 100-mL volumetrics with acid-rinsed glass pipettes, add 20 mL of La_2O_3 solution, bring to volume with reagent grade H_20, and cap (0 serves as blank; others equate to 0.25, 0.5, 1, 2 and 3 μg/mL standards).

Unknown preparation:

Place a predetermined aliquot* of unknown (ashed sample in solution) into 8-mL culture tube with Hamilton syringe, add 1 mL of La_2O_3 solution and enough H_20 to result in a total volume of 5 mL, and cover tubes

Read standards and unknowns on atomic absorption spectrophotometer (refer to instrument manual for wavelength, etc.). Results are expressed in concentration (mg/mL).

*Calculation of aliquot to use:

Estimate Ca content of unknown

Example:
If there is 0.7% Ca in sample, and a 1-g sample is used and brought to 100-mL volume, then 1 g has 0.007 g of Ca (0.007 x 1 g), and 0.007 g = 7 mg of Ca
7 mg/100 mL = 0.07 mg/mL = 70 μg/mL
70 μg/mL x 0.1 mL = 7 μg (in this aliquot)

7 μg/5 mL = 1.4 μg/mL in sample to be read. This falls in the readable range of the standards, thus, 0.1 mL (100 μL) is an appropriate aliquot. **Note**: the amount of H_2O to bring to 5 mL would be 3.9 mL. **Note**: if the sample size is larger (i.e., 2 g) or brought to volume in 50-mL volumetric, Ca will be more concentrated, and a smaller aliquot would be appropriate.

Calculations:

AA reading yields μg/mL in tube.

Multiply this value by 5 to determine the quantity of Ca in the aliquot.

Divide volume of ash in solution by aliquot taken from that solution:
Example – 100/0.1 = 1,000

Multiply amount of Ca in aliquot by this factor to determine quantity of Ca in volumetric flask.

Divide by dry sample weight:
(Total μg/g of dry sample x $1g/10^6$ μg) x 100 = % Ca, dry matter basis.

CHAPTER X

Evaluation of Cereal Grains

Vast quantities of cereal grains are used in the feeding of all classes of livestock. In the U.S., the two major feed grains are corn and sorghum, whereas barley, oats, and wheat are of considerable importance in certain regions of the U.S. and in European countries. With nonruminants like poultry and swine, most grain is simply ground before to feeding, but with ruminants, grain may be fed whole, finely or coarsely ground, or processed by a variety of methods. Because of this variety in processing techniques and the effects processing can have on animal performance, laboratory evaluation of processed grains has become increasingly important.

It is generally accepted that grain processing improves utilization compared with unprocessed grain. This is almost always true with sorghum grain, but may not be the case with barley, wheat, and corn. The effect of processing on a particular grain depends on the type and extent of processing. Numerous types of processing are available, including high moisture harvesting and ensiling, reconstitution, steam flaking, popping, micronizing, grinding, and cracking. Wagner et al. (1973) provide an excellent review of different processing methods, and portions of their review have been reproduced in Table 10-2. Expected performance responses in cattle fed processed corn grain has been summarized by Henderson and Geasler (1971), and portions of their summary are shown in Table 10-3. Another excellent review of cereal processing can be found in a 1972 text, published by the U.S. Feed Grains Council, entitled "Cereal Processing and Digestion."

To fully understand how processing affects utilization of cereal grains, it is necessary to understand the nature of cereal grain starch. Figure 10-1, adapted from Armstrong (1972), and Figure 10-2 depict the various parts of a cereal grain and the proportions of various components. Most cereal grains contain from 60 to 80% starch, primarily in the endosperm. This starch is a mixture of amylose and amylopectin, with amylopectin predominating in most grains. Amylose is a straight-chain polymer of glucose in α-D-(1$\rightarrow$4) linkages. Amylopectin, on the other hand, has short α-D-(1$\rightarrow$6) linked branch points, resulting in a highly branched or tree-like polymer. Armstrong (1972) indicates that amylose has a degree of polymerization of 1,000 to 2,000 D-glucose units, whreas the length of a unit chain in amylopectin is 19 to 26 D-glucose units. Most cereal grains contain from 22 to 28% amylose, but flint corn and waxy varieties of both corn and sorghum contain virtually no amylose, being comprised almost entirely of amylopectin.

Starch occurs in the endosperm as discrete, microscopic granules, held closely together with protein filling the intergranular spaces (Armstrong, 1972). The starch granule is characterized by a central crystalline region or crystallite, in which parallel polymer chains are held closely together by hydrogen bonding. Extending outward from the crystalline region is an amorphous mass of polymer chains held together by fewer hydrogen bonds. Because of the central crystalline region in an amorphous mass, native starch granules express the property of birefringence. This property can be observed when the granule is viewed under a microscope with polarized light. The crystalline region and the amorphous mass reflect light in different directions, resulting in a dark interference or Maltese cross. Loss of crystallinity results in loss

of birefringence.

When starch is heated in water, granules swell and eventually, crystallinity of the granule is lost. The temperature, or range of temperatures, at which swelling and loss of crystallinity are irreversible, is called the gelatinization temperature. Thus, gelatinization is equivalent to loss of crystallinity in starch granules. Heat and moisture processing methods like steam flaking result in extensive gelatinization, and because the temperature (~100°C) used in such methods is considerably greater than gelatinization temperature, extensive rupture of starch granules can occur. Other processing methods like micronizing and popping apply heat and rely on internal moisture in the grain for completion of the gelatinization process, rather than adding moisture to the grain. Typical gelatinization temperatures for a number of grains are shown in Table 10-1, adapted from Armstrong (1972).

Gelatinization of starch is significant, in that cooked or gelatinized starch is usually more digestible than raw starch. In addition, gelatinized starch is more digestible by ruminal microorganisms, resulting in a greater availability of energy to cattle fed processed grains. The effect of processing seems to be greater in cattle than sheep, primarily because sheep masticate or grind their feed more finely than cattle, and particle size reduction compensates for processing effects. Moreover, extensive processing seems to be of limited value for swine and poultry, with fine grinding being the most common method of processing.

High moisture processing results in similar improvements in animal performance to heat/moisture processing; however, very little, if any, gelatinization occurs in high moisture grains. It seems that solubilization of the protein matrix surrounding starch granules is extensive during high moisture storage, resulting in greater accessibility to starch granules by ruminal microorganisms. Solubilization of nitrogen in high moisture grains has been demonstrated by Prigge et al. (1976).

A number of techniques have been developed to evaluate processed grains. Evaluation is important, because degree of processing may differ both within and between methods. For example, steam flaked grains can vary considerably in degree of gelatinization, as a result of roller and temperature settings used in the flaking process. Generally, density or bushel weight is a reasonable index of degree of processing with flaked grains; lighter bushel weights being associated with greater degrees of gelatinization. In addition, in high moisture grains, solubilization of nitrogen may vary, depending on moisture level of the grain at the time of ensiling. These and other evaluation techniques will be described in following paragraphs.

Particle Size. The surface area available for digestion influences both rate and extent of digestion. This is especially true in nonruminants, and in the case of rate of digestion, is also true for ruminants. Extent of digestion in ruminants may not be influenced to as great an extent by particle size as in nonruminants because rate of passage or retention time may compensate somewhat for larger particle size. Thus, for ground or cracked grains, an evaluation of particle size is meaningful.

Most procedures for evaluating particle size involve using a known weight of grain and shaking it through a set of sieves with discrete screen sizes. Then the percentage of sample

remaining on a particular sieve is evaluated through an index or calculation method. The procedure of Ensor et al. (1970) describes a method for calculating the geometric mean diameter of a feedstuff.

Gelatinization. Loss of crystallinity can be measured microscopically as loss of birefringence (Schoch and Maywald, 1956) or by enzymatic methods. Sung (1960) reported a method in which starch was incubated with β-amylase. Milligrams of maltose released per gram of grain during the incubation is an index of amount of gelatinized starch.

Additional techniques have been developed that do not measure gelatinization directly but give an indication of degree of starch damage. Croka and Wagner (1975) describe a procedure in which grain is incubated with amyloglucosidase and yeast. The enzyme breaks down grain starch to glucose, and the yeast ferments the glucose to CO_2. Gas production is measured as an index of starch availability. Generally, gelatinized grains have greater gas production values than unprocessed grains. High moisture grains also have greater values, however, so the method may be considered to give an index of starch availability, rather than gelatinization per se. Other gas production methods involving ruminal microorganisms rather than enzymes have also been employed (Trei et al., 1970). More recently, Xiong et al. (1990) described an enzymatic technique in which processed grain samples were incubated with amyloglucosidase and glucose release was used to assess starch availability; this procedure is included with this chapter.

Other Methods. In vitro ruminal digestibility estimates are often used in grain evaluation, and methods generally follow those outlined in Chapter VIII. One typical modification, however, is the elimination of the second stage-pepsin digest.

Although not useful in describing processing effects, analysis of the starch content of grains is quite common. A useful procedure, patterned after MacRae and Armstrong (1968) is provided with this chapter.

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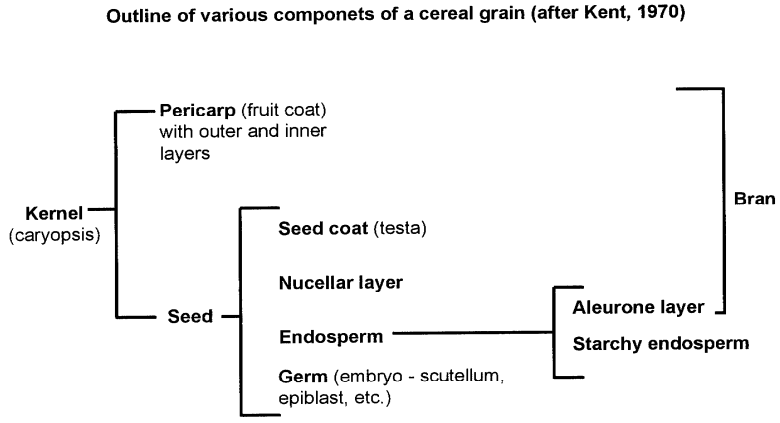

Proportions of various components (g/100 g)

	Oats		Barley				
	Whole grain	Kernel	Whole grain	Kernel	Wheat	Corn (flint)	Sorghum
Hull	25.0		13.0				
Pericarp			2.9	3.3	8.2	6.5	
and testa	9.0	12.0					8.0
Aleurone			4.8	5.5	6.7	2.2	
Endosperm	63.0	84.0	76.2	87.6	81.5	79.6	82.0
Germ	2.8	3.7	3.0	3.4	3.6	11.7	10.0

Figure 10-1. Adapted from Armstrong (1972)

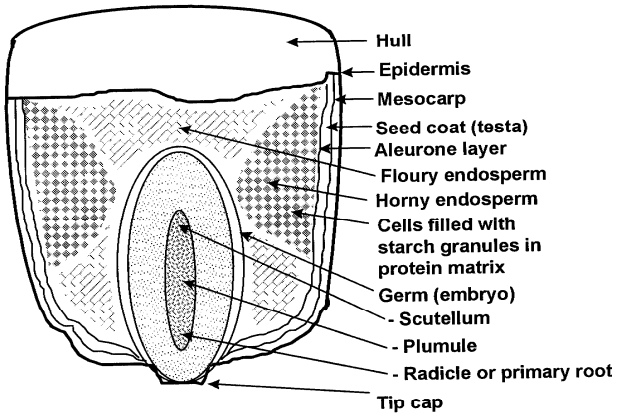

Figure 10-2. Longitudinal section of a corn kernel. Adapted from Feed Management Vol. 36, No. 7 (1985).

Table 10-1 The amylose contents and gelatinization temperature range of whole granular cereal starches.

Grain	Amylose, % in starch	Gelatinization temperature range, ^{o}C
Barley	22	59 to 64
Maize	28	62 to 72
Oats	27	-
Wheat	26	65 to 67
Maize (flint)	1	66 to 69
Sorghum	25	67 to 77
Maize gene combinations		
ae (amylomaize)	61	92*
du, wx	0	74
wx	71	71

*Birefringence end-point temperatures represent the higher of the two temperatures describing the gelatinization temperature.

Adapted from Armstrong (1972).

Table 10-2. Methods of grain processing (from Wagner, D. G., R. Totusek, and D. R. Gill, 1973.

Grain processing for feedlot cattle. Cooperative Extension Service, Great Plains Beef Cattle Feeding Handbook, GPE-2000).

Method	Description
Grinding	Usually done with hammermill. Grain ground to varying degrees of fineness. Screen size, hammermill size, power, and speed, as well as type of grain and moisture content influence final product.
Dry rolling	Grain passed through a set of grooved rollers. Particle size varies from coarse to fine and is influenced by roller weight, pressure and spacing, moisture content of grain, and rate of grain flow.
Steam rolling	Grain exposed to steam for 1 to 8 min before rolling. Also known as crimping or steam crimping. Moisture content of grain increased slightly. Offers little or no advantage in feed efficiency over grinding or dry rolling.
Pelleting	Grain is ground or rolled before steam treatment and processing through a pelleting mill. Improves feed efficiency, but benefits seldom cover pelleting cost.
Steam flaking	Grain subjected to steam under atmospheric pressure for 15 to 30 min and rolled through large, heavy rollers set at near zero tolerance. Thin, flat flake with a bushel weight of 22 to 28 pounds and moisture level of 16 to 20% is produced. Starch is gelatinized and more digestible.
Pressure flaking	Grain subjected to steam under about 50 psi for 1 to 2 min. Grain is then rolled, producing a product similar to steam flaking.

Table 10-2 (cont).

Method	Description
Popping	Air-dry grain is popped by heating it with high temperature (700 to 800°F) for 15 to 30 seconds. Product has a moisture content of approximately 3% and usually must be rolled and remoisturized.
Micronizing	Dry grain is heated with gas-fired infrared generators as the grain passes along an oscillating steel plate. Grain is then dropped into knarling rolls, producing a flake-like product. Density of the product ranges from 18 to 30 pounds per bushel.
Exploding	Grain is fed into high tensile strength steel bottles. Live steam is injected into the bottles until the pressure reaches 250 psi. After approximately 20 sec, a valve opens to let the grain escape as expanded balls with the hulls removed.
Extruding	Grain processed in extruding machine that applies heat and pressure as grain passes through a tapered screw. Process produces ribbons that break into small flakes.
Roasting	Grain passed through a roaster of the same type as used for soybeans. Grain heated to approximately 300°F and has oily, puffed, slightly carmelized appearance.
High moisture harvesting	Grain harvested at ~ 30% moisture, stored as ground product in trench-type silo, or in whole form in an oxygen-limited silo. Does not result in gelatinization, but increases soluble nitrogen content of the grain.
Reconstitution	Dry grain reconstituted to a moisture level of 25 to 30% and stored whole in an oxygen-limited silo for at least 20 d before use. Usually rolled or ground before feeding.

Table 10-3. Performance by cattle fed processed grains (from H.E. Henderson, and M.R. Geasler, 1971. Physical preparation of grain for feedlot cattle. Coop Ext. Service, Michigan State Univ. AH-BC-71).

A. Whole vs. ground, rolled, or cracked corn (summary of 13 trials)

	Average daily gain, lb	
% Concentrates	Whole	Ground, rolled, or cracked
Under 70%	2.60	2.73
70 to 80%	2.75	2.72
Over 80%	2.46	2.37

B. Whole vs. rolled high moisture corn (summary of six trials - corn varied from 40 to 70% of dry matter).

Average daily gain, lb			Feed efficiency		
Whole	Rolled	% Change	Whole	Rolled	% Change
2.09	2.11	+.1	8.32	7.72	+7.2

C. Steam flaked vs. ground, cracked, or rolled corn in high-concentrate diets (summary of nine trials).

Average daily gain, lb			Feed efficiency		
Control	Steam flaked	% Change	Control	Steam flaked	% Change
2.50	2.51	-	8.31	7.70	+7.3

D. Reconstituted whole corn vs. dry whole corn (summary of six trials).

Average daily gain improved by 2%

Feed efficiency improved by 4%

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Enzymatic Method for Estimating Starch Availability

Apparatus:

A. Thermostatically controlled water bath
B. Spectrophotometer set at 420 nm

Reagents:

A. *Buffer Solution*- Dissolve 3.7 mL of glacial acetic acid and 4.1 g of anhydrous sodium acetate in approximately 100 mL of H_2O; bring to 1L in distilled water and adjust the pH by adding acetic acid or sodium acetate, if necessary, to 4.5 ± .05.

B. *Enzyme Solution*- Dissolve 750 mg of amyloglucosidase (Sigma # A-7255, containing 12,100 unit/g) in 50 mL of distilled water. The solution contains 15 mg of crude enzyme or 180 units/mL. Prepare on day of assay. **(Adjust accordingly for enzyme containing different units/g)**.

C. *Glucose Determination Kit –* Glucose Liqui-UV Test (Endpoint) from Stanbio Laboratory (Boerne, TX). Reference # 1060-430.

D. *Glucose Standard* – Dissolve 100 mg of pure glucose in 70 mL of distilled water and dilute to 100 mL. Prepare on day of assay. **Stock solution alternative – Prepare a 0.2% (wt/vol) benzoic acid solution by adding 1 g of benzoic acid to a 500-mL volumetric and bring to volume with distilled water. Add 50 g of glucose to a 500-mL volumetric and bring to volume with the 0.2% benzoic acid solution. This stock solution will last approximately 4 mo when stored in a refrigerator**.

Procedure:

1. To 0.2 g of finely ground (1-mm screen), air-dried grain sample add 15 mL of buffer solution and then 1 mL of enzyme solution in a 25- or 50-mL graduated test tube and incubate in a 40°C water bath for 1 h. Shake the tubes initially and every 15 min during incubation.

2. At 1 h, add 2 mL of 10% $ZnSO_4 \cdot 7H_2O$, mix and then add 1 mL of 0.5 N NaOH. Dilute to 25 mL with deionized water, mix, and filter (Whatman # 40) with gravitational flow funnels in a funnel rack. **This allows many samples to be filtered in a short period of time. Care must be taken, however, to keep each tube with its corresponding funnel and filtrate. It is very important to bring every tube to exactly the same volume. Therefore, bring to 25 mL by pipetting 6 mL of distilled water to each tube with an Oxford macro-pipette.**

3. Prepare working reagent for the Stanbio kit by mixing 5 parts of buffer (R1) with 1 part of enzyme (R2). Store at 4°C and protect from light. Working reagent is stable for 90 d. Allow to equilibrate to room temperature before using.

