Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits

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Washington, DC

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2003
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Prepared by a Task Force of the

ILSI International Food Biotechnology Committee

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Foreword

The use of biotechnology to modify the genetic makeup of plants to contain insect protection, herbicide tolerance, and virus resistance traits has led to a new generation of crops, grains, and their byproducts for food and feed. Such new agricultural products have aroused an increased interest in the safety of food and feed produced from genetically modified (GM) plants. In addition, feed, livestock, and allied industries wanted the biotechnology industry to demonstrate similar performance between livestock and poultry fed conventionally and those fed genetically enhanced crops, grains, and their byproducts. As a result, the biotechnology industry has initiated and conducted many livestock and poultry studies to evaluate GM crops. Other scientists in many areas of the world have expressed a desire to conduct livestock and poultry performance studies. Because of the potential effect of the results of these studies on the future of biotechnology, it is imperative that studies be conducted with the utmost scientific rigor and sensitivity.

The purpose of this document is to recommend guidelines for the production, harvest, sampling, and analysis of GM plants containing input traits (i.e., insect protection, herbicide tolerance, virus resistance) and for the conduct of animal experiments using these plants. The rationale for this project was to provide a platform that could serve as the basis for the international harmonization of study protocols for livestock and poultry performance. This publication should be a good reference for animal scientists worldwide in academia, industry, and government desiring to conduct studies with livestock and poultry fed GM crops and their byproducts. In preparing this document, scientists with expertise in areas such as animal nutrition (various farm animal species and poultry), health, feed chemistry, statistics, and other relevant disciplines developed a process whereby guidelines were developed under the leadership of the International Life Sciences Institute (ILSI) in collaboration with the Federation of Animal Science Societies (FASS). The authors decided that the study guidelines for each animal species should be written as a stand-alone procedure to simplify their use.

This document has been reviewed in draft form by individuals internationally recognized for their diverse perspectives and technical expertise in the respective animal species areas. The authors would like to thank the following individuals for their participation in the review process and for providing many constructive comments and suggestions:

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Chapter 1: Introduction

Traditional genetic selection—the selection of seed with desirable traits from superior plants or selection of animals with desirable traits and reproducing these through breeding—has been performed for centuries. None of the current food plants resembles its wild counterpart as a result of centuries of modification to improve quality, production, and hardiness. These methods have significantly increased productivity, with maize and wheat yields approximately doubling over the past 40 to 50 years, substantial improvements in milk yield per cow, and more efficient use of feed and leaner pig meat, just to name a few. However, without continued innovation meeting the challenges to expand agricultural production at a rate exceeding population growth, starvation will be inevitable. The projected doubling of the global population will require at least a doubling of the amount of food that will be needed in the next 30 to 50 years (Kendall et al. 1997).

Biotechnology, the application of biological processes for industrial purposes, also has a long history of use by mankind. Uses include the production of foods such as bread, vinegar, cheese, yogurt, pickles, sauerkraut, soy sauce, wine, beer, tempeh and natto (fermented soybeans), belacan (fermented shrimp paste), and budu and ngoc nam (fermented fish sauce). Modern biotechnology uses the tools of genetics to add new beneficial traits to plants, animals, and microorganisms for food production or to enhance preexisting beneficial traits. The process involves adding or removing, with more precision than natural breeding, specific genes to achieve a desired trait. The ability to introduce specific DNA directly into crop plants enables a selective plant improvement process that may enhance agricultural productivity while using more sustainable and environmentally sound approaches. Numerous traits are being evaluated for their potential, for example, to protect plants against insect damage and fungal, viral, or bacterial diseases; provide selectivity to more desirable herbicides for improved weed control; directly enhance crop yields; increase nutritional value to animals and humans; reduce naturally occurring toxicants or allergens; modify the ripening process and provide superior sensory qualities; use plants to make such products as biodegradable polymers or pharmaceutical products; modify food composition for disease prevention; and reduce input of required natural resources (e.g., water, nutrients, fossil energy). Although biotechnology provides an important tool to help address many of these challenges, this tool must be effectively integrated with the best current agricultural practices that encompass the most productive and environmentally appropriate technologies around the world.

Transgenic crops developed through biotechnology, often referred to as genetically modified (GM) crops, represent a new tool in the production of food, feedstuffs, and fiber that can make a vital contribution to the ever-increasing global need. From 1996 to 2002, global adoption rates for transgenic crops increased from 2 million to 58.7 million hectares (James 2003) as a result of grower recognition of more convenient and flexible crop management, higher productivity or profit per hectare, a safer environment through decreased use and exposure to conventional pesticides and herbicides, reduced health risks, diminished environmental effects, and an even safer food and feed supply through reduced mycotoxin levels (James 2003, Masoero et al. 1999, Munkvold et al. 1999). Adoption of this technology provides a means to contribute to a more sustainable agriculture.

As the adoption of GM crops grew, the animal production industry and related associations began to receive questions about the performance and safety of farm animals fed GM crops. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations advocate the concept of substantial equivalence as the most practical approach to address the safety evaluation of foods, feeds, or food components derived from modern biotechnology (FAO/WHO 1991, 1996, 2000). In this approach, it is assumed that new food or food components that are substantially equivalent to an existing food or food component can be treated similarly with respect to safety. Substantial equivalence evaluation focuses on the product rather than the process used to develop the product. A rigorous safety assessment including the nature of the gene and expressed protein, molecular characterization, agronomic traits, nutritional and antinutritional traits, and toxicology is conducted before a crop is deemed safe and is released for commercial use. The goal of studies of substantial equivalence is to determine whether the transgenic product is substantially equivalent (in terms of chemical and nutritional composition and characteristics) to its conventional counterpart that has a history of safe use.

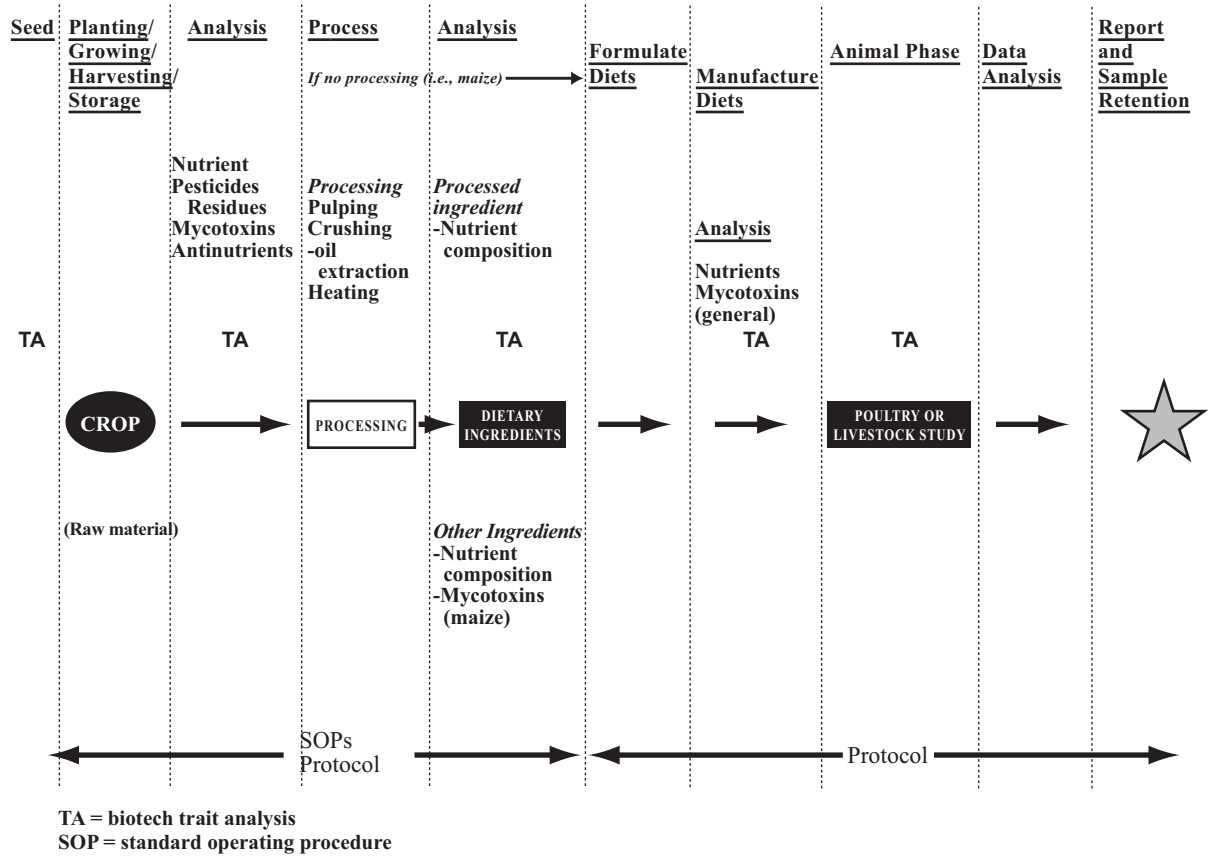
The livestock industry has expressed interest in determining whether transgenic crops and their products derived from processing are nutritionally equivalent to conventional counterparts when fed to livestock. In response, numerous studies have evaluated performance and product quality of farm animals fed transgenic crops compared with control and commercially available varieties. Aumaitre et al. (2002), Faust (2002), Clark and