4. Create a 96-well plate map including samples, starch, glucose standard(s), and blanks in duplicate wells.

5. Pipette 5 µL of sample, starch, and glucose standard (100 mg/dL; provided with the Stanbio kit) in duplicate into a 96-well plate.

6. Add 100 µL of working reagent to each well using a multi-channel pipette.

7. Shake plate in the plate reader for 20 s at 600 rpm, and incubate at 37°C for 5 min.

8. Read absorbance at 340 nm.

9. Calculate glucose concentration as follows: Glucose, mg/100 mL = Au/Ac x 100, where Au is the absorbance of the blank-corrected unknown and Ac is the absorbance of the blank-corrected calibrator (100 mg/dL glucose standard).

10. This method is linear in the range of 0 to 500 mg glucose/dL. Dilution is required for samples with glucose concentration exceeding method linearity.

11. Alternatively, include a series of standards ranging from 0 to 500 mg of glucose/dL (e.g., 0, 100, 200, 300, 400, 500 mg/dL) and determine the glucose concentration by linear regression from the standard curve.

NOTE: To express starch availability as degree of gelatinization, 0, 50, 100, 150, and 200 mg of unprocessed ground grain samples from the same source are mixed with 15 mL of buffer solution in 25 mL graduated test tubes. The tubes are then put in boiling water for 1 h. After boiling, to the respective tubes add 200, 150, 100, 50, and 0 mg of unprocessed ground grain, and mix. These mixtures represent 0, 25, 50, 75, and 100% gelatinization, respectively. Obtain standard curve for degree of gelatinization by following Steps 1 to 6.

Animal Nutrition Laboratory

Determination of Starch in Feed, Digesta, and Feces

Adapted from MacRae and Armstrong (1968)

Reagents:

1. Enzyme

    a. Amyloglucosidase (Sigma # A-7255, containing 12,100 units/g) in 50 mL of distilled water. The solution contains 15 mg of crude enzyme or 180 units/mL. Prepare on day of assay – Adjust accordingly for enzyme containing different units/g.

OR

    b. Glucoamylase (833 GAU/g) dissolve 0.3g in 50 mL of distilled water. Prepare on day of assay.

2. 0.2 *M* acetate buffer solution, pH 4.5

    a. For 2 L – Add 20.4 g of sodium acetate trihydrate and 250 mL of a 1.0 *M* stock solution of acetic acid to volumetric flask and dilute to 2 L with deionized water.

    b. To prepare 1.0 *M* acetic acid stock solution, add 57.46 mL of glacial acetic acid to volumetric flask and dilute to 1 L with deionized water.

3. Glucose determination kit: Glucose Liqui-UV Test (Endpoint) from Stanbio Laboratory (Boerne, TX). Reference # 1060-430.

Equipment:

1. Autoclave or boiling water bath to gelatinize starch samples
2. 125-mL Erlenmeyer flasks
3. Eppendorf or Hamilton pipets
4. Balances – top loading and analytical
5. 96-well plates (flat bottom)
6. Microplate spectrophotometer.

Procedure:

1. Weigh sample and add to 125-mL Erlenmeyer flask (0.2 g feed, 0.5 g ingesta, and 1.0 g feces)

2. Weigh flask and contents (top loading balance to 1 decimal place is adequate)

3. Gelatinize sample with either of the following methods:

a. Add 50 mL of deionized water and autoclave at 124ºC for 90 min. Place on slow exhaust (a cycle that permits cooling without loss of fluid). Cover with aluminum foil or a rubber stopper.

OR

b. Add 50 mL of deionized water and boil at 100ºC for 90 min. After boiling, cool flasks in cold tap water.

4. The flasks should be cooled to room temperature before adding buffer and enzyme.

5. Add 50 mL of acetate buffer solution and 1 mL of enzyme solution (or other quantity adjusted for enzyme concentration).

6. Remove aluminum foil or stopper and reweigh flask and contents.

7. Cover flask with aluminum foil or stopper Incubate flask and contents at 60ºC for 24 h.

    a. Remove aluminum foil or stopper and reweigh flask and contents.

8. Cool to room temperature.

9. Mix the sample and let settle before taking an aliquot (1 or 2 mL is sufficient) to determine glucose concentration. At this point aliquots can be stored in the refrigerator to continue with glucose determination next day.

    a. Prepare working reagent for Stanbio kit by mixing 5 parts of buffer (R1) with 1 part of enzyme (R2). Store at 4°C and protect from light. Working reagent is stable for 90 d. Allow to equilibrate to room temperature before using.

    b. Create a 96-well plate map including samples, starch, glucose standard(s), and blanks in duplicate wells.

    c. Pipette 5 µL of sample, starch, and glucose standard (100 mg/dL; provided with the Stanbio kit) in duplicate into a 96-well plate.

    d. Add 100 µL of working reagent to each well using a multi-channel pipette.

    e. Shake plate in the plate reader for 20 s at 600 rpm, and incubate at 37°C for 5 min.

    f. Read absorbance at 340 nm.

    g. Calculate glucose concentration as follows:

        Glucose, mg/100 mL = Au/Ac x 100, where Au is the absorbance of the blank-corrected unknown and Ac is the absorbance of the blank-corrected calibrator (100 mg/dL glucose standard).

h. This method is linear in the range of 0 to 500 mg glucose/dL. Dilution is required for samples with glucose concentration exceeding method linearity.

i. **Alternatively**, include a series of standards ranging from 0 to 500 mg of glucose/dL (e.g., 0, 100, 200, 300, 400, 500 mg/dL) and determine the glucose concentration by linear regression from the standard curve.

Calculations:

1. α-linked glucose polymers = GC x (V/100) x (1/W)
    a. α-linked glucose polymers are in units of mg/g
    b. GC = glucose concentration in mg/100 mL
    c. V = flask volume in milliliters assuming unit density (weight at Step 6 minus weight at Step 2). When weights at Step 6 and Step 8a are greatly different decrease volume (V) by difference (weight at Step 6 minus weight at Step 8a)
    d. W = sample dry weight

2. % starch = ([α-linked glucose polymers, mg/g]/1,110) x 100

CHAPTER XI

The Use of Indigestible Markers in Nutrition Studies

General. As discussed in Chapter VII, digestibility and intake of feedstuffs have a considerable impact on the performance of livestock. However, conventional trials for determination of digestibility and intake are time consuming and expensive, and under certain conditions, may not provide valid estimates of these measurements. One important example of a situation in which conventionally determined values may be suspect is the grazing ruminant. A conventional approach to determining digestibility and voluntary intake of grazed forages would be to harvest the forage and feed it to penned animals. Unfortunately, this method ignores the animal's ability to select specific plants or portions of plants while grazing. Thus, harvested forage may not be representative of the forage actually consumed by the grazing animal. An excellent discussion of this selectivity problem is found in the review of Harris et al. (1967).

Because of such problems, indigestible markers or reference substances have been employed extensively in grazing research for determination of digestibility and intake. In addition, markers can be used to determine the rate of passage of nutrients through the gastrointestinal tract, site and extent of digestion, and microbial protein synthesis in ruminants.

Many excellent review articles are available on the use of indigestible markers and attempts will be made in this chapter to cite several references for students who desire additional information. The principal objective of this chapter, however, is to briefly discuss situations in which markers can be used and illustrate their use by examples and calculations.

The Ideal Marker. The requirements for an ideal marker have been listed by Faichney (1975) and can be briefly stated as follows:

(1) Should be inert, with no toxic physiological effects on the animal or microflora.
(2) Should not be absorbed or metabolized within the gastrointestinal tract.
(3) Should be physically similar to or intimately associated with the material it is to mark.
(4) Should not influence gastrointestinal secretion, digestion, absorption, or motility.
(5) Should have physiochemical properties that allow for precise, quantitative analysis, and it must not interfere with other analyses.

Unfortunately, none of the markers currently in use satisfy all these criteria. However, with proper selection of a marker, based on specific experimental conditions, effective measurements can be made. For example, chromium sesquioxide can be used to measure digestibility, even though it does not remain intimately associated with either the particulate or fluid fractions of digesta because it is only necessary that it be fully recoverable (i.e., non-absorbable) for digestibility measures. It could not, however, be used as an index of digesta flow rate (Faichney, 1975).

A number of markers are presently used in nutrition studies. These can be divided into **internal** and **external** markers. Internal markers are integral components of the feedstuff, and lignin is the most widely used. Recently, there has been considerable interest in the use of acid

insoluble ash as an internal marker. Indigestible ADF and NDF also have been studied (Waller et al., 1980) and seem to have considerable promise. Procedures for measurement of these two markers are included at the end of this chapter. Students are referred to Thonney et al. (1979) and Van Keulen and Young (1977) for more information on acid insoluble ash as an internal marker. Harris et al. (1967) give information relative to lignin as an internal marker.

External markers are indigestible substances added to a feedstuff. Chromium sesquioxide (Cr_2O_3) is chief among external markers, but several other markers including cerium, dysprosium, ytterbium, and a ruthenium phenanthroline complex have received considerable attention. Many of the rare-earth element markers like Yb are made to bind with a specific feed, and as such, are quite useful in determining flow rates of particular feed ingredients. We will not attempt to discuss each of these markers in detail, but will indicate which markers are of value as we discuss applications.

A final type of marker deserves some consideration. In the case of ruminants, it is often desirable to determine the quantity of microbial protein synthesized with various diets because microbial protein makes a significant contribution to the nitrogen needs of ruminants. It is possible by using markers mentioned above, to determine the total protein passing to the lower tract in ruminants; however, it is then necessary to determine what portion of the total protein is of microbial origin. This can be estimated by using a number of markers specific to bacteria, including diaminopimelic acid (DAP), ribonucleic acid, and purines. Diaminopimelic acid is thought to be found only in bacteria, so by determining the DAP content of digesta and knowing the DAP:N ratio in bacteria, one can estimate the portion of nitrogen in the digesta of microbial origin. In the case of RNA, it is assumed that feed RNA is broken down in the rumen and only RNA of bacterial origin passes to the lower tract. Again, when the RNA:N ratio in bacteria is known, the microbial contribution to total protein in the digesta can be determined by measurement of digesta RNA concentrations. Other such markers have been described in the literature, and Stern and Hoover (1979) provide an excellent review of the subject. Students should keep in mind that DAP and RNA are not indigestible markers, but serve only to aid in determination of the microbial protein content in ruminant digesta.

Use of Markers to Estimate Digestibility. Now let us move on to specific applications of indigestible markers. Estimates of total tract or specific site digestibility are possible with many of the markers described above. The digestibility of a given nutrient can be determined by application of the following formula:

$$\text{Nutrient digestibility, \%} = 100 - 100 \times \left(\left[\frac{\text{\%marker in feed}}{\text{\%marker in feces}} \times \frac{\text{\%nutrient in feces}}{\text{\% nutrient in feed}} \right] \right)$$

This equation can be used with both internal and external markers. For example, lignin could be used as an internal marker and percentage of lignin in feed and feces could be determined, as well as the percentage of the nutrient in question. This method allows digestibility to be calculated without total collection of feed and feces; that is, grab samples of feed and feces can be used for analyses. An example of this calculation with lignin is given in Table 11-1. Similarly, acid insoluble ash, indigestible ADF, or indigestible NDF could be used.

Chromic oxide is probably the most widely used external marker for this particular application, with a known percentage of Cr_2O_3 being incorporated into the feed.

Estimates of digestibility in specific sites of the digestive tract also can be obtained using a slight modification of the equation presented above. For example, suppose a steer is fitted with a cannula in the duodenum, which allows samples to be collected as they pass out of the rumen. In this case, the equation used before can be rewritten as:

$$\text{Ruminal nutrient digestibility, \%} = 100 - 100 \times \left(\left[\frac{\text{\%marker in feed}}{\text{\%marker in duodenum}} \times \frac{\text{\%nutrient in duodenum}}{\text{\% nutrient in feed}} \right] \right)$$

An example of this calculation using Cr_2O_3 as a marker is given in Table 11-2. Lignin and indigestible ADF or NDF also could be used in this application. At present, other markers like cerium, ytterbium, and ruthenium are more frequently used for rate of passage studies than for site of digestion markers.

Before we move on to another application, it would likely be beneficial to look more closely at the mechanics of measuring digestibility with markers, rather than depending on an unquestionable faith in a series of equations. This can be done with the aid of a few schematics. First, consider the schematic in Figure 11-1:

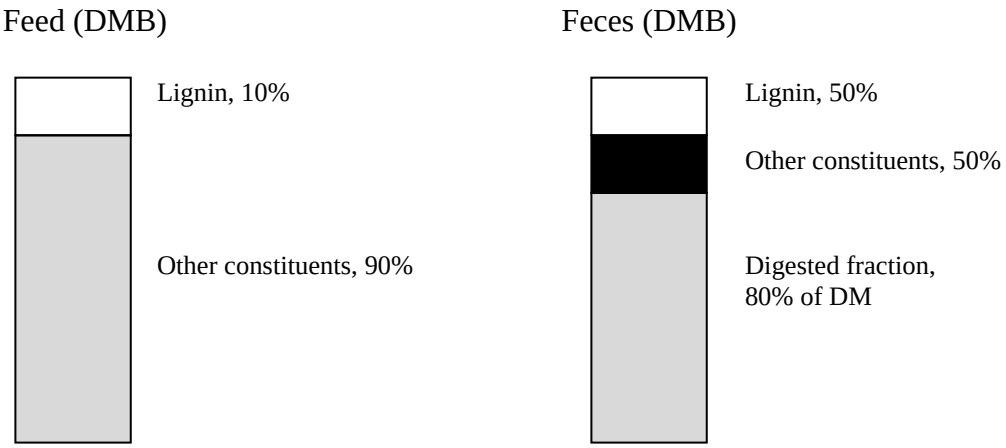

Figure 11-1. The use of lignin as a marker to estimate dry matter digestibility of a feed.

If lignin is indigestible, there is still the same amount of lignin in the feces as was in the feed (i.e., 10%). Lignin is in a 1:1 ratio with other constituents in the feces. Thus, there must be 10% lignin, 10% other and 80% of the feces that was digested, if we are to be able to reconstruct feed dry matter. This same approach can be applied to the digestibility of specific nutrients, as illustrated in Figure 11-2 with protein.

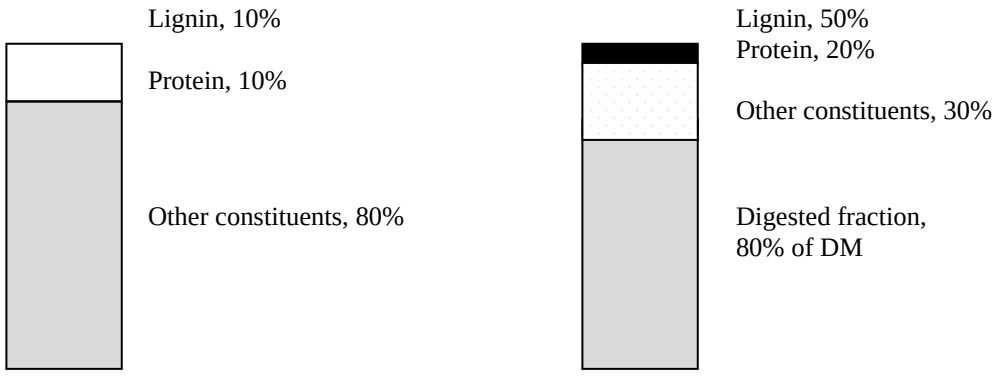

Figure 11-2. Digestibility of protein as determined by the lignin-ratio technique.

In this example, we have the same feed as above. Again, lignin is indigestible, so there is the same amount in feces as there was in the feed. The ratio of lignin to protein in the feed is 1:1, whereas in the feces, the ratio is 5:2. This means that three parts out of the original five parts of protein (i.e., 5:5 ratio = 1:1 ratio in feed) have been digested and two parts remain in feces. This means that 3/5 or **60%** of the protein was digested. Alternatively, we know that 80% of the dry matter was digested and 20% was not digested. Protein makes up 20% of this undigested dry matter. Thus, 20 x 0.2 = 4% of the original 10% feed protein remains in the feces. Thus, if we calculate protein digestion as (10 - 4)/10, we derive a value of 60% protein digestion.

Both examples shown in Figures 11-1 and 11-2 can be checked with the equations given previously. It is hoped that these examples will illustrate the principles behind the method more fully than the equations alone. Church (1975) gives additional examples of using markers to estimate digestibility.

Use of Markers to Measure Intake. As we have discussed before, intake has a significant effect on the economy of livestock production. Intake of the grazing ruminant is difficult to measure by conventional means, since harvested forage may not be representative of forage actually grazed. Thus, researchers have applied indicator methods to estimate intake.

When fecal excretion and digestibility of the diet are known, intake can be readily calculated. It is given in this calculation that the quantity of fecal excreta produced by an animal is a function of intake and indigestibility of a particular diet. This expression can be written in equation form as:

Fecal output, g = DM intake x % DM indigestibility

Rearranging this equation, one can solve for dry matter intake as follows:

Dry matter intake, g = Fecal output, g/% Dry matter indigestibility

Now the problem becomes one of estimating fecal output and digestibility. Several methods can be used to estimate digestibility, including the use of markers as described previously,

harvesting forage and feeding it in a conventional digestion trial, collecting grazed forage via an esophageal cannula and feeding it in a conventional digestion trial, or collecting grazed forage via an esophageal cannula and determining its in vitro dry matter digestibility.