Ipharraguerre (2001), Faust (unpublished, 2001), Flachowsky and Aulrich (2001), and Flachowsky et al. (2000) recently summarized data comparing performance of farm animals fed GM crops with animals fed conventional counterparts and concluded that there were no differences. There is now global interest in the conduct of livestock and poultry feeding studies with genetically enhanced crops and their products. For valid conclusions from research studies to be drawn, the quality of the product being tested must be verified and each study must be designed and conducted in a scientifically valid and rigorous manner using internationally recognized best practices (VICH 2000).

The purpose of this document is to recommend guidelines to scientists on how to produce, handle, store, and process transgenic crops containing input traits; sample and analyze the harvested and processed crop; design and conduct livestock and poultry studies; and analyze and interpret the results. This publication focuses on input traits (i.e., traits such as those that protect the crop from disease or insect damage or that provide tolerance to herbicides). These traits are of primary benefit to the producer. Output traits (i.e., traits that increase nutritional value, reduce naturally occurring toxicants, enhance flavor, or yield pharmaceutical products) will be discussed in a subsequent publication.

An overall flow diagram from the production of seed to final evaluation in animal studies is shown in Figure 1-1. Handling and disposing of unapproved GM crops and animals fed such crops should be done according to each country's regulations.

Figure 1-1. Project flow diagram for animal studies.



TA = biotech trait analysis
SOP = standard operating procedure

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Chapter 2: Production, Handling, Storage, and Processing of Crops

A key component of good design of animal performance studies is the production of high-quality test material and the appropriate control material. Commercial seed of high quality should be obtained from a reliable source. The negative control material should be produced from a near isogenic counterpart of the transgenic material and should be genetically similar except for the genetically modified (GM) trait. If resources are available, it is advisable to include several commercially available nontransgenic varieties to compare with the transgenic and its near isogenic counterpart.

Planting of Crops

Complete records should be maintained of the seed planted, including source, variety, line, event, seed population, seed type, and planting dates. Examples of the types of information to be recorded and samples of forms to record data are given in Appendix 2-1. Specific location and country guidelines for the production of certified seed or regulated plantings for spatial, temporal, or physical isolation measures should be adhered to. Careful planning should be undertaken to avoid cross-pollination of nontransgenic and transgenic crops. See Table 2-1 for examples of isolation distances for transgenic crops. The absence of cross-pollination can be confirmed by analysis for the GM trait.

The test material (GM and near isogenic counterpart) should be produced in a location that is representative of the commercial production of the crop. Test plot preparation and planting (e.g., row and plant spacing) should simulate local commercial practices for the test crop. The transgenic and its near isogenic counterpart must be produced at similar if not identical environmental locations. Soil characterization is not normally required; however, the soil type should be typical for the test crop production in the trial site area and should be recorded.

Field plots being used for the production of the test material may be planted in replicates depending on the location and amount of material needed. The production plot should be sufficiently large that the edges do not have to be harvested. Uniformity issues can be avoided by collecting samples from the interior of the plot, especially when the plots are small (less than 0.1 hectare). Each plot should be clearly and uniquely identified (e.g., labeled stakes or flags) and related to a permanent field marker.

Growing Season

Careful record keeping should continue throughout the growing of the test material. Records should include dates of pesticide treatment, visual observations relating to insect and disease infestations, and irrigation and fertilization dates and rates. Samples of forms that can be used to collect these data are given in Appendix 2-2. The sample forms are for an experiment with maize and would need to be modified appropriately for other crops.

Table 2-1. Examples of isolation distances for transgenic crops*

Crop	Isolation distance
Alfalfa	183–402 m (one company uses 275 m as its performance standard)
Canola (oilseed rape)	200 m or 10 m pollen trap of nontransgenic type that flowers at the same time as the genetically modified type (the pollen trap area must be destroyed)
Maize (open pollinated)	200 m
Cotton	200 m or a 12 m perimeter of nontransgenic cotton to act as a pollen sink for insect pollinators (this material must bebe destroyed)
Upland cotton versus Egyptian cotton	402 m
Soybeans	Space sufficient for mechanical mixing to be avoided (equipment dependent
Sugar beets	3-6 m between blocks to avoid mechanical mixing and 6 m surrounding plot area to minimize escape of material
Wheat 10 m	10 m

*Adapted from the U.S. Environmental Protection Agency (1999).

The GM trait of the test material determines the agronomic practices required during the growing season. For example, if the GM trait relates to herbicide tolerance, only the transgenic variety should be sprayed with the herbicide of interest. The treated plot must be planned so that treatment with commercial type or small plot application

equipment is possible. The near isogenic counterpart and commercial lines would not confer herbicide tolerance and would therefore not be sprayed with the herbicide of interest. If additional herbicide treatments outside scope of the herbicide tolerance trait are deemed necessary, all plots should be treated identically. The control plots should be located at least 15 m upwind (prevailing wind) and upslope from the herbicide-treated transgenic plot. If the GM trait is related to insect tolerance, identical insecticide treatments should be used on all plots. Agronomic practices (irrigation, fertilization, etc.) should be identical for all plots and careful records of all agronomic treatments should be made.

Consideration of adequate and timely moisture for normal plant growth and development throughout the test is important. All normal and prudent crop maintenance activities should be conducted to ensure normal plant growth and development.

Harvesting Grain Crops

Grain samples should be harvested at normal maturity. Grain should preferably be field-dried to a maximum of 15% moisture (85% dry matter) before harvesting. If necessary, the shelled grain should be dried at the field site to achieve a moisture level below 15% before analysis.

Precautions must be taken to preserve test material identity during harvest. Test material may be harvested by hand or by mechanical means as appropriate. Sampling details should be recorded. Special considerations and procedures may be put in place for regulated material in an effort to ensure that material has been thoroughly purged from commercial equipment. Whenever possible, all equipment should be used first for nontransgenic crops and then for transgenic crops. Harvesting equipment should be thoroughly cleaned between nontransgenic and transgenic plots and between nontreated and treated crops. A test strip of the test crop should be harvested (flush run) and discarded to ensure that harvest equipment is free of contaminants. All harvest equipment should be adjusted to remove the maximum amount of fine particles and foreign matter from the grain.

The test material chain of custody must be maintained and carefully recorded through planting, production, harvest, storage, sampling, and analysis. An example of a form for documenting chain of custody is given in Appendix 2-2.

Maintenance of Crop Transporting Equipment

Equipment and vehicles for transporting genetically modified grain and silage should be clean and visually inspected for contaminants before a crop is transported. Drivers should have clear instructions on where to deliver the crop, and the transported material should be properly identified. Whenever possible, nontransgenic crops should be transported before transgenic crops.