Fecal output is usually estimated with indigestible markers, but can be determined by fitting grazing animals with fecal collection bags and conducting a total collection of feces (Harris et al., 1967). Chromic oxide is probably the most frequently used marker to estimate fecal output, and it is normally administered as a bolus twice daily (e.g., 0800 and 1700) for a preliminary period of approximately 7 d, followed by a collection period of approximately 5 d. During the collection period, fecal "grab samples" are obtained via rectum at the same times the boluses are given. Chromic oxide can then be determined by wet ashing fecal samples followed by atomic absorption or spectrophotometric measurement of chromium content. Fecal output is then calculated from the following formula:

Fecal output, g = (Indicator consumed, g/d)/(Indicator concentration in feces,g/g of DM)

A spectrophotometric procedure for the determination of chromic oxide has been reported by (Kimura and Miller, 1957). Connor et al. (1963), stress the importance of adding fecal ash to chromic oxide standards in this procedure, to avoid overestimation of fecal excretion. An example calculation of forage intake using Cr_2O_3 as a marker is given in Table 11-3.

A potentially significant problem in determination of forage intake with Cr_2O_3 or similar markers is the diurnal excretion pattern of such markers. That is, fecal excretion is inconstant, or variable throughout the day. As such, fecal excretion of a marker may not be constant during the day and over- or under-estimation of chromium concentration in fecal grab samples can occur. Nevertheless, a large amount of data on forage intake has been collected by this method and the technique seems quite useful, especially when relative, rather than quantitative, estimates of intake are desired. Church (1975) provides a more detailed discussion of diurnal variation in fecal excretion in Chapter 8 of his text.

Use of Markers to Measure Rate of Passage. Rate of passage of particulate and fluid materials through the digestive tract is of considerable importance in livestock production. In ruminants, fluid flow rate, or more specifically fluid dilution rate (percentage of fluid volume leaving the rumen per unit time) has been shown to be related to the efficiency of microbial growth (Owens and Isaacson, 1977). Generally, the faster the dilution rate, the more efficient is microbial growth.

Rate of passage of particulate material from the rumen can have a significant effect on intake and digestibility. Grinding and pelleting of forages tends to increase rate of passage, allowing for an increased voluntary consumption of feed, as bulk fill no longer limits intake. At the same time, digestibility is often decreased by grinding and pelleting forages because particles have less opportunity to undergo fermentation. Moreover, changes in digestibility may effect rate of passage. If feed is fermented rapidly to small particles, rate of passage may increase, and subsequently, intake will increase.

Rate of passage of specific nutrients can also be calculated. This is an alternative to

partitioning digestibility as described previously, in that the passage of a specific nutrient from the rumen or other organ could be used to "back calculate" digestibility when intake is known.

Several markers are available to measure the rate of fluid passage from the rumen. Perhaps the most commonly used are chromium EDTA, cobalt EDTA, and polyethylene glycol (PEG). Methods for preparation of CrEDTA and CoEDTA are included with this chapter. Faichney (1975) provided an excellent review of how these markers can be applied to the estimation of flow rates throughout the gastrointestinal tract. One method involves continuous infusion of the marker with time sequence sampling. For example, the marker could be given in the diet or directly infused into the rumen until an equilibrium point had been reached. Equilibrium can be defined as the point when marker concentration at any sampling point is constant. When equilibrium has been reached, samples can be taken at several intervals and combined. Numerous samples are taken in order to overcome the effects of individual "nonrepresentative" samples. The flow rate of digesta past a given sampling point can be calculated as:

Flow rate at a sampling site = Infusion rate/Marker concentration at sampling point

For example, if 10 g of PEG are infused into a sheep and the marker concentration at the abomasum is 0.01 g/mL, then the flow rate past the abomasum (from the rumen) would be calculated as 10 g/d/0.01 g/mL = 1,000 mL/day. The flow rate of a given constituent of the fluid, could be calculated by multiplying the flow rate of the fluid by the concentration of the constituent in the fluid at the sampling site. This method is quite useful; however, it does not allow the volume of a specific pool to be calculated, simply the flow rate.

A means by which both flow rates and volume can be calculated is the single-dose method with time sequence sampling. In this technique, a known amount of marker is dosed into a particular segment of the digestive tract, and its concentration in digesta is determined by sampling at time periods following the dose. If, for example, one wished to determine the fluid volume and outflow rate of the rumen, CrEDTA could be administered through a ruminal cannula and ruminal fluid samples taken at periods following the dose. Rumen volume is assumed to remain constant, with inflow equal to outflow. When the Cr concentration in the fluid is plotted against time, a non-linear curve will result. Plotting the natural log of the Cr concentration against time, however, will yield a linear relationship. The slope of this log curve equals the fractional dilution rate or, in other words, the percentage of volume passing per hour. Extrapolation to concentration at time zero and division of this value into the dose yields an estimate of the volume. An example of this method is shown in the Table 11-4 using CrEDTA as a marker. Students should note that this method can be applied to estimate the volume and fractional dilution rate of any pool (e.g., plasma free fatty acids, body glucose, ruminal VFA, and so on) if an appropriate marker is chosen. Hungate (1966) provides an excellent review of this method in Chapter 5 of his text. An alternative calculation method to log transformation is the use of non-linear regression techniques.

Measurement of particulate passage rates can be accomplished by the same methods described above. In other words, a continuous or single dose of a particulate phase marker can be given and flow rates and/or volumes calculated as described above. Chromic oxide has been used for this purpose; however, it has the disadvantage of not being intimately associated with

the particulate phase. More recently, ytterbium, dysprosium, cerium, and a ruthenium phenanthroline complex have been used to estimation of particulate turnover rates. These materials seem to bind tightly with particulate matter and closely meet the criteria for ideal markers described previously. In the case of rare-earth elements, feedstuffs are usually allowed to soak with the rare earth for some time, followed by washing off of unbound rare earth. Treated feedstuff can then be fed or dosed for estimation of passage rate. Many of these markers are used as the radioactive form, in an effort to facilitate measurement; however, animals treated with radioactive substances fall under strict federal guidelines. Recently, Grovum and Williams (1973) and Ellis et al. (1979) have proposed two-compartment models to describe the fecal excretion of particulate markers, which allows determination of particulate passage rates.

Internal markers like indigestible ADF can also be used to determine rate of passage. As yet, the author is not aware of studies in which such markers as acid insoluble ash have been applied to the estimation of rate of passage.

An example of the use of ytterbium-labeled feed to measure rate of passage is given in Table 11-5. In addition, methods of treating forage with ytterbium and extracting ytterbium for analysis are attached to this chapter.

Markers for Measurement of Microbial Protein Synthesis. As discussed earlier in this chapter, it is often desirable to determine the amount of microbial protein synthesized by ruminants. This can be accomplished with the use of such markers as diaminopimelic acid, RNA and purines. Perhaps the simplest way to describe this method is by example, and Table 11-6 shows how microbial protein synthesis can be estimated by the use of purines as a microbial marker.

Summary

Indigestible markers are extensively used in nutrition studies and their use will continue to grow with time. A full understanding of their use and utility is necessary for students of nutrition. This chapter is designed to provide a brief introduction to the subject. Interested students should carefully read the literature citations included in this chapter for a more thorough treatment of the subject.

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Table 11-1. Example calculation of digestibility using lignin as an indigestible marker

A researcher conducted an experiment to estimate the digestibility of range forage. Forage samples were collected using esophageally cannulated cows and were found to contain 5% lignin (DMB) by using the ADL procedure. Five cows were used to collect feces by grab sampling at 0800 and 1700 daily for 5 d. These samples were composited within cows, and the average fecal lignin content for one of the five cows was 12% (DMB). Crude protein content of the forage was found to be 10% (DMB), and the CP content of the cow's feces described above was 12% (DMB). Dry matter and CP digestibility were calculated as follows:

DM digestibility = 100 - 100 x ([5%/12%] x [100%/100%])

= 100 - 100 x (0.41667)

= 58.34%

Protein digestibility = 100 - 100 x ([5%/12%] x [12%/10%])

= 100 - 100 x ([0.4166 x 1.2])

= 100 - 49.99

= 50.01%

Table 11-2. Example calculation of ruminal digestibility using Cr_2O_3 as an indigestible marker

An experiment was conducted with four abomasally cannulated steers to determine digestibility of DM and starch, ruminally and postruminally. Steers were housed in metabolism crates and fed a diet containing .2% Cr_2O_3 for 14 d at the rate of 5,000g per day (DMB). Feces were quantitatively collected for 4 d and composited, and abomasal samples were taken on two consecutive days and composited. Data from one steer are listed below:

DM intake = 5,000 g/d
% Cr_2O_3 in feed, DMB = 0.2%
% starch in feed, DMB = 70.0%
% starch in feces, CMB = 40.0 %
Fecal output, DMB = 500 g/d
% Cr_2O_3 in abomasum, DMB = 0.8%
% starch in abomasum, DMB = 60.0%

Total tract DM digestibility, % = ([5,000 - 500]/5,000) x 100 = 90.00%

Ruminal DM digestibility, % = 100 - 100 x ([0.2%/0.8%] x [100%/100%]) = 75.0%

% of DM entering intestine digested = 5,000 x 0.75 = 3,750 g digested ruminally
5,000 - 3,750 = 1,250 g entering intestine
([1,250 - 500]/1,250) x 100 = 60.0% of that entering intestine digested

Total tract starch digestibility,% = (([5,000 x 0.70] - [500 x .40])/[5,000 x 0.70]) = 94.28%

Ruminal starch digestibility,% = 100 - 100 x ([0.2%/0.8%] x [60%/70%]) = 78.58%
% of starch entering intestine digested = 3,500 g of starch intake x 0.7858 = 2,750.3 g digested ruminally
3,500 - 2,750.30 = 749.7 g of starch entering intestine
([749.7 - 200]/749.7) x 100 = 73.32% of that entering intestine digested

Table 11-3. The use of Cr_2O_3 to estimate intake of grazed forage

A steer grazing native range grass was dosed with 15 g of Cr_2O_3 for a 7-d preliminary period and 5-d collection period. Fecal grab samples were taken at 0800 and 1700 during the 5-d collection period and composited. The Cr_2O_3 concentration in feces was determined to be .005 g/g of fecal DM. Esophageally collected forage samples were analyzed using Tilley and Terry procedures and were found to have an IVDMD of 50.0%. Fecal output and intake were calculated as follows:

Fecal output of DM, g/d = 15 g/0.005 g/g of DM = 3,000 g

DM intake, g/d = 3,000 g/0.50 = 6,000 g, where 0.50 = 50% indigestibility

Table 11-4. Calculation of ruminal fluid volume and dilution rate with CrEDTA as a marker

Procedure: A steer was dosed with 200 mL of CrEDTA and samples of ruminal contents (fluid strained through cheesecloth) were obtained at 2, 5, and 24 h after dosing. Ruminal fluid samples were centrifuged at 10,000 x *g*, and clear fluid was decanted and saved. Atomic absorption Spectroscopy was used to measure the Cr conc in fluid samples and a 1:500 dilution of the original CrEDTA solution.

Results:

AA Readings

Standard concentration, mg/L	Absorbance
1	0.05
2	0.10
3	0.21
4	0.32
5	0.40
Ruminal fluid @2 h	0.39
Ruminal fluid @ 5 h	0.27
Ruminal fluid @ 24 h	0.10
1:500 CrEDTA	0.15

A regression equation was prepared from the standard values above:

$Y = 10.73261783 x + 0.6817545497$

Predicted concentration in ruminal fluid samples is:

2 h = 4.867 mg/L
5 h = 3.579 mg/L
24 h = 1.755 mg/L

Concentration in 1:500 CrEDTA = 2.29 mg/L
Thus, Cr conc in CrEDTA = 1,145 mg/L, and if there were 1,145 mg/L, there would have been 229 mg/200 mL dose

Table 11-4 (cont)

Now that we have these data, we can estimate ruminal fluid volume and dilution rate as follows:

X, time in h	Y, ln Cr concentration
2	1.58247
5	1.27508
24	0.56247

$Y = -0.043210X + 1.58651$

The slope is the fractional dilution rate. Ignoring the sign, the dilution rate is 4.32 %/h

Ruminal fluid volume can be calculated as follows:

Antilog of the intercept is: 4.88689 mg/L = concentration at T_0

Volume = dose/T_0 concentration = 229 mg/4.88689 mg/L = 46.8619 L

Total turnover time = 1/fractional dilution rate = 1/0.0432 = 23.14 h

Outflow from the rumen in L/h = 46.8619 x 0.0432 = 2.02 L/h.

Summary:

Fluid dilution rate	4.32 %/h
Fluid flow rate	2.02 L/h
Ruminal fluid volume	46.86 L
Turnover time	23.14 h

Table 11-5. Measurement of particulate passage rate with Yb-labeled forage

Procedure: A ruminally cannulated ewe was dosed with 20 g of a Yb-labeled forage sample. The sample contained 16.5 mg Yb/g of dry matter. Fecal samples were obtained at the times shown above. Samples were dried (50^0C), ground, and Yb was extracted with an EDTA solution. The Yb concentration was determined with an AA (nitrous oxide/acetylene flame).

Sample time	Yb concentration, mg/g of fecal DM
4	0
8	0
12	112.85
16	371.77
20	630.47
24	542.59
30	398.47
36	279.39
48	182.74
60	90.60
72	51.05

Estimation of particulate passage rate:

1. Calculations of Grovum and Williams, Br. J. Nutr. 30:313 (1973)

Model assumes two compartments with rate constants k_1 and k_2, where k_2 represents passage through the cecum and proximal colon. Passage from the rumen is represented by k_1.

To calculate k_1, do the following:

1. Examine data
2. Find **peak** marker concentration (in our example, the peak is at 20 h)
3. Regress ln marker conc vs. time from the peak

Time from peak, h	ln Yb concentration
20	6.446
24	6.296
30	5.988
36	5.633
48	5.208
60	4.506
72	3.932

$Y = 7.4359 - 0.0484$, where $7.4359 = A_1$

Thus, k_1 = 4.84%/h; r = 0.9986

To calculate k_2, do the following:

1. From regression used to estimate k_1, predict concentrations for times before the peak
2. Regress ln (predicted minus actual) vs. time

Time, h	Pred ln conc	Pred conc	Actual conc	Pred - Actual
12	6.855	948.56	112.85	835.71
16	6.661	781.54	371.77	409.77

Regression of ln (Pred - Actual) on time:

Y = 8.867 - 0.1782; r = 0.9999, where 8.867 = A_2
Thus, k_2 = 17.82%/h

To calculate TT, which represents the time from dosing to first appearance of marker in feces:

$TT = (\ln A_2 - \ln A_1)/(k_2 - k_1) = (8.867 - 7.4359)/(0.1782 - 0.0484) = 11.02$ h

2. Calculations from Ellis et al., Fed. Proc. 38:2702 (1979)

These workers also calculate two rate constants, but interpretation is different than Grovum and Williams (1973). The model is a two-compartment, time-dependent, time-independent model. Rate constant k_1 represents mixing in the rumen, and rate constant k_2 represents passage from the rumen

Thus, k_2 in the Ellis et al. (1979) model is equivalent to k_1 in the Grovum and Williams model.

Ellis and coworkers also have provided a one-compartment model. The rate constant k_1 x .59635 is equivalent to k_2 in their two-compartment model.

Calculations from the models of Ellis et al. are most conveniently done by the non-linear regression program (PROC NLIN) on SAS. The SAS model statements for the one- and two-compartment models follow:

```
DATA TWOCMPT;
INPUT TIME 1-3 CONC 5-8;
TP = TIME;
Y = CONC;
CARDS;

PROC NLIN ITER = 200 METHOD = MARQUARDT;
PARAMETERS K0 = 1000 K1 = 0.15 K2 = .050 TAU = 10;
BOUNDS K0>0, k1>0, K2>0, TAU>0;
T = TP-TAU;
IF T<0 THEN GO TO ALPHA;
E1 = EXP (-K1*T);
E2 = EXP (-K2*T);
DIFF1 = K2-K1;
DIFF2 = DIFF1**2;
DIFF3 = DIFF1**3;
BRAC1 = ((K1**2)*T/DIFF1)-(K1**2)/DIFF2;
BRAC2 = (K1**2)/DIFF2;
ONE = T*(K1**2)/DIFF2;
TWO = 2.0*(K1**2)/DIFF3;
MODEL Y = K0*(E1*BRAC1+E2*BRAC2);
DER.K0 = E1*BRAC1 + E2*BRAC2;
DER.K1 = K0*(K1*T*(2.-K1*T)/DIFF2.*K1*(K1*T-1.)/DIFF2-TWO)*E1+E2*
(2.*K1*K2/DIFF3)*K0;
DER.K2 = K03(TWO-ONE)*E1 + E2*(-ONE-TWO)*K0;
DER.TAU = K0*(E1*(( (K1**3)*T-K1**2)/DIFF1-(K1**3)/DIFF2)+E2*K2* (K1**2)/DIFF2);
GO TO BETA;
ALPHA:
MODEL Y = 0.0;
DER. K0 = 0.0;
DER.K1 =0.0;
DER.K2 = 0.0;
DER.TAU = 0.0;
BETA: ;
OUTPUT OUT=POINTS PREDICTED = YHAT RESIDUAL=YRES PARMS=K0 K1 K2
TAU;
PROC PRINT DATA= POINTS;
PROC PLOT;
PLOT YHAT*TP  = '*' CONC*TP = 'Y'/OVERLAY;
PLOT YRES*TP;
```

```
DATA ONECMPT;
INPUT TIME 1-3 CONC 5-8;
TP = TIME;
Y = CONC;
CARDS;
PROC NLIN ITER = 50 CONVERGENCE = 0.0001 METHOD = MARQUARDT;
T = TP-TAU;
PARAMETERS K0 = 20000 K1 = 0.10 TAU = 12;
BOUNDS K0>0, K1>0, TAU>0;
IF T < 0.0 THEN GO TO ALPHA;
E1 = EXP(-K1*T);
ONE = T*(K1**2)*E1;
MODEL Y = K0*ONE;
DER.K0 = ONE;
DER.K1 = T*K1*K0*E1*(2-K1*T);
DER.TAU = K0*(K1**2)*E1*(K1*T-1.0);
GO TO BETA;
ALPHA:
MODEL Y = 0.0;
DER.K0 = 0.0;
DER.K1-0.0;
DER.TAU = 0.0;
BETA:;
OUTPUT OUT = POINTS PREDICTED = YHAT RESIDUAL = YRES PARMS = K0 K1
TAU;
PROC PRINT DATA = POINTS;
PROC PLOT;
PLOT YHAT*TP = '*' CONC*TP = 'Y'/OVERLAY;
PLOT YRES*TP;
```

Results of the SAS PROC NLIN analysis for the Ellis One- and Two-Compartment Models was as follows:

Parameter	Two-compartment model	One-compartment model
k_0	779.6210	15,225.6563
k_1	0.951567	0.098437
k_2	0.049143	-
Tau	14.25356	11.27302

Note that k_1 from the one-compartment model should be multiplied by .59635 to make it equivalent to the k_2 parameter from the two-compartment model (in this case the multiplied value equals .05837).