Maintenance of Grain Storage Locations

Storage locations should be cleaned carefully and visually inspected before storage of the GM crop material. Legs, pits, conveyors, augers, and all other grain- or silage-handling equipment should be cleaned and inspected. Spilled grain around storage locations should be removed to reduce contamination and rodent problems. Storage locations should be inspected for structural soundness; open areas that can lead to grain spills and entry points for water, insects, and rodents should be sealed. If storage locations are infested with insects, fumigation and residual insecticide applications (using only products approved for bin and grain treatment) may be necessary.

Storage locations, which should be in a secure area, must be clearly labeled to identify the GM crop material. Storage locations should be properly sealed after harvest to reduce contamination from other grains or silage.

Unloading of Grain

Before grain is unloaded, procedures should be reviewed and all equipment should be inspected and approved. The unloading supervisor should inspect all incoming grain to ensure that purity and quality mandates are met. A sample of each load should be retained for quality assurance. A complete record of all transfers, by bin and silo, should be maintained.

Storage of Grain

Grain should be cleaned of fine particles and foreign matter before storage. It is much easier to store good-quality, clean grain than cracked and broken grain. Grain should be checked for moisture to assess whether drying is necessary to achieve the desired moisture content as indicated in Table 2-2.

Table 2-2. Maximum recommended storage moisture contents for oilseed and aerated grain

Crop	Maximum recommended storage moisture, %	
	Storage ≤1 year	Storage ≥1 year
Maize and sorghum	14	13
Soybean	12	11
Cottonseed	9	9
Canola	8	8
Small grain (wheat and barley)	13	13

Moisture and temperature are the main determinants of how well grain keeps in storage. Aeration will help keep the grain at the desired moisture and temperature. Stored grain should be inspected every 2 weeks to verify grain temperature and to assess whether control of insects is necessary.

Sample Removal from Storage

Before samples are removed from storage, identity preservation procedures should be reviewed and all equipment should be inspected. Molecular analysis can be used to verify the identity of the test material at this time.

Crop Processing

Grain and oilseeds should be processed at locations known to produce high-quality products or in experimental facilities using pilot or small-scale equipment. Whenever possible, nontransgenic crops should be processed before transgenic crops. The processing plant and equipment should be cleaned and inspected before and after the GM grain or oilseed is processed. All processing conditions (time, temperature, moisture, etc.) should be recorded and filed with the crop records. To ensure similar end products, both the near isogenic control crop and the GM crop need to be processed identically. The final processed product should meet the industry quality standards of the country in which it is produced. Samples of the processed crops should be retained for quality assurance. Processed crops should be stored in clearly labeled, cleaned, and inspected storage containers.

Reference

U.S. EPA (U.S. Environmental Protection Agency) (1999) Isolation Standards per 7 CFR 201.76: for regulated GM crops.

Appendix 2-1. Example of Plot and Planting Information for an Experiment with Maize Planting Design

Planting Design

1. Each entry will be planted to an area of approximately 1.2 hectares per entry (Figure 2-1A).
2. Seed spacing within each row will be approximately 20 to 30 cm apart.
3. Spacing between rows will be approximately 75 cm.
4. The control near-isogenic counterpart plot will be planted before the test plot.
5. All remnant seed will be removed from the equipment before and after planting each entry.
6. Inspection and cleaning of equipment must occur at the field (release) site to prevent potential dispersal of regulated seed.
7. The two plots will be separated by a minimum distance 200 m (660 feet USDA/APHIS requirement). A minimum 200-m maize-free buffer will also be maintained between each plot and any other open pollinated maize.
8. If the minimum distance cannot be maintained and documented, the plots must be destroyed before flowering occurs. Full details of planting and maintenance will be recorded promptly in the field notebook.
9. For material regulated under USDA/APHIS, the release site listed must not be planted before the date specified in the notification or permit.

Maintenance of Field Plots

1. Normal pest control and maintenance practices, consistent with maize production for the area, will be used to produce the crop.
2. All maintenance practices (irrigation, fertilizer, herbicide, etc.) will be applied uniformly to the entire trial area.
3. The sponsor must approve the composition of maintenance chemicals before application.
4. If irrigation is necessary and available, it will be applied to produce a successful crop.
5. Details of all maintenance practices will be recorded in the field notebook (raw data).

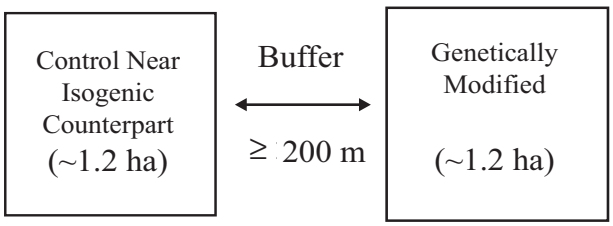
Agronomic Performance

To evaluate the agronomic performance of each hybrid entry, the following agronomic traits will be measured and recorded in the field notebook:

1. Early population (number of plants emerged per 10 m of row at full emergence)
2. Approximate time to silking (accumulated heat units* and date when approximately 50% of plants are at silk stage)
3. Approximate time to pollen shed (accumulated heat units and date when approximately 50% of plants are shedding pollen)
4. Plant height (height to tip of tassel measured for 10 plants at physiological maturity)
5. Ear height (height to base of primary ear measured for 10 plants at physiological maturity)
6. Stalk lodging (approximate percentage of plants lodged at the stalk region at physiological maturity)
7. Root lodging (approximate percentage of plants lodged at the root region at physiological maturity)
8. Final population (number of viable plants remaining per 10 m of row at physiological maturity)
9. Stay green (overall plant health evaluated at physiological maturity)
10. Disease incidence (any obvious disease incidence at physiological maturity)
11. Insect damage (any obvious insect damage at physiological maturity)

*Heat Unit = [(MAX + MIN)/2] – 10. All units are in degrees Celsius. If MAX temperature is greater than 30, use 30. If MIN temperature is less than 10, use 10. Accumulated heat units are calculated for each growing day and summed to give a total value. If a daily heat unit is negative, use 0 (zero).

Figure 2-1A. Example trial design



Appendix 2-2. Samples of Forms Used to Collect Data for an Experiment with Maize Planting Design

Seed Transfer Receipt

Line Identification	Lot/Batch No.	Amount Received (g, kernals, etc.)	Date Received	Received by (initials)
Example		2 packs of 40 kernels each	01Feb01	GD

Condition upon receipt (intact, torn, wet, etc.)	
Date/Initials	

Seed Storage

Storage Location		Initial_____ Date_____
Storage Temperature (°C)*	Min: _____ Max: _____	Initial_____ Date_____
Was storage location secured and how: Yes:_____ No:_____	(locked cabinet, room, etc.)	

Test Site Location and Area Map

(Example: State, County)_____

Distance and direction to nearest town:_____

_____

Initial_____ Date _____

Site Map

Attach (using glue) a copy of a local map, showing the location of field plot, north direction, and major roads. This map must be sufficiently detailed to allow an inspector to reach the actual field site without additional information; it must be clear enough to photocopy without loss of detail.

Initial_____ Date _____

Plot Plans and Dimensions

Attach (or draw) the plot plan including all the following information:

1. Plot dimensions, including dimension of buffer areas.
2. Plot orientation and relation to permanent local landmarks.
3. Entry identification for each plot.
4. Row direction and north direction.