Table 11-6. Estimation of microbial protein synthesis using purines as a marker of bacteria

Assume an experiment was conducted as described in Table 11-2 with abomasally cannulated steers. Recall the pertinent data were as follows:

DM intake = 5,000 g/d
Ruminal dry matter digestion = 75%

Given the following additional data:
% protein in abomasum = 40.0% (DMB)
Purines in abomasal samples = 2.5 mg/g of DM

The microbial protein synthesized can then be calculated as follows:

Dry matter passing out of the rumen per day is calculated as 5,000 g x 25% ruminal indigestibility = 1,250 g

The protein passing out of the rumen can then be calculated as 1,250 g x 40% protein in abomasum = 500 g

500 g protein passing = 80 g of N passing/day

If purines are assumed to be in a constant ratio with microbial N, the portion of this 80 g that is of microbial origin can be calculated as follows:

If there were 1,250 g passing out of the rumen per day, and this contained 2.5 mg/g of purines, then there was 1,250 g x 2.5 mg/g = 3,125 mg or 3.125 g of purines passed out of the rumen per day.

If purines are in a ratio of 1:10 with microbial N (1 part purines per 10 parts microbial N – *in practice, this value should be measured, not assumed*), there would be 3.125 g x 10 = 31.25 g of microbial N passing out of the rumen per day.

Thus, out of the 80 g of total N passing out of the rumen per day, 48.75 g were of feed origin and 31.25 g were of microbial origin.

Microbial protein synthesized would be = 31.25 g x 6.25 = 195.31 g/d

The percentage of total N that was feed escape N would be 48.75/80 = 60.93%.

Procedure for Preparation of CrEDTA for Use as an Indigestible Marker

Reference: Binnerts et al. (1968) - Vet Rec 82:470

Weigh 14.2 g of pure chromium trichloride ($CrCl_3 \cdot 6\ H_20$) in an 800 mL beaker and dissolve in 200 mL of distilled H_2O. Then dissolve 20 g of the disodium salt of ethylene diaminotetracetic acid (EDTA) in 300 mL of distilled H_2O and add to the first solution. Heat the combined solution to boiling with a few boiling chips, then cover with a watch glass and boil gently for about 1 h. The solution will gradually assume a deep violet color as the 1:1 complex of EDTA and Cr is formed. After heating, neutralize excess EDTA with 4 mL of a 1.0 M calcium chloride solution. Then bring the pH to between 6 and 7 with a small amount of NaOH or HCl and make up to 1 L.

Procedure for Preparation of CoEDTA for Use as an Indigestible Marker

FROM: E. C. Prigge and G. Varga, West Virginia, University. See Uden et al. (1980) J. Sci. Food Agric. 31:625

Weigh the following:

25 g of cobalt (II) acetate$\cdot 4\ H_2O$
29.2 g of ethylene diaminetetracetic acid
4.3 g of $LiOH \cdot H_2O$

Put all ingredients into a 2-L beaker.

Add 200 mL distilled H_2O and dissolve with heating if necessary.

Cool and add 20 mL of 30% hydrogen peroxide solution.

Allow to stand 2 to 3 h at room temperature or overnight.

Add 300 mL of 95% ethanol and store overnight under refrigeration.

Filter through Whatman filter paper (fast) and wash with about 1 L of 80% ethanol.

Dry crystal in 100^0C oven overnight.

Resuspend crystal in 1 L of distilled H_2O, and measure Co concentration on an atomic absorption spectrophotometer.

Animal Science Nutrition Lab

Procedure for Extracting Rare Earth Metals

Reference: Hart, S. P. and C. E. Polan. 1984. Simultaneous extraction and determination of ytterbium and cobalt ethylenediaminetetra-acetate complex in feces. J. Dairy Sci. 67:888.

1. Weigh out .2 g of dried, ground sample (2-mm screen) in duplicate.
2. Place sample into a 30- to 50-mL screw cap tube.
3. Add 20 mL of a .05 M EDTA solution.
4. Cap the tubes and shake for 30 min.
5. Filter sample through a Whatman #1 filter paper twice.
6. Read the sample on the atomic absorption spectrophotometer.

PREPARATION OF STANDARD SOLUTIONS

1. Standard concentrations of the element in question should be made as follows:

    a. Yb: 0, 1, 2, 3, 4, 5 mg/L
    b. Dy: 0, 10, 20, 30, 40, 50 mg/L

2. Place a 10 g of Time 0 composite fecal sample into a 2-L volumetric flask.
3. Add 1,000 mL of .05 M EDTA solution and stopper flask.
4. Shake for 30 min then filter as described above.
5. Composite all the filtered solution.
6. Place the proper amount of known standards into 100-mL volumetric flasks.
7. Bring to volume with the composited solution.
8. Read on the atomic absorption spectrophotometer.
9. To measure dose, treat it as a sample as far as extraction is concerned. Preparation of standards for reading dose is the same except that 10 g of hay is used to a produce solution for bringing standards to volume (i.e., make a separate set of standards to measure amount of Yb in the hay that was used to dose the animal).

PROCEDURE FOR PREPARING 0.05 M EDTA

1. Weigh out 58.4 g of EDTA (ethylenediaminetetraacetic acid) and 15.3 g of KCl (potassium chloride).
2. Place both EDTA and KC1 in a 4-L flask. Bring to volume with deionized H_20. Save approximately 100 mL of deionized H_20 if total volume is 4-L.
3. While mixing, adjust the pH to 6.5 with concentrated reagent grade NH_4OH (ammonium hydroxide). Record volume of NH_4OH used. Subtract that volume of NH_4OH used from 100 mL of deionized H_20 saved in previous step. Pour remaining volume of deionized H_2O into a 4-L flask containing dissolved EDTA/KCl.

Animal Science Nutrition Laboratory

Ytterbium and Dysprosium Analysis - Non-boiling method

Reference: Ellis, W. C., C. A. Lascano, R. Teeter and F. N. Owens. 1982. Solute and particulate flow markers. In: F. N. Owens (Ed.). Protein Requirements for Cattle: Symposium. pp. 37-55, Oklahoma Agric. Exp. Sta. MP109.

Reagents:

3 M HCl (241.2 mL HCl/L)

3 M HNO_3 (190.8 mL HNO_3/L)

Remaining volume of acid solutions is deionized water. Be sure to add some water first. Mix equal portions of these two 3 M acids to produce ACID MIXTURE.

KC1 solution: dissolve 38.2 g of KCl/L - bring to volume with deionized water.

Sample Preparation:

Ash 2-g sample in duplicate (1 g for dose). Place residue in 100-mL beaker.

Add 20 mL of acid mixture and cover with watch glass; let stand 12 h.

Filter into acid-rinsed 50-mL volumetric through #1 or #4 Whatman filter paper; bring to volume with deionized water.

Note: 10 mL acid brought to volume in a 25-mL volumetric can be used if one needs to concentrate the sample.

Standard Preparation:

Extract a 24-g ashed fecal sample (0 h or unmarked) in 240 mL of acid mixture for 12 h; filter and bring to 600 mL with deionized water; use this to bring standards to volume.

Place 0, 100, 200, 300, 400, and 500 μL of stock Yb solution (1,000 μg/mL) into acid-rinsed 100-mL volumetric flasks with Hamilton syringes; these correspond to 0, 1, 2, 3, 4 and 5 μg/mL (ppm) standards. Add 10 mL of KCl solution and bring to volume with 0-h fecal extract.

Unknown Preparation:

Place 1 mL of KC1 solution and 9 mL of unknown extract in scintillation vial (1.1 dilution factor).

Read standards and unknowns on atomic absorption spectrophotometer--refer to IL manual for instrument setup. Results are expressed in ²g/mL.

If readings are too low, resolubilize a larger amount or concentrate in smaller volumetric. If readings are too high (overflow), standards can be remade as above, except 40 mL of deionized water are added to the volumetric before bringing it to volume with fecal

extract. Unknowns are made from 1 mL of KC1, 5 mL of unknown extract, and 4 mL of water (dilution factor = 2).

Dose Measurement:

Ash 1 g of unlabeled dose material; place residue in 100-mL beakers, and add 10 mL of acid mixture; let stand 12 h.

Filter and bring to 25 mL with deionized water. Use this solution to bring standards to volume for reading doses.

Ash 1 g sample of dose (in duplicate); place residue in 100-mL beakers; add 10 mL acid mixture; let stand 12 h.

Filter and bring to 25 mL with deionized water.

Transfer 0.25 mL of dose extract to an acid-rinsed 50-mL volumetric.

Add 5 mL of KCl solution and bring to volume with deionized water (dilution factor = 200).

Prepare standards by adding 0, 50, 100, 150, 200, and 250 μL of Yb stock solution to acid-rinsed 50-mL volumetrics.

Add 5 mL of KCl solution and .25 mL of the unlabeled material extract; bring to volume with deionized water.

Read standards and doses on atomic absorption spectrophotometer. Results are expressed as μg/mL.

Calculations:

mg Yb/g (DMB) = (μg/mL x dilution factor x volume of ash in solution, mL)/dry sample weight, g

Procedure for Preparation of Ytterbium Labeled Feedstuffs for Use as an Indigestible Marker

FROM: E. C. Prigge and G. Varga, West Virginia University

Dissolve 2.5 g of $YbCl_3 \cdot XH_20$ in distilled H_20 and dilute to 1 L. This solution is poured onto 50 g of feedstuff (chopped hay, grain, etc.) and allowed to soak for 24 to 48 h, during which time it is stirred three to four times each day. This mixture is then filtered and washed six times with water* over a 6-h period, after which the forage can be dried at 50 to 60^0C. The Yb concentration can then be determined by atomic absorption spectroscopy.

*Washings can be checked by mixing them 1:1 with phosphate buffer. If a precipitate forms, Yb is still being washed off. Washing should be continued until all excess Yb is washed off.

Animal Science Nutrition Lab

Indigestible ADF Procedure

1. Run 96-h in vitro and 48-h acid-pepsin digest on feed, digesta, or feces (use standard in vitro procedure as outlined in this laboratory manual with 0.5-g samples).
2. Empty tube contents into Berzelius beaker (without centrifugation), and rinse tube with ADF solution. Add a total volume of 100 mL of ADF solution.
3. Run standard ADF procedure (Whatman #541 filter paper may be used instead of tared crucibles).

% Indigestible ADF =

(({dry residue - [pan + filter paper wt]} - {blank - [pan + filter paper wt]})/dry sample wt) x 100

Indigestible NDF

1. Run 96-h in vitro and 48-h acid-pepsin digest on feed, digesta, or feces (use standard in vitro procedure as outlined in this laboratory manual with 0.5 g samples).
2. Empty tube contents into Berzelius beaker (without centrifugation), and rinse tube with NDF solution. Add a total volume of 50 mL of NDF solution.
3. Run procedure for slow-filtering NDF, (i.e., use amylase). Whatman #541 filter paper is used for filtration.

% Indigestible NDF =

(({dry residue - [pan + filter paper wt]} - {blank - [pan + filter paper wt]})/dry sample wt) x 100

CHAPTER XII

Radioactivity and Liquid Scintillation Counting

General. As discussed in the previous chapter, radioactively labeled materials are sometimes used as markers in nutrition studies. This use allows rapid and precise measurement of the markers. Moreover, carbon-14 and tritium, as well as other biologically important compounds, emit β particles and can easily be quantified by liquid scintillation counting (LSC). Before we discuss LSC, however, it is appropriate to briefly consider radioactivity in general.

Although most elements have stable nuclei, it has been known since the beginning of the 20th century that some nuclei exist which spontaneously transform themselves into other nuclear species by the emission of particles or energy (Beiser, 1966). Elements with such nuclei are termed radioactive and the radiation emitted by them is of three types; alpha particles, beta particles and gamma rays.

To understand why radioactive decay occurs, one must consider the stability of a nucleus. Generally, as the number of protons there are in the nucleus increases, the number of neutrons must increase at a greater rate to maintain stability of the nucleus. This is because neutrons are needed to offset electrostatic repulsion between protons. In fact, an element of a given atomic number has only a very narrow range of numbers of protons and neutrons in which to be stable (Beiser, 1966). Figure 12-1 taken from Beiser's (1966) text effectively illustrates this concept.

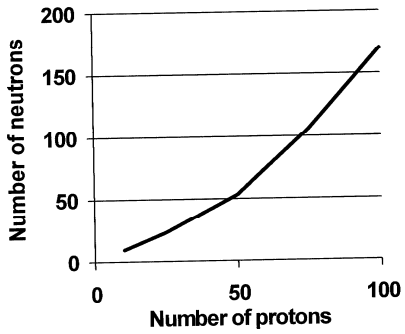

Figure 12-1. Number of neutrons vs. number of protons in stable nuclei. Note that the larger the nucleus, the greater the number of neutrons. Adapted from Beiser (1966).

Radioactive decay occurs as nuclei attempt to reach a stable configuration. Suppose there are too many neutrons in the nucleus. If a neutron transforms itself into a proton, a negative electron or beta particle (β) must be emitted to conserve electrical neutrality. The residual nucleus may be left with excess energy as a consequence of shifted binding energy and releases this energy in the form of a gamma (γ) ray (Beiser, 1966).

If a nucleus has too few neutrons, a proton can become a neutron with the emission of a positron. This is also classified as β decay (Beiser, 1966). A third way of achieving stability is emission of an alpha particle (α), which consists of two neutrons and two protons. Generally, a particles are emitted by elements of heavy mass. Often, a succession of α, β, and γ decays are necessary for nuclei to achieve stability (Beiser, 1966).

As a general rule of thumb the energy associated with or penetrating power of the types of decay is in the order $\gamma > \beta > \alpha$. These types of decay also carry a certain amount of energy, usually measured in electron volts. Other common measurements of particle energy are Kev (thousand electron volts) and Mev (Million electron volts). Gamma and β emissions have sharply defined energy spectra while β particles have a continuous energy spectrum. The continuous nature of the β particle's spectrum is related to the difficulty the charged particle has escaping from the atom.

The basic unit of radioactivity is the disintegration, which corresponds to the decay of a single nucleus. Activity can then be expressed as rate of decay or disintegrations per unit time (i.e., dpm = disintegrations/min). Activities are often so high, that another unit of activity, the Curie is used. The Curie is defined as the activity of l g of radium and its value is set at 3.7×10^{10} disintegrations/sec. The millicurie and microcurie (10^{-3} and 10^{-6} respectively) also are used. Specific activity is another commonly used term and is essentially a concentration unit (e.g., dpm/mole or dpm/g).

Another important term in dealing with radioactive materials is half-life. Half-life is defined as the time required for half the original sample of radioactive material to decay. Some radioactive isotopes (isotopes are different forms of the same element) have short half-lives (seconds), whereas others are thousands of years. Half-lives of some biologically important radioisotopes are shown in Table 12-1.

Table 12-1. Half-lives of some biologically important radioisotopes. Adapted from Kobayashi and Maudsley (1974)

Radioisotope	Particle	Half-life
^{3}H (Tritium)	β-	12.3 yr
^{14}C	β-	5,568 yr
^{40}K	β-, γ, X-rays	1.25×10^{9} yr
^{45}Ca	β-	164 d
^{51}Cr	γ, X-rays	27.8 d
^{135}I	γ, X-rays	60 d
^{35}S	β-	86.7 d
^{32}P	β-	14.22 d

Liquid Scintillation Counting. Liquid scintillation counters are frequently employed in nutrition studies, primarily because the isotopes of major interest (^{3}H, ^{14}C, ^{32}P, etc.) emit β particles that can be easily counted in these instruments. The following description of a liquid scintillation counter is taken primarily from the instruction manual for a Packard Model 3255 Tri-carb Liquid Scintillation Spectrometer (1977).

As we discussed before, a β-emitting radioisotope decays by release of a β particle. Becasue these particles have mass, inertia, and charge, they will interact with matter (Packard Manual, 1977). If the particle can be made to indicate its presence, the activity of a sample can be determined. In a liquid scintillation counter, the radioactive sample is dissolved in a solvent. Energy from β particles is transferred to molecules of the solvent, which in turn pass their energy on to a fluor that has been added to the mixture. The excited fluor returns to the ground state and in the process, emits a photon of light. This light then can be detected and quantified. The total light produced is the scintillation and its intensity depends on the energy of the β particle emitted, as this influences the number of collisions the particle has with solvent molecules (Packard Manual, 1977). The solution in which this process takes place is called the scintillation cocktail.

To understand more about the instrument, it is helpful to consider the function of its parts. A simplified diagram of a liquid scintillation counter based on the components described above is shown in Figure 12-2. We will start our discussion with the photomultiplier tubes.

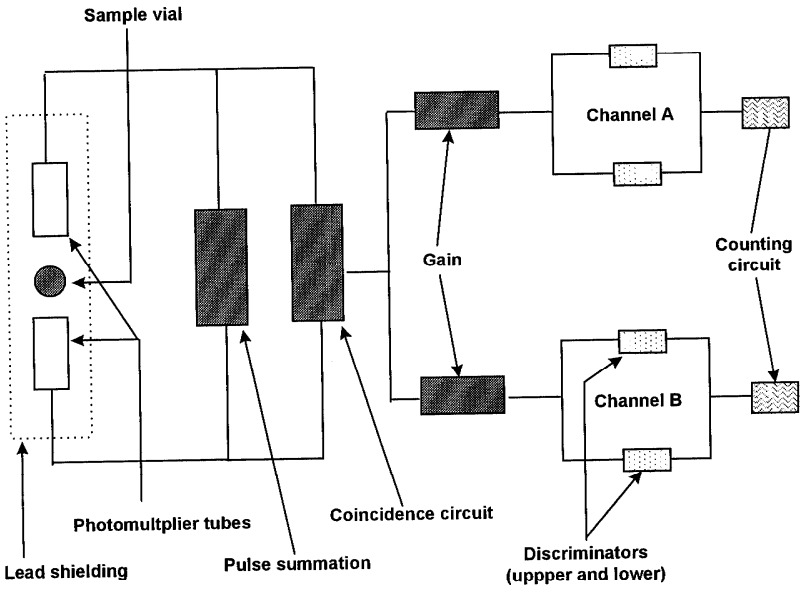

Figure 12-2. Basic components of a liquid scintillation counter.

Photomultiplier Tubes. Generally, the radioactive sample is mixed with solvent and fluor in a small vial. This vial is then placed between two photomultiplier (PM) tubes in order to detect the light resulting from b particle emission. We have previously discussed the use of photomultiplier tubes in the Chapter VIII. An electrical pulse is the output of the PM tubes, the amplitude of which is proportional to the number of photons from the scintillation that struck the PM tube (Packard Manual, 1977).

Noise and Background. An electrical pulse emerging from the PM tube may not necessarily arise from scintillations alone. They can result from electronic noise in the PM tube and from radiation from sources other than the sample. Two PM tubes are used rather than one to distinguish between electrical noise and sample pulses. Because electrical noise is random, the probability of two PM tubes producing electrical noise pulses in both PM tubes at the same time is small. In contrast, β emissions will produce pulses in both PM tubes at the same time. A coincidence circuit is used in the counter, so that only an electrical pulse detected from both PM tubes at the same time is counted. Thus, the coincidence circuit cuts the background resulting from PM tube noise down to extremely low levels.

Summation. With two PM tubes, pulses from both tubes can be added together before analysis of pulse amplitude. Because the amplitude of a summed pulse does not depend on the number of photons of light that reach one or the other PM tubes, it more accurately reflects the energy of emitted β particles (Packard Manual, 1977).

Pulse Height Analysis. As indicated previously, radiation from other sources besides the sample may influence the pulses measured by the PM tubes. To minimize this effect, two methods are employed. One is to shield the counting chamber with lead. The other is to analyze the pulses and reject those that are outside the energy range of β emitters. Thus, the lead shielding eliminates all but very high energy radiation, which can then be eliminated by pulse height analysis. Exclusion of counts is done by use of discriminators, which can be set to exclude pulses of certain amplitudes. Most instruments have an upper (for high amplitude pulses) and a lower (for low amplitude pulses) discriminator setting. Moreover, the amplitude of a pulse can be altered by adjusting the gain control of the instrument. With higher gain settings, a pulse is amplified to a greater extent. The gain and discriminator controls make up a channel of analysis (Packard Manual, 1977). Appropriate use of gain and discriminator settings allows the instrument to be used to separate pulses from two different radioisotopes in the same sample. Once appropriate gain and discriminator settings are achieved, pulses can be counted and summed. Most instruments currently available on the market have channels that have been pre-set at the factory for particular isotopes, eliminating the need for adjustable gain and discriminator settings.

Preparation of the Sample for Counting. The scintillation cocktail consists of the sample, solvent, and fluor(s). Generally, the solvent is an aromatic hydrocarbon, with toluene frequently used for non-polar samples. The fluor is most commonly PPO (2, 5-phenyl oxazole). Sometimes, however, the emission wavelength of the primary fluor is not optimized with PM tube sensitivity, and a secondary fluor is used. The compound POPOP (1, 4-di-[2-(5-phenyloxazolyl)]-benzene) is often used, as is di-methyl POPOP. In aqueous samples, 1, 4-dioxane is often added to make the sample miscible with toluene. Several commercial scintillation mixtures are available.

Counting Efficiency and Quenching. The number of counts recorded by a liquid scintillation counter is always less than the number of emissions by the sample. This is because the counter is not 100% efficient. Quenching is a major factor influencing counting efficiency, and quenching can be defined as any decrease in efficiency resulting from the sample or substances in the scintillation cocktail. In applications where the specific activity of a sample must be known, quenching or loss in efficiency must be determined and the data adjusted accordingly.

There are several types of quenching. Chemical quenching occurs when chemicals in the cocktail absorb radiation, preventing excitation of the fluor. Chloroform and carbon tetrachloride are powerful chemical quenching agents. Color quenching occurs when colored solutions absorb light emitted by the fluor, preventing its detection by the PM tubes. Red solutions tend to be fairly potent color quenchers. Quenching also may occur when radioactive material is absorbed onto the surface of the counting vial and is no longer miscible with the solvent.

Quench correction involves correcting the sample count for loss in efficiency caused by quenching. Several methods are used for this correction, and we will briefly discuss three of them.

Internal Standardization. To begin with, the sample is counted and its counts recorded. A known amount (dpm) of a standard source is then added to the sample, and the sample is recounted. The efficiency of counting can then be determined by subtracting the count of sample alone from sample plus standard and dividing by the known dpm of the standard as shown below:

Efficiency = 100 x ({[Sample count + standard] - sample count}/known dpm of std).

This method is somewhat difficult, in that very precise measurements of internal standard added must be made, and frequent handling of radioactive materials is required.

Sample Channels Ratio. Quenching effects both the number and energy of photons of light produced by the scintillation mixture (Packard Manual, 1977). This will cause a shift in pulse height, which is employed in the channels ratio method to determine efficiency.

A set of standards is prepared and counted. Each standard contains a known dpm and a quenching agent to provide a range in quenching. One channel is set to have counts lower than that of the other channel. As degree of quenching increases in the standards, counts will decrease in both channels and the ratio of the counts will increase because energy of pulses falls below the discriminator setting of the "low count" channel. Counting efficiency can then be plotted against the ratio of counts in the two channels to provide a quench correction curve. Unknown samples can then be counted and the channels ratio used to determine counting efficiency.

External Standard Channels Ratio. When a γ source is placed adjacent to the counting vial, the γ radiation will produce Compton electrons, which behave as β particles and produce scintillations. The pulse produced by Compton electrons also is decreased by quenching. This effect can be monitored by changes in samples channels ratio to determine counting efficiency. Most liquid scintillation counters are fitted with a small quantity of γ emitter, which when desired, can be placed adjacent to the counting vial for use in external standardization measurements.

Once counting efficiency has been determined by one of the methods described above, observed counts can be corrected for changes in efficiency. This is done by simply dividing observed counts by efficiency expressed as a decimal. It should be emphasized again that some type of correction for loss in efficiency is necessary if specific activity of an unknown sample must be known.

References

Beiser, Arthur. 1966. Modern Technical Physics. Cummings Publishing Company, Menlo Park, California.

Kobayashi, Y., and D. V. Maudsley. 1974. Biological Applications of Liquid Scintillation Counting. Academic Press, New York, NY.

Packard Model 3255 Liquid Scintillation Spectrometer System Instruction Manual. 1977.

Section 3 - Principles of Operation. Packard Instrument Co., Inc., Downer's Grove, IL.

Radioimmunoassay of Serum Progesterone (Revised July, 1979)

New Mexico State University
Endocrinology Laboratory
D. M. Hallford

References: Kittok et al., 1973; J. Anim. Sci. 37:985
Hallford et al., 1975; J. Anim. Sci. 41:1706
Gibrrori et al., 1977; Endo. 100:1483

1. Introduction

a) The Endocrinology Laboratory contains many dangerous compounds, including organic solvents and radioactive materials. Care must be taken at all times to prevent accidents.

b) RIA procedures are very useful, but their sensitivity and accuracy depend on **strict attention** to detail and technique.

c) Individuals may use equipment in the Endo Lab only after receiving proper instructions.

2. Preparation

a) The assay is conducted primarily in disposable glassware. Any non-disposable glassware should be special washed, rinsed with methanol, and dried before use.

b) Begin thawing serum samples, antibody, and competitor. After thawing, replace in refrigerator until use.

c) Each assay usually contains a wether sample, a wether + 5 ng of progesterone sample, and 14 unknowns. The following outline will detail the assay procedure for a single sample.

d) Number three (A, B, C) extraction vials (20 mm x 150 mm) for each sample.

e) Label two scintillation vials as Total Count 1 (TC 1) and Total Count 2 (TC2).

f) Label a scintillation vial for each serum sample as Recovery (R1, R2, etc.)

3. Extraction

a) Pipette approximately 3,000 dpm of repurified ^{3}H progesterone (tracer) into TC1, extraction vial A and TC2, in sequence. Place tracer back in freezer immediately. Thoroughly rinse 10-mL Hamilton syringe, and pipette an appropriate amount of a serum sample (usually 0.25, 0.5 or 1.0 mL) into extraction vials A, B, and C.

i) **Note**: In a complete assay, the syringe is washed 10 times in each of two distilled water rinses between samples and is equilibrated 15 times in each sample.

b) Vortex to mix serum and tracer, and allow to equilibrate for 15 min.

c) Add 5 mL of hexane:benzene (2:1; vol:vol) to extraction vials A, B, and C. Place caps securely on extraction vials.

d) Extract by vigorous inversion for 15 min.

e) Place extraction vials in freezer for 1 to 2 h or until the serum is completely frozen.

f) While the serum is freezing, number a series of 12 mm x 75 mm disposable culture tubes.

i) **Note**: In a complete assay, 48 tubes are used. Tubes 1 through 8 and 41 through 48 contain standards, whereas tubes 9 through 40 contain extracts from the serum samples.

g) After the serum is frozen, decant the extract from extraction vial A (containing tracer) into a properly labeled recovery vial.

h) Decant the extract from extraction vials B and C into properly labeled culture tubes.

i) After all extracts have been decanted, allow the total count and recovery vials to dry overnight, then add 10 mL counting fluid to each.

j) Culture tubes containing extracts B and C are then dried under nitrogen.

k) While tubes are drying, continue with IV.

4. Addition of Standards

a) Wash "standard" syringe (100-mL) 30 times in methanol before and after pipetting standards.

b) Standards are prepared in such a way that 100 mL of fluid contains 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, or 1.6 ng progesterone.

c) To disposable tubes 1 through 8, and 41 through 48, add 100 mL of the appropriate standard in ethanol.

5. Example

a) Tubes 1 and 41 receive 100 mL of the 0-ng standard.

b) Tubes 2 and 42 receive 100 mL of the 0.1-ng standard.

c) Start with 0 ng and progress through 1.6 ng.

d) After pipetting 0 ng into tubes 1 and 41, expel the contents of the syringe needle (but do not wash the syringe) before equilibrating the syringe in the .1-ng standard.

e) Dry culture tubes under nitrogen.

6. Addition of Antibody and Competitor

a) Wash the "Antibody-Competitor" syringe 30 times in distilled water before adding the antibody.

b) Add 200 mL of anti-progesterone in PBS + Gel to each 12 mm x 75 mm disposable culture tube.

c) Vortex lightly.

d) Incubate at room temperature for 15 min.

e) While tubes are incubating, wash syringe 30 times in distilled water.

f) Add approximately 40,000 dpm of ^{3}H progesterone (competitor) in 200 ml PBS + Gel to each tube.

g) Votex lightly.

h) Incubate 12 to 20 h at 4°C (in a plastic bag).

i) Wash syringe 30 times in distilled water and 30 times in methanol after adding competitor.

7. Addition of Charcoal Dextran

a) Place rack and 250 mL of charcoal dextran in an ice water bath for 10 min.

b) While stirring, rapidly add 1.0 mL of charcoal dextran to each tube, and vortex in less than 2 min.

c) Incubate in an ice water bath for 7 min.

d) Centrifuge at 2,500 rpm for 15 min at 4°C.

e) Store automatic syringe with water in barrel.

8. Addition of Counting Fluid

a) Decant supernatant from each disposable tube into a corresponding scintillation vial.

b) Add 10 mL of counting fluid to each vial. Rinse automatic pippetor with distilled water after use.

c) Count each vial for 10 min.

i) **Note**: There will be a total of 66 scintillation vials for each assay containing 16 serum samples.

CHAPTER XIII

Gas Chromatography

Chromatography can be defined as a physical method for separation of components in a mixture. There are several types of chromatography, including paper, thin layer, liquid and gas. We will confine our present discussion to gas chromatography, as it has wide application to the analysis of organic samples commonly used in nutrition studies.

The basis of gas chromatography is the separation column. The column has both a stationary phase, which may be either solid or liquid on a solid support, and a carrier gas mobile phase. Gas liquid chromatography (GLC), which is most widely used, employs a liquid stationary phase on solid support. When a volatile sample is injected onto the column, it encounters two opposing forces. One force, the carrier gas, pushes the sample through the column, whereas the second force, the interaction of components with the stationary phase, retards movement of the sample through the column. Individual components of the sample can be solubilized partially in the liquid stationary phase or absorbed to the solid support. Physio-chemical aspects of each component determine extent of interaction with the stationary phase, and because components have different properties, they are eluted from the column at different rates, resulting in separation of components.

The efficiency with which a column separates materials is obviously of concern and depends in part on the column temperature and gas flow rate. Efficiency can be determined from mathematical calculations and can then be used to determine optimum column conditions. A detailed description of these calculations, however, is beyond the scope of our discussion, and students are referred to Fritz and Schenk (1979) and Zweig and Sherma (1972) for a more detailed treatment. The remainder of our discussion will focus on the components of a gas chromatograph and the use of GLC in quantitative analysis of organic molecules.

Basic Components of a Gas Chromatograph. Figure 13-1 depicts the components found in most gas chromatographs. We will briefly discuss each component.

Gas supply and flow regulators provide a means of obtaining a uniform rate of gas flow (Zweig and Sherma, 1972). The carrier gas chosen will vary with the detector used, but should be inert and inexpensive. Nitrogen gas is a popular choice when a flame ionization detector is used (Fritz and Schenk, 1979).

The injection port is a heated chamber into which a liquid sample is injected. Injection is accomplished with a syringe through a self-sealing septum (Zweig and Sherma, 1972). Normally microliter quantities of the sample are injected, and the injection port temperature is maintained at a level that will immediately volatilize the sample. The flow of carrier gas then pushes the sample onto the column.

The column is held in an oven that maintains the desired temperature for analysis (Fritz and Schenk, 1979). Columns may be constructed of glass or metal (usually stainless steel). Column packing (solid support and liquid phase) varies depending on the nature of the substance

analyzed. Several commercial firms are capable of assisting the analyst in choosing the proper column and typically provide pre-packed columns or column packing material for purchase. Length and diameter of the column also vary depending on the nature of the instrument and sample to be analyzed.

The detector sits at the end of the column. As components of the sample exit from the column, the detector functions to sense their presence and send out a signal proportional to the concentration of the component in the carrier gas (Fritz and Schenk, 1979). Different kinds of detectors are used depending on the nature of the material being analyzed. The detector also is heated to maintain sample components in a gaseous state.

The three most widely used detectors are the flame ionization detector, the thermal conductivity detector, and the electron capture detector. The **flame ionization detector** is essentially a micro-burner surrounded by an electrode that collects ions produced when a sample component is burned (Fritz and Schenk, 1979). The flame is usually provided by a flow of hydrogen and air into the detector, and as an organic sample enters the flame with carrier gas, it is burned, resulting in the formation of ions. These ions migrate to the electrode, and the electrical current is amplified and sent to the recorder.

The thermal conductivity detector works on the principle that the rate of heat loss of a hot wire is proportional to the molecular weight of the gas surrounding the wire (Fritz and Schenk, 1972). If Helium (MW = 4) is used as a carrier gas, sample components in gaseous form will be of higher molecular weight and thus decrease thermal conductivity. Heated metal filaments in the detector form a Wheatstone bridge that measures the differences in thermal conductivity of pure carrier gas and carrier gas plus sample components. As a sample component passes over a filament, its temperature increases because of the lower thermal conductivity of the sample component compared with the carrier gas. This heating increases the resistance of the Wheatstone bridge, and the resistance needed to restore the balance between the two filaments (carrier gas filament and carrier gas + sample filament) is measured (Fritz and Schenk, 1979).

The electron capture detector is very useful for detecting organic halogen compounds and finds considerable use in pesticide analysis. The detector contains a β-emitting radioactive substance (usually ^{63}Ni). Carrier gas reacts with β particles, producing a stream of electrons. Electrons are attracted to an anode in the detector, providing a baseline electric current. As sample components come off the column, they interact with β particles and decrease the baseline current, providing a signal that can be detected (Fritz and Schenk, 1979). Common applications with these three types of detectors are shown in Table 13-1.

The amplifier and recorder are used to multiply detector response and provide a permanent record of detector output, respectively. Many new gas chromatographs have mini-computer attachments that integrate peaks as they come off the column and actually calculate concentrations of components. Such computational instruments have largely eliminated the use of recorder-produced chromatograms in quantitative analysis of GLC data.

Other Important Terms in GLC. Before we move on to quantitative analysis by GLC, it seems appropriate to define a few commonly used terms. **Retention time** is the time lapse

between sample injection and emergence of any component. Retention time varies depending on column packing, temperature, and gas flow rates. It is important that sample components have distinct retention times so separation can be effective. Generally, sample components are identified by comparing retention times of standard samples with retention times of unknown sample components.

Peak area is quantitatively related to the amount of component present in a sample and is thereby of considerable importance. As indicated previously, peak area can be determined with the aid of computerized integrators. It also can be determined manually by a process known as triangulation. This process entails transforming a peak into a triangle and determining peak area as peak height times peak width at half height.

Resolution is a term that indicates degree of separation. It depends primarily on the column packing but also may be influenced by gas flow and column temperature. Resolution is indicated by degree of peak separation and sharpness; good resolution is highly desirable in any GLC analysis.

Quantification of GLC Data. As suggested previously the nature of components in an unknown sample can be determined by comparing their retention times with the retention times of known components in a standard mixture. Once it is known "what the component is", peak area can be used to determine the concentration of components in the sample.

The basic idea behind quantitative GLC analysis is that peak area is proportional to the concentration of components in the sample. However, detector response varies with different components, and slight corrections are needed to ensure that a given peak area is equivalent to a given molar concentration. Usually this correction is small, and often ignored; however, computerized integration systems are usually capable of correcting for variations in detector response.