Initial_____ Date _____

Field Characteristics

Soil Type:	
Soil Series:	
Percent organic matter (approximate):	
Soil pH:	
SOURCE OF INFORMATION:	

Initials:_____ Date:_____

Plot Plan and Dimensions

Number of rows per plot (not including borders):	
Number of border rows on each side of each plot:	
Number of plots:	
Row width:	
Row length:	
Seeding rate (No. seeds planted/row):	
Hectares planted:	

Initials:_____ Date:_____

Field History (2 previous years)

Year/Crop	Product(s)/Formulation	Active Ingredient (ai) and Concentration	Rate (kg ai/ha)
Example: 1997/Maize	Bullet/4F 4.48	Alachlor 0.36 kg ai/L + Atrazine 0.12 kg ai/L	4.48
	Dual II/7.8EC	Metolachlor 84.4%	2.24
Source of information:		Initials:_____ Date:_____	

Site Preparation (activities before planting)

a. Tillage Practices/Cultivation

Operation/Implement	Date	Date Documented	Initials
Example: Field Cultivator	23Oct99	01Nov99	HCD
Source of Information:			

b. Fertilizer Application (before planting)

Composition (% N-P-K)	Product (kg/ha)	Active Ingredient (ai) (kg ai/ha)	Date	Date Documented	Initials
Example: 46-0-0	434	200-0-0	17May99	19May99	HCD
Source of Information:					

c. Herbicide Application (this season, before planting)

Product(s)/ Formulation	Acrtive Ingredient (ai) and Concentration(s)	Rate (kg ai/ha)	Date Applied	Date Documented	Initials
Example: Bullet/4F	Alachlor 0.36 kg ai/L + Atrazine 0.12 kg ai/L	4.0	18May98		HCD
Source of Information:					

Planting Data

Planting Date:		
Air Temperature (°C):		Thermometer ID:
5 cm. Soil Temperature (°C):		Thermometer ID:
Soil Surface Moisture:	Dry/Moist/Muddy (circle one)	
Planted by:		
Other Factors Affecting Planting:		
	Initials:_____ Date: _____	

Plot Maintenance Practices

a. Pesticides/Herbicides (during or after planting)

Product/ Formulation	Active Ingredient(s) Concentrations	Rate (kg ai/ha)	Target Pest	App. Date	Applied by:
Example: Pounce 2 EC	Permethrin	0.12 kg or 120 ml	Cutworms	10Dec99	*LMP*
Degree Extra	Acetochlor, Atrazine	0.9 kg 0.45 kg	Weeds	12Dec99	*LMP*

b. Fertilizer application (during or after planting)

Composition (% N-P-K)	Product (kg/ha)	Active Ingredient (ai) (kg ai/ha)	App. Date	Applied by:
Example: 13-13-13	300	39-39-39	18Mar99	LMP

c. Cultural Practice (example: cultivating, hand weeding)

Operation/Implement	Depth (cm)	Date Performed	Performed by:
Example: Cultivating/cultivator	5.0	30Nov99	*LMP*

d. Irrigation

Type	Amount (cm)	App. Date	Applied by:
Example: Overhead sprinkler	5.0	30Nov99	*LMP*

Silking (accumulated heat units when approximately 50% of plants are at silk stage). Refer to protocol for accumulated heat unit formula.

Entry	Accumulated Heat Units	Initials	Date

Pollen shed (accumulated heat units when approximately 50% of plants are shedding pollen.) Refer to protocol for accumulated heat formula.

Entry	Accumulated Heat Units	Initials	Date

Silking Notes: _____

Initials:_____ Date:_____

Pollen Shed Notes: _____

Initials:_____ Date:_____

Height

Evaluate plants at physiological maturity (55 to 65 days after silking)

Hybrid ID	Plant No.	Plant Height (cm)	Ear Height (cm)	Notes
	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	Avg.		Avg.	
	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	Avg.		Avg.	
	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	Avg.		Avg.	
	1			
	2			
	3			
	4			
	5			
	6			
	7			
	8			
	9			
	10			
	Avg.		Avg.	

Scale = height (cm) to the base of the primary ear of 10 individual plants per plot.

Ear Height Notes: _____

Initials:_____ Date:_____

SAMPLE TRANSFER FORM (Chain of Custody Form)

FROM: TO:

Study No.:____ Crop: ____ Tissue: ____ Total No. of Samples ____ Approx. Total Wt. ____

Index No.	Shipped (✓)	Sample ID	Sample Description	Lot No.	Amount	Received (✓)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						

Shipper Information:

Samples Shipped By: _____ Date: _____ Number of Boxes: _____

Method of Shipment: _____ Condition of Samples: _____

(e.g., Truck, FedEx, Air) (e.g., frozen on dry ice, cold on wet ice, ambient temperature)

Storage conditions required for samples upon receipt: _____
(TO BE COMPLETED BY SHIPPER) (e.g., -80ºC, -20ºC, ambient)

Receiver Information (THE FOLLOWING INFORMATION AND THE "RECEIVED (✓)" COLUMN ABOVE SHOULD BE COMPLETED BY RECIPIENT):

Samples Receipt Date: _____ Received and Checked By: _____

Conditions of Samples Upon Receipt: _____

(e.g., frozen, thawed, damaged container)

Storage Upon Receipt: Location: _____ Temperature: _____

Return Signed Original to Study Coordinator

Chapter 3: Sampling and Analysis of Harvested and Processed Crop Material

It is important to determine the nutrient and antinutrient content of transgenic crops as well as to confirm whether the forage, grain, or other feed products contain a genetic modification. In addition, accurate feed analyses are needed to formulate diets so that the correct feedstuff is fed to the proper class of livestock; feedstuff value, animal performance (growth, lactation, and reproduction), and farm profitability are maximized; and environmental waste is minimized.

Sampling—the most important factor affecting the accuracy of feed analyses—is the process by which inference is made about the whole by examining a part. Therefore, obtaining the most representative sample of the whole is the most important step in achieving accurate analysis, yet proper sampling is the step most often taken for granted. Results obtained from improper sampling have led to decisions resulting in poor livestock performance and health, grain trade problems, environmental waste, and negative economic effects.

Basic principles of obtaining a representative sample include collecting several samples from different areas of the lot; combining these samples to form a single sample; considering the size of the sample needed for analysis; and completely mixing, blending, and subsampling the final sample. This section will discuss sampling of grain, hay, fresh forage, pasture, silage, and total mixed diets and proper sample handling. Additional information about sampling principles and practices is provided in a book edited by Pfost (1976), the Association of Official Analytical Chemists manual (AOAC 2000), Bell (1997), Doidge (1999), Jones (1980), Potter (2000), Schneider and Sedivec (1993) and U.S. Department of Agriculture (1995).

Sample Lot

The validity of a testing program rests on obtaining a sample that accurately resembles the entire lot of product. Each sample must represent only one lot of forage, grain, or feed product.

A lot of forage may consist of forage harvested from one field at the same cutting and maturity within a 48-hour period. The most important consideration when identifying a lot is uniformity. All forage from the same lot should be similar in terms of type of plants, field (soil type), cutting date, maturity, variety, weed contamination, type of harvest equipment, weather during growth and harvest, preservatives, drying agents, additives, curing and storage conditions, and pest or disease damage. Variation in any of these characteristics can cause substantial differences in nutrient content. When these characteristics differ, a new lot should be designated and sampled.

To identify different lots of silage, several small bales of straw or shredded paper can be fed through the blower when the last load from each lot is ensiled. For grain, lots may be a field, truckload, rail car, barge, bin, silo, or a specific amount of one source acquired from the same source at the same time. Each lot should be sampled and analyzed.

Grain Sampling

Tailgate Sampling

Tailgate sampling is the use of a simple container to sample grain from a moving stream of grain. Tailgate sampling will draw a reasonably representative sample as grain is unloaded from a combine to a truck or wagon or from a truck or wagon to a bin. The grain should flow from the carrier (truck, combine, bin) for a few seconds before the first sample is taken. The last 100 to 200 kg flowing out of the container is to be avoided. The sampling device is held so that it is at one side of the grain stream. Then the tailgate sampler (e.g., a can attached to a pole) is pulled through the grain stream in a continuous motion. Each sample is emptied into a clean, dry container. There should be a minimum of three samples per carrier; taking more samples will result in a more representative composite sample.

Probe Sampling

Sampling with a hand probe is the only effective method for obtaining a representative sample from grain or feed at rest in a truck, bin, or other container. There are two types of hand probes-an open-throat probe and a compartmented probe. The open-throat probe does not contain compartments, which allows the sample to be poured directly from the probe into a sample container. The open-throat probe tends to draw more grain from the top portion of the lot. Results obtained with an open-throat probe may differ from those obtained with a compartmented probe. The compartmented probe may have 11, 12, 16, or 20 compartments and generally gives a more representative sample. When the slots are aligned, grain or feed can enter into and be emptied from the compartments. Hand probes are constructed of brass or aluminum

Table 3-1. Probe sampling

Carriers & containers	Probe length (m)	No. of compartments
Barges and bay boats	3.7	20
Gravity flow (hopper) cars	3.0 – 3.7	20
Boxcars	1.8	12
Trucks	1.5 – 1.8	11 or 12
Gravity flow, bottom-	1.8	12
unloading (hopper-bottom)	2.4	16
trucks	3.0	20
Sacks and bags	1.5	11
Other containers	Use grain probes that will reach the bottom of the container.	

and come in standard sizes (1.5 to 4.0 m long). The sample is most representative of a lot if the probe reaches from the top to the bottom of the container. The depth of the carrier or container dictates the length of probe that is used to draw the sample. See Table 3-1 for recommended probe lengths and number of compartments for different carriers and containers.

Sampling Canvas or Trough

Sampling canvases, which are usually made out of flat duck cloth or similar material, must be longer than the probe used to draw the sample. This extra length is needed so the canvas can hold the grain from the entire length of each probe without any being spilled. Half sections of pipe or troughs (e.g., rain gutters) may be used instead of sampling canvases. Troughs must also be longer than the probe used to draw the sample.

Sampling Bag

Sample bags for grain must be constructed from heavy cloth or canvas, have a drawstring closure, and be large enough to contain at least 4 kg of grain. A plastic liner inside the sample bag will prevent a change in moisture or odor. The sample identification, chain of custody, and other records should be inserted between the liner and the bag, not directly in the sample. Containers such as metal buckets or plastic cans may be used instead of sample bags if they are clean and dry. Plastic bags with twist ties or Whirl-Paks may be used instead of cloth or canvas.

Sacked or Bagged Grain or Feed

A double-tubed, compartmented grain probe is the best tool for sampling sacked or bagged grain or feed. The number of sacks or bags in each lot is counted. At least five or six sacks from each lot should be sampled (0.5 to 1 kg) and the samples should be mixed thoroughly. A representative sample (0.5 to 1 kg) is then placed in a plastic bag, excess air is excluded, and the bag is tightly sealed and submitted for analysis.

To collect a sample, a sack is stood on end and the probe is inserted into a top corner. The probe, with the slots closed and facing upward, is pushed diagonally through the sack until the end of the probe touches the opposite bottom corner. The probe is then opened, two up-and-down motions are made, and then the probe is closed and removed. The contents of the probe are emptied into a clean dry container or onto a canvas. This procedure is repeated with the other randomly selected sacks.

If all of the probe samples have a similar composition, the samples are combined and placed into one bag. When a sample is transferred from the canvas or container to the sample bag, care must be taken not to spill any portion of the sample or allow fine material to be blown away. If examination of the probe samples indicates that the condition of the lot is not uniform, a sample should be drawn from each of the different parts in addition to the sample as a whole.

Bulk Concentrates

Commodity feeds should be analyzed as a composite of 10 to 15 areas of a given feed. When the composite is mixed, segregation by particle size must be avoided or the true sample may be distorted. A 0.5- to 1-kg sample should be sent to the laboratory.

Grain or Feed in Bin

Ideally, if a bin can be accessed from the top, a grain probe should be used to obtain at least 3 samples of grain. If the bin cannot be accessed from the top, 12 to 20 random samples are collected when the grain is discharged (see tailgate sampling above) and combined in a clean plastic bucket. For flat storage, 12 to 20 samples are taken (preferably using a grain probe) from various sites and

combined in a clean plastic bucket. The composite is thoroughly blended and 0.5 to 1 kg is placed in a plastic bag for analysis.

Trucks

The locations in the container to be probed are determined; sampling in the spout stream should be avoided. With the slots on the probe closed, the probe is inserted at a slight angle (10 degrees). With the slots facing upward, the probe is opened and moved up and down in two short motions to fill the compartments. The probe is then closed and withdrawn from the grain, and the grain is emptied onto a canvas or trough that is slightly longer than the probe. As the sample is drawn, the grain is checked for general condition as well as for objectionable odor, insect infestation, large stones, pieces of metal or glass, and any other potentially harmful contaminants.

Figures 3-1 through 3-3 present examples of three different types of carriers and suggested locations to insert the probes. In all cases, the probe should be inserted at a 10-degree angle in the direction of the arrow.

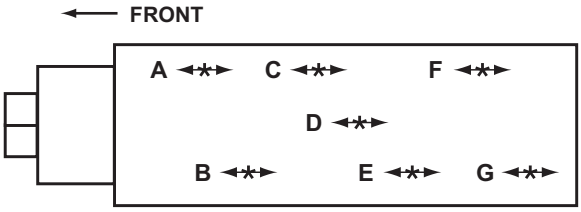


Figure 3-1. Flat bottom trucks or trailers containing more than 1.5 m deep or eight filled probe compartments.

Site A: Approximately 60 cm from the front and side. *Site B*: The opposite side of site A, approximately halfway between the front and center of the carrier and approximately 60 cm from the side. *Site C*: The same side as site A, approximately 75% of the distance between the front and center of the carrier and approximately 60 cm from the side. *Site D*: The center of the carrier. *Site E*: The side opposite site C, approximately 75% of the distance between the rear and center and approximately 60 cm from the side. *Site F*: The side opposite site E, approximately one-half the distance between the rear and center and approximately 60 cm from the side. *Site G*: The same side as site E, approximately 60 cm from the rear and side of the carrier.

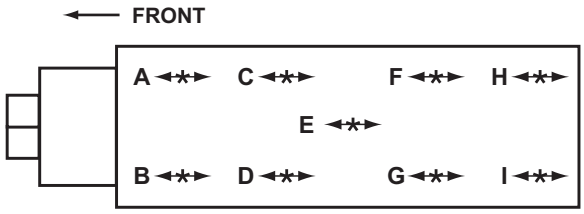


Figure 3-2. Flat-bottom trucks or trailers containing grain less than 1.5 m deep or fewer than eight filled probe compartments.

Site A: Approximately 60 cm from the front and side. *Site B*: The opposite side of site A, approximately 60 cm from the side. *Site C*: The side as site A, approximately 75% of the distance between the front and center of the carrier and approximately 60 cm from the side. *Site D*: The same side as site B, approximately 75% of the distance between front and center and approximately 60 cm from the side. *Site E*: The center of the carrier. *Site F*: The same side as site C, approximately 75% of the distance between the center and rear of the carrier and approximately 60 cm from the side. *Site G*: The same side as site D, approximately 75% of the distance between the center and rear of the carrier and approximately 60 cm from the side. *Site H*: The same side as site F, approximately 60 cm from the rear and side of the carrier. *Site I*: The same side as site G, approximately 60 cm from the rear and side of the carrier.

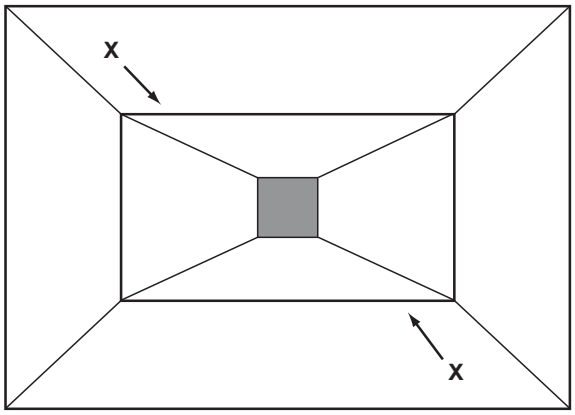


Figure 3-3. Sampling pattern for gravity-flow, bottom-unloading, hopper-bottom container (view of the inside of the container from the top).