A further problem in quantification of GLC data is that variations in temperature, gas flow, and so on affect peak area. For this reason, most quantitative techniques employ some type of internal standard addition to adjust for such variations. An example of GLC quantification of volatile fatty acid concentrations is shown in Table 13-2. In the Animal Nutrition Laboratory, a digital, computing integrator is used for GLC analysis. The procedure for quantification of volatile fatty acids in ruminal fluid is attached to this chapter.

In summary, GLC is extremely important in quantitative analysis of a number of organic compounds. Applications include the measurement of fatty acids, amino acids steroids, pesticides, and a large number of other biologically important compounds.

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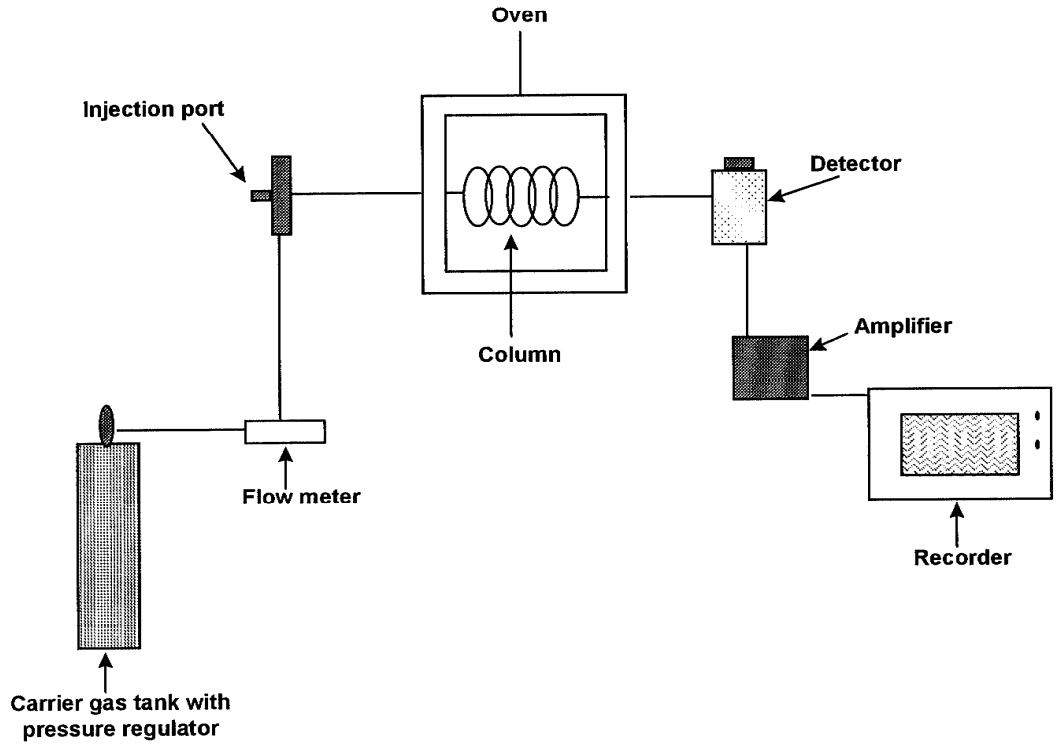

Figure 13-1. Basic Components of a gas chromatograph. Adapted from Zweig and Sherma (1972).

Table 13-1. A comparison of detectors used in gas chromatographs. Adapted from Fritz and Schenk (1979)

Detector	**Output**	**Uses**
Flame ionization	Output proportional to ions produced when sample is burned in H_2 + air flame.	Used for organic compounds. No response to CO_2, H_2, CO, H_2S, and NH_3. Useful for analysis of water extracts.
Thermal conductivity	Output is difference in thermal conductivity between pure carrier gas and carrier gas plus sample.	Used for both inorganic and organic compounds. Sensitive to changes in gas flow and termperature.
Electron capture	Output proportional to change in current of carrier gas/β particle interaction caused by sample components.	Used for O-, P-, S-, NO_2- and halogen-containing compounds. Weak response to ethers and hydrocarbons. Use ful in pesticide and insecticide analysis.

Table 13-2. Example of quantitation of gas liquid chromatography data for volatile fatty acids

Procedures outlined in the Animal Nutrition Laboratory handout for volatile fatty acid analysis were followed, using 2-ethyl butyric acid as an internal standard. Standards and unknown samples were injected on the GLC and peak area values were determined with a computing digital integrator. Values for acetic acid are shown below.

Standard acetate area* = 10,000

Standard 2-ethyl butyrate area = 5,000

Unknown acetate area = 8,000

Unknown 2-ethyl butyrate area = 5,000

Weight of acetate in standard = 3.5 g/L

Weight of 2-ethyl butyrate in standard = Weight of 2 ethyl butyrate in unknown

Calculate area ratios as follows:

1. Acetate/2EB area ratio in std = 10,000/5,000 = 2

2. Acetate/2EB area ratio in unknown = 8,000/5,000 = 1.6

Given that the amount of 2-ethyl butyrate is the same in both unknown and standard, we can set up the following proportion:

(x/3.5 g/L) = (1.6/2), where x = the concentration of acetate in unknown samples.

x = 2.8 g/L of acetate

This same procedure could be used for other acids in the sample. The key point in this procedure is that the amount of 2-ethyl butyric acid is **exactly the same** concentration in standards and unknown sample.

*Peak area values are corrected for variation in detector response.

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Analysis of Volatile Fatty Acids in Rumen Fluid

M. L. Galyean

1. Reagents

   a) 25% (wt/vol) metaphosphoric acid solution containing 2 g/L of 2-ethyl butyric acid (exact amount of 2EB must be known).

      i) 2.165 mL of 2-EB = 2.00 g (use Hamilton syringe).

   b) VFA standard

      i) Add the following volumes of acids to a 100-mL volumetric flask, and fill to volume with deionized H_2O. Use a Hamilton syringe for all measurements. Store in refrigerator when not in use.

a)

		MW	Vol., μL	Conc., g/L
(1)	Acetic	60.05	330	3.46
(2)	Propionic	74.08	400	3.97
(3)	Isobutyric	88.10	30	0.29
(4)	Butyric	88.10	160	1.53
(5)	Isovaleric	102.13	40	0.375
(6)	n-valeric	102.13	50	0.471

2. Sample and Standard Preparation

   a) Centrifuge strained ruminal fluid at 10,000 x *g* for 10 min.

   b) Mix 5 mL of ruminal fluid supernatant with 1 mL of meta-phosphoric acid-2EB solution.

   c) Stand in cold (ice bath) for > 30 min.

   d) Centrifuge for 10 min. at 10,000 x *g*.

   e) Take supernatant fluid for GLC injection.

   f) **Standard preparation** - to ensure 2-EB amount is the same in standard and sample, take 5 mL of standard prepared in Section I and add l mL of the metaphosphoric:2-EB solution. Mix completely, and keep in cold.

3. Equipment and analytical conditions

   a) Shimadzu GC-14A gas chromatograph

   b) Shimadzu Computing Integrator

      i) See instructions in Integrator Manual for calculations methods.

   c) Column

      i) Supelco 1-1965 10% SP-1200/1% H_3PO_4 on 80/100 Chromosorb W

ii) Temperature

a) Column - 135^0C or 140^0C

b) Inlet - 180^0C

c) Detector - 175^0C (flame ionization)

iii) Gas flow

a) Carrier = 40 cc/min

b) H_2 and air – Set a manufacturer's specifications for the flame ionization detector

iv) Injection size - 1 or 2 μL for samples and standards

d) Calculation

i) Integrator will calculate desired units (g/L, mmole/L, etc.) of VFA and total concentration

ii) Calculate molar % as: 100 x (concentration of individual acid/total VFA concentration)

CHAPTER XIV

Microscopy

The compound microscope is a common instrument in animal nutrition laboratories. It can be used for histological or hematological procedures, as well as evaluation of gastrointestinal tract (GIT) microorganisms. The observation, classification, and quantification of GIT bacteria and protozoa can be performed with the aid of light microscopes.

The compound microscope has two lenses, the objective lens, which is placed close to the object being viewed, and the ocular lens, which is close to the eye of the observer (Brock, 1970). Image enlargement is primarily a function of the objective lens; however, the total magnification of a compound microscope is the product of the magnifications of the objective and ocular lenses. A diagram of a typical compound microscope, adapted from Brock (1970). is shown in Figure 14-1.

One important property of microscopes is the ability to separate and distinctly reveal two points that are close together. This property is known as **resolution** or resolving power. Generally, microscopes with high resolution are good for viewing small structures (Brock, 1970). Resolution is a function of the wavelength of light and the **numerical aperture** of the objective lens. Numerical aperature (NA) is used to compare the resolving power of objective lenses, and the larger the NA, the greater the resolution because the wavelength of light is usually fixed.

Two commonly used objective lenses are achromatic and planoapochromatic lenses. Achromatic lenses partially correct for lens aberration resulting from the separation of light into component spectra (chromatic aberration) and aberration resulting from the inability to focus different colors of the spectrum in one place (spherical aberration). Planoapochromatic lenses completely correct for both spherical and chromatic aberration and are quite useful in photomicrography (Davis, 1980). The NA of objective lenses can be affected by the medium through which light passes. When light passes through air between the object being viewed and the objective lens, the lens can never have a NA greater than l.0, and oils are often used as a medium between object and lens to obtain NA greater than 1.0. Many high-power objective lenses are oil-immersion lenses and have quite high NA of 1.2 to 1.4 (Brock, 1970).

The light available for viewing in a microscope is a function of the light source and the condenser lens system. The condenser lens serves to focus light on the object being viewed and contains an iris diaphragm, which controls the diameter of the circle of light as it leaves the condenser. As such, the iris ensures that the light passing through the object will be equal to the opening in the objective lens, thereby eliminating problems of stray light and glare (Brock, 1970). Several types of optical systems are available on microscopes. Perhaps the most common system is the **bright-field microscope**, in which direct light is presented to the viewer. Generally, staining procedures of various types are used to provide contrast between the object being viewed and the surrounding medium. A common example is the use of dyes to stain microorganisms. The path of light in a typical bright-field microscope is shown in Figure 14-2.

Other types of optical systems allow observation of specimens without staining. The **phase-contrast microscope** works on the principle that specimens differ in refractive index from their surrounding medium, and this difference can be used to produce an image with a high degree of contrast (Brock, 1970). Light is passed through an annular ring in the condenser. Light passing through the specimen is retarded when the specimen's refractive index is different from the medium. A phase ring or stop in the objective lens shifts the phase of light. Light passing through the slide that is unretarded by the specimen appears as normal white light to the viewer, whereas light refracted by the specimen has a longer light path and reaches the viewer out of phase with unretarded light. Interference between the retarded and unretarded light produces an image of the specimen. Usually, the image is dark on a bright background (Brock, 1970). Phase-contrast microscopy is quite useful for observing specimens in the living state, avoiding problems that may be encountered with staining procedures. The light path in a phase-contrast microscope is illustrated in Figure 14-2.

The **dark-field microscope** has a condenser system that contains a central stop, which does not allow light to shine directly into the objective lens. Specimens placed in the path of this oblique light will deflect some of it into the objective lens, producing a bright image on a dark background. This system is illustrated in Figure 14-2. Dark-field microscopy also is useful for observing live specimens without staining.

Another useful type of microscope is the fluorescence microscope, which is used to observe objects that fluoresce, either from natural fluorescence or the use of fluorescent dyes. Fluorescent microscopes are often used in immunological studies, in which an antibody labeled with a fluorescent dye can be used to identify specific cells. This method also has been used in counting specific strains of bacteria in ruminal contents (Jarvis et al., 1967).

Students who desire further reading on the specific applications of microscopy to counting procedures with ruminal bacteria and protozoa should consult Hungate (1966) and Warner (1962). A bacterial and protozoal counting procedure employed by the Animal Nutrition Laboratory is attached to this chapter.

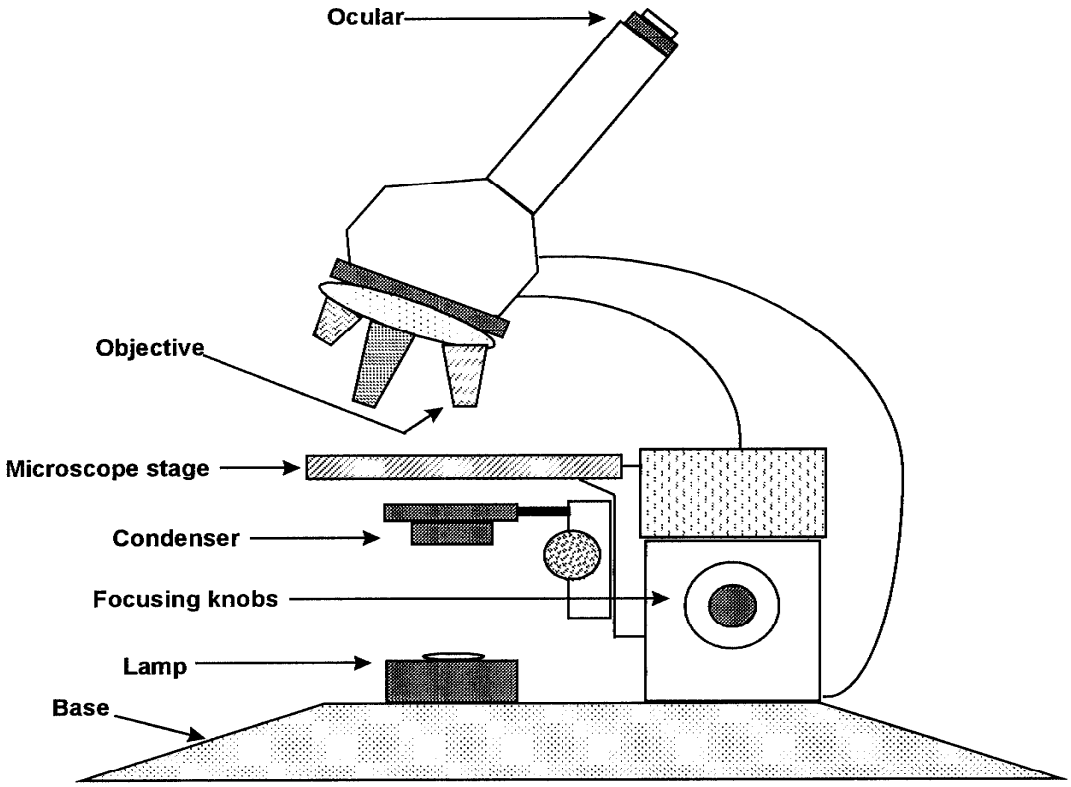

Figure 14-1. Diagram of a compound light microscope. Adapted from Brock (1970).

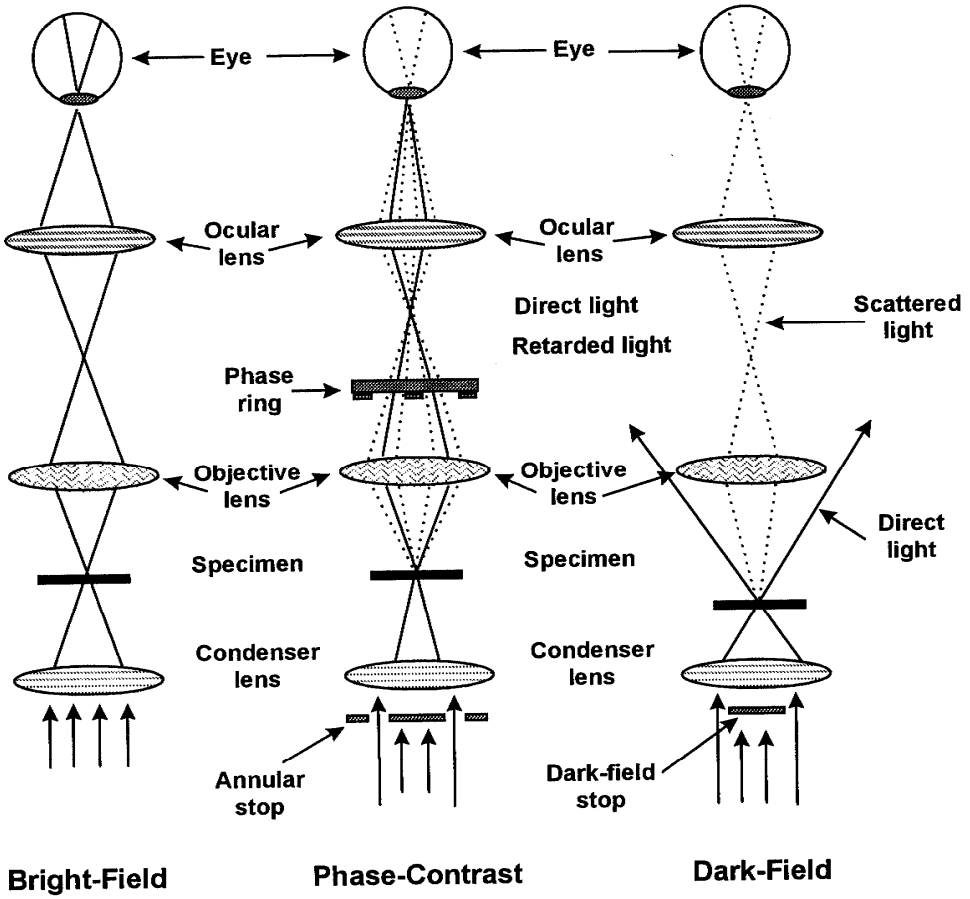

Figure 14-2. Light paths of bright-field, phase-contrast, and dark-field microscopes. Adapted from Brock (1970).

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Animal Nutrition Laboratory

Procedure for Total Count of Ruminal Protozoa and Bacteria

1) Collect about 20 mL of ruminal fluid; stirring contents and collecting from top, middle, and bottom of fluid phase.
2) Using large-bore pipette (so protozoa will fit), add 1 mL of fluid into previously prepared solution (P.P.S. is 9 mL of 0.85% [wt/vol] saline solution with 10% formalin [vol/vol]). This will kill protozoa and bacteria. This step yields a 1:10 dilution. After diluting, keep samples refrigerated.
3) For protozoa counts, use a Sedgewick-Rafter chamber, and add cover slip. Add grid to eye piece of microscope. Count 25 large squares (randomly selected), divide number of protozoa counted by 25 to find the average per square. A higher dilution may be necessary. Counting at 100 power will probably be sufficient.
4) For bacterial counts, use a Petroff-Hausser chamber. Count 1 set of 25 squares, sum the counts, and divide by 25 to obtain average number of bacteria per square. Use phase contrast at 600 power.