Hay Sampling

Hay is harvested and preserved in a number of different forms-as pellets, cubes, small two-wire or string bales, small three-wire bales, large square bales (900 kg), large round bales, or stacked as loose hay. The most commonly used sampling tool for baled or stacked hay is a core sampler that uses a stainless steel hollow tube (probe) with a drill attachment at one end and a cutting edge at the other. Many different core samplers have been developed. The inside diameter of the coring device must be between 0.95 and 2 cm. The cutting edge must be sharp and must not separate stems from leaves; a dull tip may reduce the amount of stem material sampled. The drill should be run at slow speeds because high speeds heat the probe and can damage samples. A hand brace may be used in place of the drill. The shaft on the coring device should be long enough to take a core of at least 30 to 45 cm from the bale.

Manually pulling hay out of a bale or selecting loose flakes of hay will not result in a representative sample. If a corer is not available, a small section of hay is removed by hand from each of 15 to 20 bales. The hay is cut into 8-cm lengths with shears or a hatchet. This is a less desirable technique because leaves will be lost. Therefore, every effort should be made to include the appropriate proportion of leaves and stems in the sample. Samples are then mixed and random handfuls of the chopped material are taken for the lot sample.

Bales of hay are not uniform, because the initial windrows were not uniform and the baling process affects the distribution of leaves and stems within the bale (the bale structure). The bales should be probed so that the various concentrations of stems and leaves are sampled. At least 20 cores (one core per bale) should be taken at random (bales not selected by location, color, leafiness, smell, etc.) and combined into one composite sample per lot. Techniques to guard against nonrandom sampling include sampling every fourth or fifth bale in a stack or truckload at various heights, sampling every fourth or fifth bale in a row in the field, and taking at least five random samples from each of the four sides of a haystack.

Rectangular Bales

Rectangular bales, regardless of size, are sampled using a hay probe centered in the end of the bale perpendicular to the face of the bale. The core is drilled horizontally into the bale. Decayed or moldy hay or other portions that will not be fed or will likely be refused by the animals when fed free choice need to be discarded. However, deteriorated material that will be ground, sold, or purchased should be included. The entire sample is placed in a plastic bag, excess air is excluded, and the bag is sealed tightly.

Round Bales

Round bales are sampled by drilling horizontally into the curved side of the bale. The core is taken in an upward direction to reduce spoilage from moisture. The corer should be long enough to reach the center of the bale. Samples are placed in a plastic bag, excess air is excluded, and the bag is sealed tightly. Deteriorated hay from the exterior of the bale should not be sampled if it will not be fed to animals or the animals will choose not to eat it. Baled hay stored outside should be sampled within 2 to 4 weeks of feeding so that continued deterioration does not substantially change the quality of the bale from that of the sample. Collecting samples by hand is not recommended.

Loose Hay

For loose hay, the probe should be at least 75 cm long and have an internal diameter of at least 2 cm. A total of 15 to 20 random locations around and on top of the stack are chosen, and the corer is drilled deep into the stack. Compressed loaf stacks require six sampling locations: top front, top side, top rear, lower front , lower side, and lower rear. Alternate sides should be used as different stacks are sampled. When the top is sampled, the probe is held vertically and the hay is drilled at the spot where it is compressed by the weight of the operator. When sides are sampled, a slight downward angle with the probe is used to avoid sampling parallel to stems in the stack. Any weather-damaged surface layer that would not be included in the portion being fed should be discarded. Hay stored outside should be sampled within 2 to 4 weeks of feeding so that continued deterioration does not substantially change the quality of the bale from that of the sample. Samples are placed in a plastic bag, excess air is removed, and the bag is sealed tightly.

Hay Cubes or Pellets

Hay cubes or pellets are sampled by collecting several hay cubes or handfuls of pellets from 15 to 20 locations in each sample, for a minimum of 40 cubes or 1 kg of pellets selected.

Chopped or Ground Hay

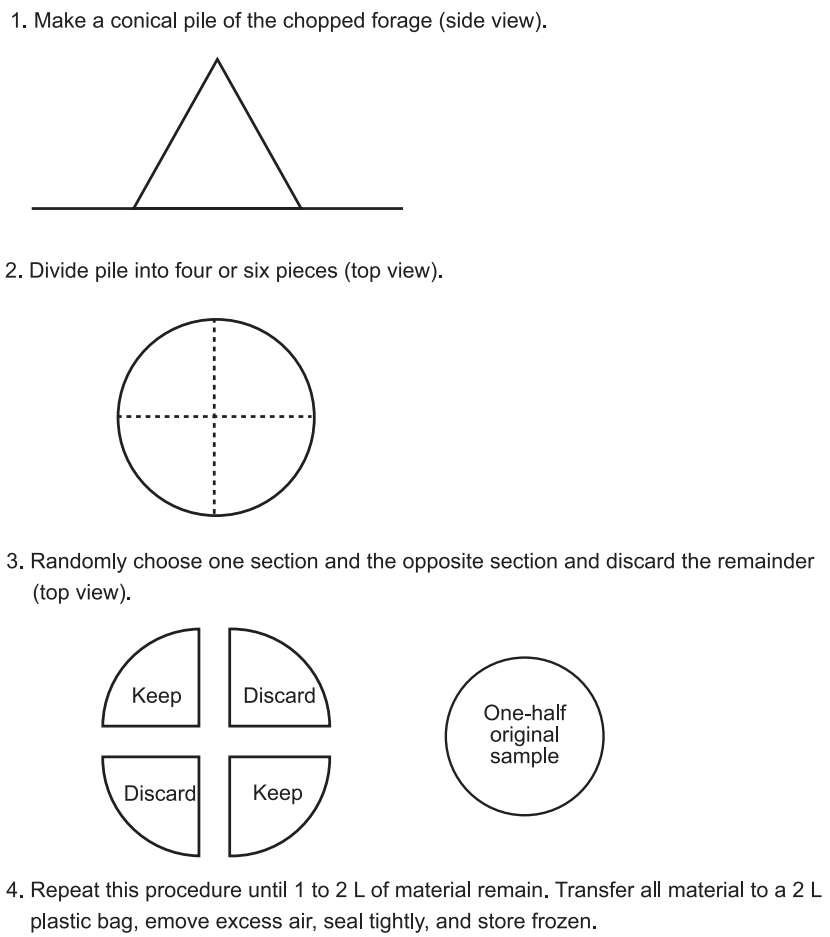
Chopped or ground hay is sampled by periodic collection of 10 small samples from each sample lot of hay during grinding. All samples are placed in one plastic bag and the bag is sealed tightly. Previously ground or chopped hay should be sampled from beneath the surface. About 25% of the samples are collected from the upper half of the pile and 75% from the lower half. Care should be taken so that fine particles do not sift between fingers.

Green Chopped Forage Sampling

Green chopped forage should be sampled as it goes into the silo. If green chopped forage is fed directly without ensiling, it should be sampled as it is delivered to the animals. One handful is taken from every fourth or fifth wagon or truckload and placed in a clean plastic bucket or bag. The container is closed between samples to prevent moisture loss. This is done continuously throughout the day for each load. At the end of the day or field or when the silo is full, the contents of the bucket or bag are mixed and at least three handfuls of forage are withdrawn and placed in a plastic freezer bag. Information is clearly marked on the sample bag, excess air is removed, and the bag is sealed tightly. The sample is stored in a freezer to prevent spoilage. When the silo is filled, all the subsamples collected for the silo are thawed and mixed together in a clean, dry plastic bucket or bag. A two-to-three-handful sample is placed in a labeled plastic bag, excess air is removed, and the bag is sealed tightly. The sample should be sent immediately for analysis.

The following quartering procedure can be used for reducing the sample size while maintaining a representative sample (Figure 3-4). The entire sample should be mixed thoroughly before being poured into the conical pile. Hay samples should not be quartered because leaf loss can drastically affect analytical results.

Figure 3-4. Quartering (subsampling) procedure.



Silage

Silage samples should represent several locations within the silo (upright, horizontal, pit clamp, stack, or silo bag) to ensure an adequate representation of the silage. The sample must be tightly packed in a plastic bag with excess air removed and sealed tightly. The samples can be shipped cold to the laboratory or frozen and then shipped to the laboratory for analysis. Samples should be taken after the fermentation process is complete, preferably 45 to 60 days after filling.

Upright Silos at Feeding

A 0.5- to 1-kg sample is collected from the silo unloader while it is operating or a comparable amount of material is collected from 20 different locations in the feed bunk by hand while animals are feeding. Contamination with old feed or supplements must be avoided. Sampling silage that has been exposed to the air for several hours should also be avoided. The sample is mixed and subsampled as described. The material is placed in a plastic bag, excess air is removed, and the bag is sealed tightly and stored frozen.

Horizontal Silos

A total of 15 to 20 or more subsamples are collected from the face of the silo to represent the entire exposed surface. Sampling should be to the depth that is removed during daily feeding. A sample from the bunk may be easier to obtain and may provide an equally representative sample if the silage is not mixed with other ingredients at the time of feeding. The sample is mixed and subsampled as described. The material is placed in a plastic bag, excess air is removed, and the bag is sealed tightly and stored frozen.

Pasture Sampling

Pasture sampling is difficult. Fertility and moisture differences in a single paddock add to the problem. In one method, 8 to 10 sites with similar moisture and fertility history are selected at random. The samples must not be collected from areas that are not being grazed and are overgrown. If the entire pasture is used, sample locations should be distributed uniformly. Forage from a 1000-cm^2 area is removed at grazing height (or to a standard height of 4 cm) with stainless steel scissors. Samples from all sites are chopped into about 7-cm pieces, mixed, and quartered as described to reduce sample size. A 1-kg sample is placed in a plastic freezer bag, excess air is removed, the bag is sealed tightly, and the sample is frozen immediately. Freezing will help reduce chemical changes due to respiration or fermentation.

A second method is to take forage being selectively grazed by animals at several locations for the sample. This is a preferred method in unimproved pasture where selective grazing is evident. However, it can be difficult to determine accurately how much of which forage to sample. With a little practice, an experienced manager can accurately identify the species being consumed at the time of sampling.

Another method is to take two pregrazing estimations of herbage yield at random from each daily allocation of grass (Roche 1995).The number of cuts depends on allocation size. The pregrazing cuts should be cut to a height of 40 mm with a mower that has a cutting blade width of 0.965 m. The cut should be taken for a distance of 9 m. The length of the strip must be measured accurately. The cut sample is weighed and a large subsample (>200 g) is retained for laboratory analysis; 100 g of this subsample is dried at 90°C for 16 hours to determine the dry matter. The yield per hectare of the paddock is calculated as follows:

Yield/hectare (above 40 mm) = [10000/(length (width)] × weight × dry matter

Mixed Diet Sampling

Mixed diets are difficult to sample because they are seldom homogeneous or well mixed. When it is unlikely that a sampling method can produce a representative sample, it is recommended that the components of the diets be sampled and analyzed individually. Only freshly blended diets or total mixed rations should be sampled; 12 to 20 handfuls of the mix are removed from different locations in the feed bunk. Each handful should contain the top, middle, and bottom portions of the pile in the feed bunk. All subsamples should be mixed in a bucket or on a canvas to form a composite. A 0.5- to 1-kg sample from this composite is placed in a plastic freezer bag, excess air is removed, and the bag is sealed tightly and stored frozen.

Many factors can result in samples of mixed diets not being representative of the lot, making the results of analyses meaningless. These factors include the use of multiple ingredients of various particle sizes that are prone to separation; lack of moisture in the diet, which can lead to separation of ingredients; scales not working properly; operator adding ingredients at rates other than those indicated on the batch mix sheet; inadequate mixing time; animals allowed to feed before the samples were taken; and nutrient composition of ingredients different from those used

in the formulation. Mixed diet analyses are most commonly used as a check to determine whether the mix is meeting nutrient specifications and to evaluate whether the diet is being properly mixed.

Handling of Sample

Proper handling of the sample between farm or research facility and laboratory ensures that a result will be representative of the lot. It is good practice to divide the sample in half and send one half for analysis and retain the other. Each sample should be 0.5 to 1 kg. The samples should be labeled with the date, sample number, study number (if appropriate), supplier's name and address, and description. Samples should be sent to the laboratory as soon as possible after collection. Moist samples such as silage, fresh forage, and high-moisture grain should be frozen before shipping. Frozen samples should be shipped by express mail or express package service and should be protected from thawing by packing in dry ice or other suitable material.

Analysis of Harvested and Processed Crop Material

Analysis of the preprocessed and processed components of the animal diet is important, even after careful production, handling, and processing methods have been followed. Two areas should be considered in the proper characterization of animal feed. First, concentration of the introduced and expressed traits must be established in both the preprocessed and processed components. Second, the chemical composition (e.g., of pesticides, mycotoxins, nutrients, and antinutrients) must be analyzed in both the genetically modified (GM) and control material.

Figure 3-5 indicates the critical points for sampling and analysis throughout the project timeline.

Analysis of the Introduced Trait

It is important that the seed being planted to generate the test material is obtained from a reputable source. Before being planted, the GM and control seed could be tested by DNA techniques such as polymerase chain reaction to ensure identity (Sambrook et al. 1989). The GM material is commonly tested at harvest, after processing of the test substance, and after manufacture of the prepared feed mixture to ensure that the protein that confers the trait of interest is expressed. Depending on the test material being incorporated into the animal diet, representative samples should be analyzed throughout the process (Figure 3-5). Proteins of interest can be quantified using enzyme-linked immunosorbent assays (Reen 1994, Tijssen 1985).

Pesticide, Mycotoxin, Nutrient, and Antinutrient Analysis

After harvest and storage and before processing and expected use, grain should be checked for pesticide residues, mycotoxins, and nutrient and antinutrient content. The pesticide residues to be evaluated are determined by the pesticides that were sprayed on the crop during the growing season. If pesticide residues exceed the locally accepted tolerance levels, the feedstuff is not suitable for animal studies.

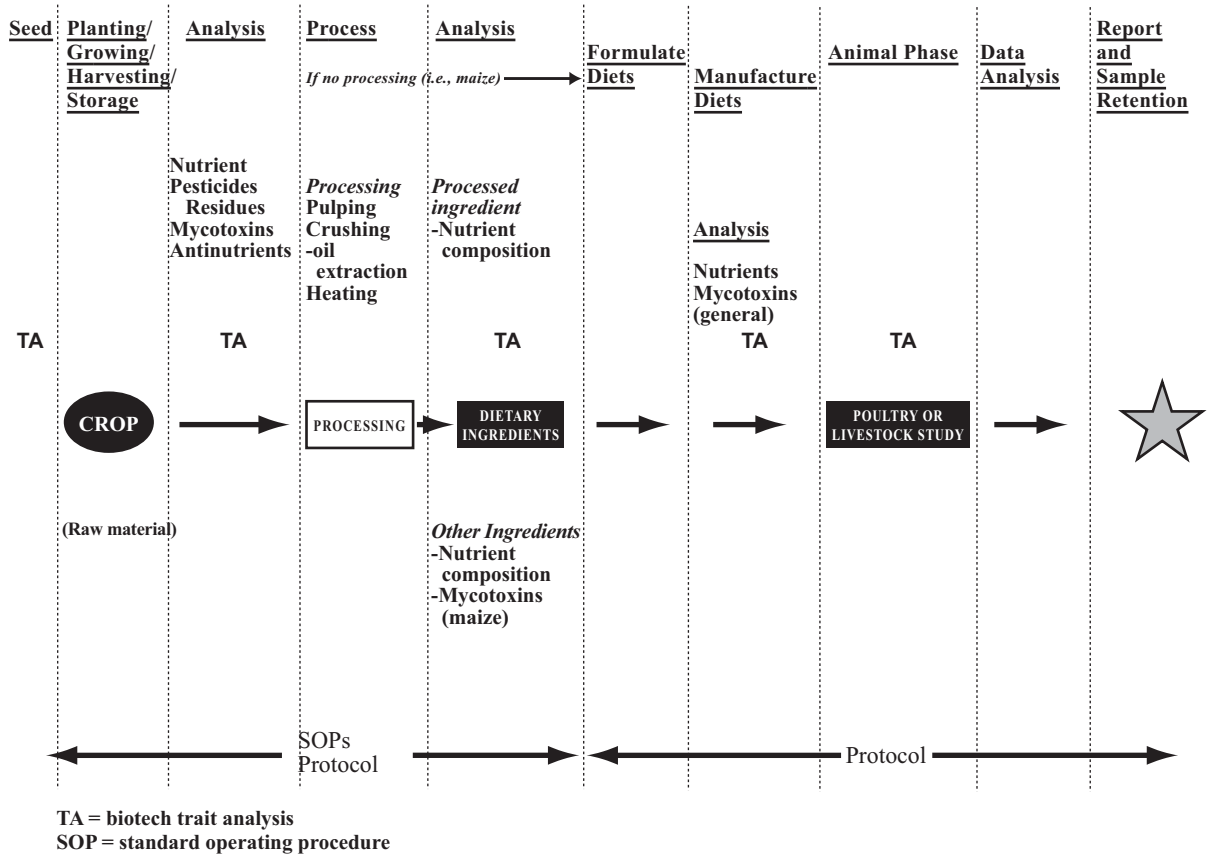
Grain and coproducts should be tested for mycotoxins that can affect animal health and reduce animal performance. Mycotoxins may be evident immediately after harvest and can increase with storage depending on conditions. Therefore, the ideal time to test for mycotoxins is just before use. Table 3-2 contains a list of mycotoxins that should be considered for analysis. The toxins that will need to be considered in a specific study will be influenced by geography (local prevalence), crop, climatic conditions, moisture, degree of pest infestation, and storage time, among others (CAST 2003). Analytical methods are listed in Appendixes 3-1 and 3-2.