CHAPTER XV

Liquid Chromatography

As previously discussed in Chapter XIII, chromatography is a physical method for separation of components in a mixture. Liquid chromatography has long been an important analytical technique, but its use has grown rapidly in recent years with the development of high-performance systems. Thus, this chapter is designed to provide a brief overview of this rapidly expanding analytical technique.

As with gas chromatography, there are both stationary and mobile phases in any liquid chromatography system. The mobile phase is the liquid, whereas the type of stationary phase depends on the system. In thin-layer and paper chromatography, the stationary phase is spread as a layer, whereas in column chromatography, it is packed as a column in a relatively narrow tube (Yost et al., 1980). Our discussion will deal primarily with column chromatography.

Types of Liquid Chromatography. Yost et al. (1980) defined four modes of liquid chromatography, including: 1) **Adsorption chromatography** - the stationary phase is an adsorbent, and separation based on repeated adsorption-desorption steps; 2) **Partition chromatography** - separation is based on partition between mobile and stationary phases; 3) **Ion-exchange chromatography** - the stationary bed has an ionically charged surface of opposite charge to the sample. This method is used with ionic or ionizable samples. The mobile phase is an aqueous buffer, with pH and polarity used to control elution time; 4) **Size exclusion chromatography** - the column is packed with a precisely defined pore size material. The sample is screened or filtered according to molecular size differences.

With the first two modes, it is not always clear whether adsorption, partition, or both are the dominant processes (Yost et al., 1980); thus, the relative polarity of mobile and stationary phases are considered, and the terms normal-phase and reversed-phase chromatography are more frequently used than absorption and partition.

In **normal-phase chromatography**, the stationary phase is very polar (e.g., silica), and the mobile phase is nonpolar (e.g., n-hexane or tetrahydrofuran). Thus, the column retains polar samples longer than less polar or nonpolar samples (Yost et al., 1980).

In **reversed-phase chromatography**, the situation is exactly opposite of normal-phase. The stationary phase is nonpolar (hydrocarbon), and the mobile phase is a polar liquid (water, alcohol). Thus, the more nonpolar a sample component is, the longer it will be retained on the column (Yost et al., 1980).

In both normal- and reversed-phase chromatography, the mobile phase can be modified to adjust its polarity. More polar substances can be added in normal-phase, and less polar substances in reversed-phase. Figure 15-1, adapted from Yost et al. (1980), provides a graphical depiction of normal- and reversed-phase chromatography.

High-Performance Liquid Chromatography. As previously stated, recent advances in the

field of liquid chromatography have markedly increased the use of this technique in recent years. Classical liquid chromatography is characterized by gravity flow of the mobile phase, with collection of fractions from the column and subsequent quantification of sample components. Classical techniques are often slow and require considerable manual operation. Newer, high-performance techniques have been developed with the goal of overcoming some of the problems of classical methodologies. According to Yost et al. (1980), modern (high-performance) liquid chromatography differs from classical liquid chromatography in the following ways:

1. Small diameter (2 to 5 mm), reusable columns
2. Small particle (3 to 50 μm) column packing material
3. Relatively high inlet pressures and mobile phase flow control
4. Precise introduction of small sample sizes
5. Sensitive, continuous detector systems
6. Automated, standardized instruments
7. Rapid, high-resolution analysis

Mobile phase flow control is achieved with high-pressure pumps, resulting in greater accuracy and precision than gravity-flow systems. Because of the use of high-pressure pumps, the term "high-pressure liquid chromatography - "HPLC" has been used to describe the technique. Pressure as such, does not significantly improve the separation process, and the term "high-performance liquid chromatography" is preferred.

Components of a High-Performance Liquid Chromatograph. The basic components of modern HPLC systems include a pump, sample injection device, column (stationary phase), detector, and recorder or data handling system. Figure 15-2 is a graphical representation of these components.

Before we discuss each component, some general discussion of the mobile and stationary phases is necessary. In HPLC, the mobile phase usually plays an active role in separation (except for size-exclusion chromatography). Thus, selection of the mobile phase is an important aspect of analyses with HPLC (Yost et al., 1980). A single substance or a mixture of two or more substances may be used as the mobile phase. Moreover, the operator can use a constant mobile phase during analysis (**isocratic operation**) or change mobile phase composition during analysis (**gradient elution**). Gradient elution is useful when sample components vary widely in polarity, and a change in polarity of the mobile phase can improve separation (Yost et al., 1980). Gradients can only be achieved with the use of more than one pump and some type of gradient programmer and controller. Figure 15-3 illustrates a typical system for gradient elution. Selection of the appropriate mobile phase is beyond the scope of our discussion; however, students are referred to Snyder and Kirkland (1979) and Yost et al. (1980) for more details.

Stationary phases in today's HPLC systems are usually chemically bonded to support particles. These **bonded phases** are prepared by chemical reaction between surface hydroxyl groups of silica particles and a linear organic molecule or an organosilane (Yost et al., 1980). The most widely used bonded phases are nonpolar and are therefore used in the reversed-phase mode. These phases usually consist of an alkyl chain (e.g., octadecyl, C-18) bonded through the silica atom of the aklylsilane (Yost et al., 1980). Particle size of the support particles also is an

important consideration, and most modern packings range from 5 to 15 μm. Small particle sizes allow for the sample to be exposed to the maximum amount of packing surface (Yost et al., 1980). Students are referred to Yost et al. (1980) for more detail on stationary phases.

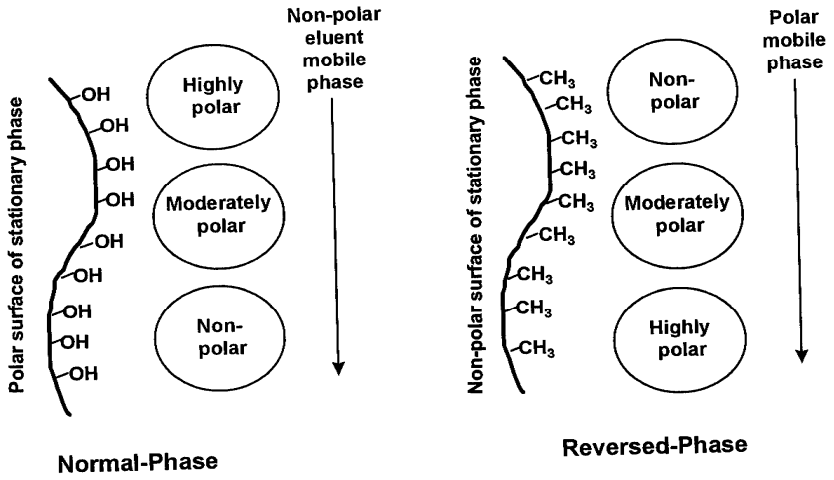

Figure 15-1. Graphical illustration of normal- and reversed-phase liquid chromatography. Adapted from Yost et al. (1980).. The circles represent the types of compounds present in the sample; their relative position to the direction of the mobile phase flow indicates their order of elution.

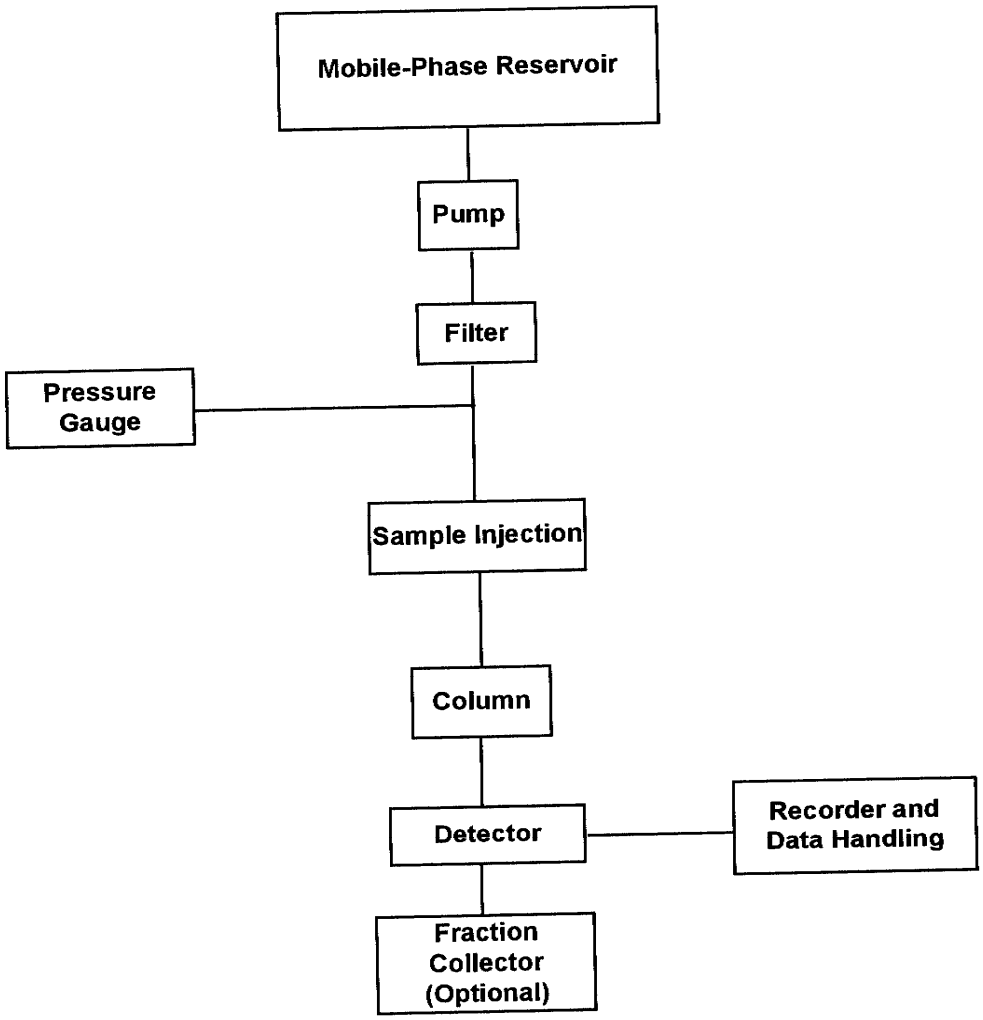

Figure 15-2. Basic components of a HPLC system. Adapted from Yost et al. (1980).

We will now briefly discuss each of the components of an HPLC system. Mobile phase flow control can be achieved by various types of **pumps**. Detailed descriptions of the types of pumping systems currently in use is beyond the scope of our discussion; however, all systems are characterized by precise control of mobile-phase flow. Typical flow rates range from 1 to 2 mL/min with 2- to 5-mm i.d. columns (Yost et al., 1980). Pressure increases as flow rate increases and can sometimes be a major problem because of increased leaks in system plumbing.

Sample injection in most modern HPLC systems is usually accomplished by specially designed injection loops. Samples can be injected with a microliter syringe into a loop that is not directly in the mobile phase flow line. Valves can then be used to direct mobile phase flow through the injection loop. Loop systems are advantageous because direct introduction of sample into the mobile phase flow line can be difficult because of the high pressure of the mobile phase.

Columns typically range from 15 to 25 cm in length and 2- to 5-mm i.d. in analytical work. Larger columns are often used in preparative HPLC work, where the operator desires to separate and collect fractions from the column. Recently, microbore HPLC has received considerable attention. Microbore columns are usually shorter than standard columns, with smaller internal diameters. Faster analysis time is one advantage of microbore HPLC. As previously indicated, selection of column packing material depends on the nature of a particular analysis. Many HPLC systems are equipped with column temperature regulators. Column temperature control may offer separation advantages in some cases and may improve overall column performance. Students are referred to Yost et al. (1980) for more details on temperature control.

Optical **detectors** are the type of detector most commonly used in modern HPLC systems. Usually, flow from the column is passed through a flow cell (small volume) in the detector, the presence of sample components is detected and a signal sent to recorder/data handling systems. Perhaps the most versatile detector in HPLC systems is the UV absorption detector (Yost et al., 1980). Variable wavelength UV/visible spectrophotometers equipped with flow cells are useful for the vast majority of analyses by HPLC. However, particular analyses may require the use of refractometers (sugar analyses) or fluorometers (analyses with fluorescent compounds or tags).

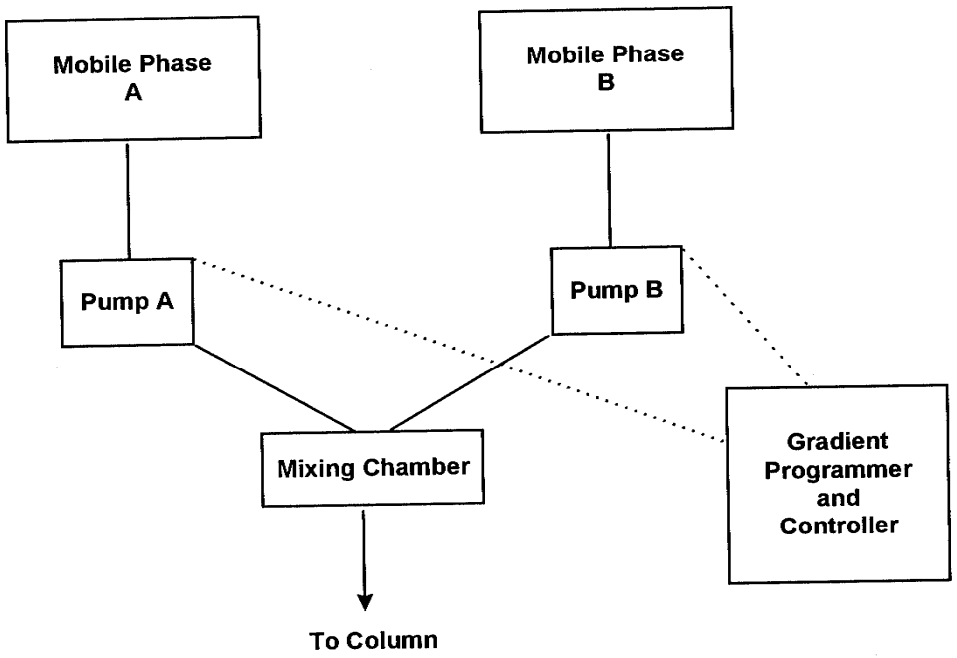

Figure 15-3. Basic components of a gradient elution system in HPLC. Adapted from Yost et al. (1980).

Recorder/data handling systems have greatly expanded quantitative data analysis by HPLC. Typically, recording, calculating integrator systems similar to those described in Chapter XIII for gas chromatography are used. Quantitative analysis in HPLC is usually achieved in the same manner as with gas chromatography, and the internal standard method is used frequently. Students are referred to Chapter XIII for consideration of the terms retention time, resolution, peak area, etc. that are as important in quantitative and quantitative analyses in HPLC as they are in gas chromatography.

In summary, this chapter is designed to provide a brief introduction to high-performance liquid chromatography. Students with additional interest in the subject are referred to Snyder and Kirkland (1979) and Yost et al. (1980) for additional references on the topic. Modern liquid chromatography is a technique with rapidly expanding applicability to the field of animal nutrition. Analysis of toxicants, carbohydrates, proteins, amino acids, nucleic acids, several vitamins and a host of other compounds can be achieved by HPLC. A procedure for analyses of phenolic monomers in forages by HPLC developed at the Animal Nutrition Laboratory is attached to this chapter.

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Animal Nutrition Laboratory

PHENOLIC MONOMER ANALYSIS BY HPLC

General. Phenolic monomer analysis may be a useful tool to evaluate forage quality. Free phenolics, or phenolics as components of tannins and lignin are potentially major anti-quality factors in forages. The following procedure was developed in the New Mexico State University Animal Nutrition Laboratory to be used with the Beckman Model 334 HPLC in the laboratory. The procedure can be easily adapted to other HPLC systems.

Equipment: Model 334 Beckman HPLC
Hitachi Variable Wavelength UV/VIS Detector
SpectraPhysics Model 4270 Recording Intergrator
Ultrasphere-ODS 5 μm 4.6-mm i.d. x 15-cm column
Ultrasphere-ODS 5 μm guard column

Reagents: HPLC-grade methanol
HPLC-grade acetonitrile
Reagent grade glacial acetic acid
Reverse osmosis-purified water, or double-distilled water

All reagents should be degassed and filtered before use in the HPLC.

Preparation of solvent. Mix water, acetonitrile and glacial acetic acid in the ratio of 425:65:10. Degas before use.

Sample Preparation. Reference - Jung et al. (1983) J. Anim. Sci. 57:1294. Weigh 500 mg of air-dried sample, and extract with 20 mL of 1 N NaOH under nitrogen. This can be accomplished by using screw-cap or tightly stoppered vials and flooding the sample with nitrogen as it is stoppered. Shake vigorously for 24 h in a darkened room. Samples are then filtered (Whatman No. 541), washed with 20 mL of water, and filtered again. Acidify filtrate to pH 2.5 with concentrated HCl. **Be careful**. Not much HC1 will be needed. Use a pH meter to follow the pH change. Next, saturate the sample with NaCl (approximately 7 g), and extract three times with 50-mL portions of ethyl ether. Use a separatory funnel for these extractions. **The process is very messy, so do not give up because things are looking bad**. Discard the aqueous phase from each extraction. Combine the ether fractions, and wash the ether with anhydrous sodium sulfate (approximately 4 g per sample). Allow the ether to evaporate to dryness. There will be a dark, tar-like substance remaining after the ether has evaporated. Resuspend this substance with 5 mL of methanol. Add the appropriate amount of internal standard to each sample at the time the methanol is added. The internal standard is p-anisic acid. Ensure the same amount of internal standard is added to samples and standard.

HPLC Conditions. The procedure involves an isocratic separation of phenolics. The solvent (water:acetonitrile:glacial acetic acid) is pumped at a rate of 1.5 mL/min. The column temperature is maintained at 30°C. Wavelength is set at 280 nm. Attenuation on the recorder is set at 8 and chart speed at .5 cm/min.

Standard Composition:

Component	Concentration	Approximate retention time, min
Protocatechuic acid	0.0001 M	3.5
4-OH Benzoic acid	0.0001 M	4.9
Vanillic acid	0.0001 M	5.8
Syringic acid	0.0001 M	6.3
4-OH Benzaldehyde	0.0001 M	6.7
Vanillin	0.0001 M	8.5
p-Coumaric acid	0.001 M	9.8
Umbelliferone	0.0001 M	l0.6
Ferulic acid	0.001 M	l2.4
Salicylic acid	0.001 M	l4.9
p-Anisic acid	0.001 M	l9.3

Twenty microliters of samples and standards are injected into the HPLC. After each sample is off the column, pure methanol is injected and allow to run for the same time as samples; this allows for purging (eliminates ghost peaks).

Syringaldehyde can be separated by the procedure, but only when p-coumaric is present in small amounts. Large concentrations of P-coumaric (0.001 M) will overpower and mask the syringaldehyde peak. Thus, syringaldehyde is not included in the standards. Sinapinic acid also can be separated, but seems to either not occur or to occur in very small amounts in samples; hence, this acid has not been included in the standards.

Quantification of peak data. Use of the DIALOG function on the SpectraPhysics recording integrator (or similar function on other integrators) will allow quantification of data.

APPENDIX

PROCEDURES

Animal Nutrtion Laboratory

DETERMINATION OF SILICA

MATERIALS:

1. 250-mL beakers
2. 6N HCl
3. Volumetric flasks (50-mL or 100-mL)
4. Funnels
5. Tared crucibles
6. Whatman No. 541 filter paper

CAUTION: Wear gloves, lab coat, and safety glasses for this procedure (**STRONG ACID**)

PROCEDURE:

1. Weigh out a 1-g sample, and ash for 6 h in a 600^0C muffle furnace.
2. Place the ashed sample into a clean and clearly labeled 100-mL Pyrex beaker, and add 50 mL of 6 N HCl (can use 1:3 HCl dilution with water).
3. Place the beakers on a hot place under the hood.
4. Boil off the acid. **CAUTION**: Do not leave your samples unattended, or the beakers can crack.
5. After removing from heat, add 25 mL of 6 N HCl to put the dehydrated ash back into solution.
6. Filter the liquid contents of each beaker into a funnel holding a piece of No. 541 filter paper, and collect the liquid into a labeled volumetric flask (save when mineral analysis is to be done). Bring to volume using deionized H_2O.
7. Place the filter paper into a previously tared crucible, and ash in a muffle furnace for 6 h at 600^0C. (Do not use aluminum pans - they will corrode.)
8. Weigh back residue as $Si0_2$.

CALCULATION: % silica = ({[wt of crucible + ash] - [crucible wt]}/sample wt) x 100

Animal Nutrition Laboratory

PURINE ASSAY

REFERENCE: Zinn, R. A., and F. N. Owens. 1982. Rapid procedure for quantifying nucleic acid content of digesta. In: F. N. Owens (Ed.). Protein Requirements for Cattle: Symposium. Oklahoma State Univ. MP:109:26.

PROCEDURE:

1. Weigh 0.5-g digesta sample and approximately 0.2 g of bacterial or standards (discussed later) into 25-mL screw cap culture tubes.
2. Add 2.5 mL of $HC10_4$ (70% perchloric acid) and tightly cap tube. Vortex until sample is wet. Incubate in 90^0 to 95^0C water bath for 15 min, remove from bath, revortex, and place in water bath for an additional 45 min.
3. Measure out 17.5 mL of dilute buffer (0.0285 M $NH_4H_2PO_4$). Add half the 17.5 mL volume, vortex vigorously to break up any black, charred mass, and add remaining half of the volume. Make sure no black clumps are sticking to the sides of the culture tubes. Vortex and reinsert tubes into 90 to 95^0C water for 10 to 15 min. Filter through Whatman No. 541 filter paper into disposable glass culture tubes.
4. Transfer 0.5 mL of filtrate to 15 mL centrifuge tubes, add 0.5 mL $AgNO_3$ (0.4 M), 9 mL buffer (0.2 M $NH_4H_2PO_4$) and allow to stand overnight.
5. Centrifuge for 10 min at approximately 1,000 x *g* and draw supernatant liquid.
6. Wash pellet with pH 2-adjusted H_2SO_4 (make this by adding enough sulfuric acid to water to bring the pH down to 2). Use 6 mL of acid for washing. Repeat Step 5. Disturb the pellet as little as possible during this step.
7. Add 10 mL of 0.5 N HCl, and vortex until thoroughly mixed.
8. Cover tube with a marble and allow to incubate in 90 to 95^0C water bath for 30 min.
9. Vortex and recentrifuge.
10. Standards are prepared by weighing 0.2 g (dry matter basis) of Torula yeast RNA and treating exactly like a sample. The clear liquid in Step 9 can be diluted to the appropriate range as follows:

Standard, mL	0.5 N HCl, mL	RNA, mg
0.25	9.75	5
0.50	9.50	10
0.75	9.25	15
1.00	9.00	20

11. Read absorbance of standards and samples in quartz cuvettes at 260 nm. The blank is 0.5 N HCl. Read the clear liquid fraction of samples obtained after centrifugation in Step 9; do not disturb or resuspend the pellet.

REAGENTS:

1. 0.2 M $NH_4H_2PO_4$ = 23 g of monobasic ammonium phosphate/L.
2. 0.0285 M $NH_4H_2PO_4$ (dilute buffer) = 143 mL/L of 0.2 M $NH_4H_2PO_4$.
3. 0.4 M $AgNO_3$ = 6.9 g/100 mL of pH 2-adjusted H_2SO_4. This may not go into solution very well. When used in Step 4 of the procedure, be sure to stir while adding. Keep $AgNO_3$ solution covered with aluminum foil.
4. 0.5 N HC1 = 40.2 mL ofconcentrated HCl/L.

CALCULATIONS:

1. Regress standard concentration on absorbance at 260 and compute milligrams of RNA. Divide milligrams of RNA by dry sample weight to calculate % RNA.

Animal Nutrition Laboratory

VANILLIN/HCL PROCEDURE FOR CONDENSED TANNINS

PROCEDURE:

1. Weigh 0.5-g samples in duplicate.
2. Place in test tubes (15-mL polystyrene tubes with caps).
3. Extract with 10 mL of 1% (vol/vol) HCl in methanol for 20 min (place tubes in rack and cap, then invert by hand for 20 min).
4. Centrifuge at 1,000 x *g* for 10 min.
5. Prepare standards (use 10-mL volumetrics). Add 0, 0.5, 1, 1.5, and 2 mL of standard to volumetrics and bring to volume with methanol.
6. Pipette 0.5 to 1 mL of unknowns and standards into test tubes (20-mL disposable culture tubes) in duplicate (two tubes for each extract tube, one serves as blank).
7. To one tube, add 5 mL of vanillin/HCl reagent. To the other tube, add 5 mL of 4% (vol/vol) HCl in methanol (background correction).
8. Place in 30^0C H_2O bath
9. After 20 min in H_2O bath, vortex, and read on spectrophotometer at 500 nm.

REAGENTS:

1. 1% HCl in methanol - add 10 mL of HCl to 990 mL of methanol (use volumetric).
2. Standard solution - add 100 mg of anhydrous catechin to 50-mL volumetric and bring to volume with methanol. Prevent light exposure (use opaque volumetric). If catechin has one water of hydration, use 106.2 mg of catechin. This solution is good approximately 2 wk.
3. 8% HCl in methanol - 80 mL of HCl brought to 1 L with methanol.
4. 4% vanillin in methanol - 40 g of vanillin (assume anhydrous) in 960 mL methanol. This solution is good approximately 2 wk in opaque container.
5. Vanillin/HCl reagent - mix equal volumes of solutions prepared in Steps 3 and 4. Make fresh **immediately** before use.
6. 4% HCl in methanol - 40 mL of HCl brought to 1 L with methanol.

CALCULATIONS:

1. Standard curve. Zero serves as blank. The 0.5, 1, 1.5, 2 mL volumes equate to 0.1, 0.2, 0.3, and 0.4 mg/mL of catechin equivalents (stock has 2 mg/mL, and standards are diluted to 10 mL). Absorbance from these serve as X and 0.1, 0.2, 0.3, 0.4, and 0.8 serve as Y in standard curve background correction for standards usually is not necessary.
2. Unknowns. Absorbance of unknown minus absorbance of unknown blank is entered into standard curve to predict concentration. Divide result by sample weight and correct for dry

matter factor. Results are expressed as catechin equivalents (mg of CE/g of dry sample)

Animal Nutrition Laboratory

PROCEDURE FOR TOTAL PHENOLICS

PROCEDURE:

1. Weigh 0.5-g samples in duplicate into 125-mL erlenmeyer flasks.
2. Add 50 mL of 1% (vol/vol) HC1 in methanol, and seal with rubber stopper.
3. Place in shaker bath for 24 h extraction.
4. Add 0, 0.5, 1, 2, 3 and 4 mL of tannic acid stock solution to 100-mL volumetrics containing 75 mL of H_2O (for standard curve).
5. Add 1 mL of extract of unknown samples to 100-mL volumetric flasks that contain 75 mL of H_2O.
6. Add 5 mL of Folin-Denis reagent to standards and unknowns.
7. Add 10 mL of saturated Na_2CO_3 solution to standards and unknowns.
8. Bring to 100 mL with H_2O, and mix well.
9. Allow to set for 30 min, **mix well**, and read on spectrophotometer at 760 nm.

REAGENTS:

1. 1% HC1 in methanol - add 10 mL of HCl to 1-L volumetric and bring to volume with methanol.
2. Tannic acid stock solution (0.1 mg/mL) - dissolve 100 mg of tannic acid in 1 L of H_2O. Prepare fresh immediately before use.
3. Folin-Denis Reagent - add 100 g of $Na_2WO_4 \cdot 2H_2O$ to 750 mL of H_2O, mix well. Add 20 g of phosphomolybdic acid (correct for waters of hydration if necessary), and mix well. Add 50 mL of H_3PO_4, mix well. Reflux for 2 h (vigorous boil), allow to cool, and dilute to 1 L.
4. Saturated sodium carbonate solution - add 700 g of anhydrous Na_2CO_3 to 2 L of H_2O. Dissolve at 70 to 80^0C, cool overnight. Seed supersaturated solution with crystal of $Na_2CO_3 \cdot 10\ H_2O$, allow to crystalize for 24 h, then filter through glass wool.

CALCULATIONS:

1. Standard curve. Zero serves as blank. Stock contains 0.1 mg/mL. Concentration of standards is 0.05, 0.1, 0.2, 0.3 and 0.4 of mg tannic acid. These concentrations serve as X and the absorbance is Y for standard curve.
2. Unknowns. Absorbance of unknowns is used to predict concentration from curve. These values are multiplied by 50 (because 1 mL of original 50-mL extract was used). Divide by sample weight, then by dry matter factor to obtain mg of phenolics/g of dry sample.

Animal Nutrition Laboratory

SWAINSONINE ASSAY

Doug Kiehl and G. Stan Smith, New Mexico State University

REAGENTS:

1. 0.2 M Acetate buffer, pH 4.5
   a) 9.8 g of anhydrous sodium acetate
   b) 7.3 mL of glacial acetic acid
   c) Dilute to 1 L with deionized H_2O
   d) Check pH; adjust to 4.5
2. 0.013 M Glycine buffer, pH 10.7 to 11.0
   a) 10 g of glycine
   b) 4 g of NaCl
   c) 8.8 g of sodium bicarbonate
   d) Dilute to 1L with deionized H_2O, adjust pH with 50% (wt/vol) NaOH
3. Substrate
   a) 65 mg of p-nitrophenyl a-D-mannopyranoside (Sigma N-2127); add 200 mL of 0.2 M acetate buffer, and heat gently to dissolve
4. Enzyme stock (0.07 U/mL)
   a) Add 1 mL of α-D-mannosidase (Sigma M 7257) to 159 mL of .2 M acetate buffer and mix gently by inversion. This solution is typically good for 3 wk.
5. Swainsonine standard solution
   a) Stock solution (500 ng/μL)
      i) 500 μg of swainsonine (Sigma S-0264)
      ii) 1.0 mL of HPLC-grade methanol
   b) Working solution (500 ng/mL)
      i) 100 μL of stock
      ii) 100 mL of .2 M acetate buffer
      iii) mix gently
   c) **Note**: When not in use, store all reagents in refrigerator at 2 to 4^0C.

ASSAY:

1. Remove reagents from refrigerator, and allow warming to room temperature.
2. Set up standards as follows:

Swainsonine, ng	Working standard, μL	Acetate buffer, μL	Substrate, mL
Blank	0	900	1.4
0	0	600	1.4
50	100	500	1.4
100	200	400	1.4
150	300	300	1.4
200	400	200	1.4
250	500	100	1.4
300	600	0	1.4

3. Prepare each standard in duplicate (only one blank needed)
4. Set up samples as follows: add 1.4 mL of substrate. Sample volume should be ≤ 600 μL. Add sufficient acetate buffer to bring final volume to 2.0 mL.
5. Prepare each sample in duplicate, one blank for each sample (prepare identical to samples, but add extra 300 μL of acetate buffer).
6. Place all standard and samples in water bath (37^0C) for 1 h.
7. Add 300 μL enzyme solution to each tube with mixng (vortex). It is easiest to add to an identical pair of tubes, then allow 30 s between addition to tube pairs. **DO NOT ADD TO BLANK**.
8. Following enzyme addition, incubate all tubes at 37^0C for 1 h.
9. After incubation, stop reaction by adding 250 μL of reaction mixture to 3.0 mL of glycine buffer in a separate tube. Mix will by vortexing.
10. Record absorbance at 410 nm of each set of standards and samples after the spectrophotometer is set to zero using the corresponding blank.
11. Read sample concentrations from standard curve, or interpolate values from a best fit through standard point data.
12. Yellow color is stable for 12 h. As swainsonine concentration increases, an inhibition of yellow color formation will occur.

Animal Nutrition Laboratory

DETERMINATION OF SERUM OR PLASMA FREE FATTY ACIDS

REFERENCE: Smith, S. W. 1975. A new salting out technique for calorimetric free fatty acids assays. Analytical Biochem. 67:531-539.

REAGENTS:

1. Redistilled chloroform - keep refrigerated in brown bottle - use within 7 d.
2. Chloroform:Heptane:Methanol - 4:3:2 ratio - make up on the day of analysis.
3. 0.035 N HCl - 3 mL of HCl diluted to 1 L with water.
4. Stock salt solution - 200 g of Na_2S0_4 + 100 g of Li_2SO_4 + 40 g of $CO(NO_3)_2 \cdot 6\ H_20$ - make to 1 L with water - can be stored indefinitely.
5. Salt reagent - make up day of analysis - add 8 mL of triethanolamine to 100 mL of stock salt solution - let stand 15 min before use.
6. Indicator solution – 0.2 g of 1-nitroso-2-naphthol in 1 L of 95% ethanol. Store in brown bottle at room temperature.
7. Standards:

   Blank = heptane

   Stock solution - 2,000 μmoles of palmitic acid/L = 0.5128 g of palmitic acid/L of heptane

   Standards - dilute stock solution with heptane to yield 1,000, 500, and 250 μmoles/L

EQUIPMENT**:**

1. 12 mm x 100 mm screw-cap culture tubes with teflon-lined caps
2. Shaker
3. Centrifuge
4. Spectrophotometer at 435 nm

PROCEDURE**:**

1. To culture tube add 100 μL of heptane (blank), standard, or plasma (run samples in duplicate).
2. Add 4 mL of chloroform:heptane:methanol solution and cap.
3. Shake for 5 min.
4. Add 1 mL of 0.035 N HCl and recap.
5. Shake for 3 min.

6. Centrifuge for 4 min at 1,000 x *g*
7. Aspirate and discard the upper aqueous methanol supernatant fluid - **NOTE** - do not disrupt the protein layer at the interface of the supernatant and lower phase.
8. Add 2 mL of cobalt reagent and recap.
9. Shake for 3 min.
10. Centrifuge at 1,000 x *g* for 6 min.
11. Transfer 2 mL of upper supernatant liquid to clean culture tube as soon as possible (**less than 30 min - IMPORTANT!**).
12. Add 1 mL of indicator and vortex.
13. Let stand for 30 min for color to develop. Color is stable for approximately 1 h.
14. Read at 435 nm in spectrophotometer.

STANDARDS AND CHEMICALS:

1. $CHCl_3$ - Wash with 5% (vol/vol) H_2S0_4, H_20, and then distill.
2. Standards: Stock: 2,000 μmoles of palmitic acid/L = 0.5128 g/L of $CHCl_3$.

   1,000 μmoles/L = 50 mL/100 mL of $CHCl_3$

   500 μmoles/L = 25 mL/100 mL of $CHCl_3$

   250 μmoles/L = 12.5 mL/100 mL of $CHCl_3$

   or

   1,000 μmoles/L = 5 mL/10 mL of $CHCl_3$

   500 μmoles/L = 2.5 mL/10 mL of $CHCl_3$

   250 μmoles/L = 1.25 mL/10 mL of $CHCl_3$

SUGGESTION FOR ANALYSIS F.F.A.:

1. Use indicator within 2 d.
2. Use chloroform within 1 wk or redistill.
3. Make sure tubes are **very** clean.
4. Use a high pool and low pool in each run (high and low serum FFA).
5. Separation after cobalt addition may not occur - this requires a rerun.
6. 54 tubes at a time, which equates to 20 samples in duplicate.

TYPICAL DATA:

Day 1		Day 2	
mM	Mean absorbance	mM	Mean absorbance
0.125	0.238	0.125	0.243
0.250	0.419	0.250	0.401
0.500	0.864	0.500	0.807
1.000	1.373	1.000	1.379

Day 1: Concentration, mM = 0.7570 x Absorbance - 0.078969

Day 2: Concentration, mM = 0.76125 x Absorbance - 0.069874