Antinutrient analysis is crop and coproduct dependent (OECD 2002, 2001). Table 3-3 lists examples of grains and coproducts and their antinutrients. Analytical methods are listed in Appendix 3-1.

Table 3-2. Mycotoxins to be considered for analysis

Aflatoxin B_1	Fusarenon X
Aflatoxin B_2	Deoxynivalenol (DON)
Aflatoxin G_1	15-Acetyl-DON
Aflatoxin G_2	3-Acetyl-DON
Ochratoxin A	Nivalenol
Citrinin	Zearalenone
T-2 Toxin	Fumonisin B_1
HT-2 Toxin	Fumonisin B_2
Diacetoxyscirpenol	Fumonisin B_3
Neosolaniol	

Figure 3-5. Project flow diagram for animal studies



TA = biotech trait analysis
SOP = standard operating procedure

Table 3-3. Examples of antinutrients in crops

Crop or Product	Antinutrient
Soybeans, soybean meal	Trypsin inhibitors, lectins
Canola, canola meal,	Glucosinolates
Cotton, cottonseed, cottonseed meal	Gossypol, cyclopropenoid fatty acids

Analysis of feedstuffs for toxicants such as excess nitrates, molybdenum, and selenium is determined by locality. Drinking water provided to animals throughout the performance study may need to be analyzed for toxicants as well as for microbial contamination.

Nutrient content needs to be analyzed after harvest, before and after processing for oilseeds and sugar beets, and after manufacture of the feed. The nutrients to be analyzed are those that are important for meeting the requirements of the recipient livestock and poultry species. Nutrient deficiency or imbalance may result in decreased animal performance. Knowing the nutrient content is critical for formulating the final prepared feed. Table 3-4 contains a list of crops and nutrient analytes to be considered for each type of crop.

The relevant components of proximate analysis (dry matter, crude protein, ether extract, and ash) are shown in Table 3-4. The two other components of proximate analysis (crude fiber and nitrogen free extract) are not included. The analysis of crude fiber is being discontinued in many areas. Neutral detergent fiber and acid detergent fiber are analyzed instead because they are better measures of fiber in animal nutrition.

If a GM crop was designed to alter the content of a particular nutrient such as a vitamin, amino acid, oil, or fatty acid, additional compositional analysis is warranted. References for analytical methods are provided in Appendix 3-1.

Table 3-4. Recommendations for nutrient analysis

Crops/grain/coproducts	Livestock type	Analyte*
Grain: maize, wheat, barley	Nonruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, ash, starch, lysine, methionine, cystine, threonine, tryptophan, isoleucine arginine, phenylalanine, histidine, leucine, tyrosine, valine
Oilseed meals: soybean, linseed, cottonseed, canola meal, full-fat oilseeds	Nonruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, ash, fatty acids (full-fat oilseed), lysine, methionine, cystine, threonine, tryptophan, isoleucine, arginine, phenylalanine, histidine, leucine, tyrosine, valine
Grain: maize, wheat, barley	Ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, starch, ADIN, soluble protein, NPN, degradable protein, NDICP, ADICP
Oilseed meals: soybean, linseed, cottonseed, canola meal	Ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, ADIN, soluble protein, NPN, degradable protein, NDIN
Seeds: soybean, cottonseed, sunflower	Ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, ADIN, soluble protein, NPN, degradable protein, NDIN
Silage: maize, grass, legumes	Ruminants	DM, CP, EE, ADF, NDF, ADIN, ADL, DNDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, soluble protein, NPN, degradable protein, NDIN, starch, sugar, pH, organic acids such as lactic, acetic,butyric, isobutyric
Fresh/dry forages: grass, legumes	Ruminants	DM, CP, EE, ADF, NDF, ADIN, ADL, DNDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, soluble protein, NPN, degradable protein, NDIN, starch, sugar

*ADF, acid detergent fiber; ADIN, acid detergent insoluble nitrogen; ADL, acid detergent lignin; CP, crude protein; DM, dry matter; DNDF, digestible neutral detergent fiber; EE, crude fat; NDF, neutral detergent fiber; NDIN, neutral detergent insoluble nitrogen, NPN, nonprotein nitrogen.

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OECD (Organization for Economic Collaboration and Development)(2002) Environment, Health and Safety Publications, Series on the Safety of Novel Foods and Feeds, No 6. 2002. Consensus document on compositional considerations for new varieties of maize (Zea mays): key food and feed nutrients, anti-nutrients and secondary plant metabolites. ENV/JM/MONO(2002)25. Organization for Economic Co-operation and Development, Paris

OECD (2001) Environment, Health and Safety Publications, Series on the Safety of Novel Foods and Feeds, No 2. 2001. Consensus document on compositional considerations for new varieties of soybean: key food and feed anti - nutrients. ENV/JM/MONO(2001)5. Organization for Economic Co-operation and Development, Paris

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Potter B. The fine art of forage sampling. Ontario Ministry of Food and Agriculture. [cited 2003 Feb 13]. Available from http://www.gov.on.ca/OMAFRA/english/livestock/dairy/facts/info_sampling.htm

Reen DJ (1994) Enzyme-linked immunosorbent assay (ELISA) Methods Mol Biol 32:461-466.

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Schneider JW, Sedivec K (1993) Sampling feed for analysis. North Dakota State University. NDSU Extension Services AS-1064, September 1993. Available from http://www.ext/nodak.edu/extpubs/ansci/livestock/as1064w.htm.

Tijssen P (1985) Processing of data and reporting of results of enzyme immunoassays. In, Practice and theory of enzyme immunoassays. Laboratory techniques in biochemistry and molecular biology, V. 15, Elsevier Science Publishers, Amsterdam, The Netherlands, pp 385-421

USDA (U.S. Department of Agriculture). Cooperative Extension Service Dairy Guide. Testing alfalfa for its feeding value. 408.01-408.03 Grain Inspection Handbook - Book I Grain Sampling. 1995. Available from http://www.usda.gov/gipsa/reference-library/handbooks/grain-insp/grbook1/gihbk1.htm

Appendix 3-1. Analytical Methods: Chemical Analyses

Note: This list of analytical methods is not all inclusive. Other validated methods may also be used.

ANTINUTRIENTS

Glucosinolates

International Organization for Standardization. Rapeseed - Determination of gluconsinolates content. Part 1: Method using high-performance liquid chromatography, ISO 9167-1:1992(E). ISO, Geneva, Switzerland.

Phytic Acid

Lehrfeld J (1994) HPLC separation and quantitation of phytic acid and some inositol phosphates in foods: problem and solutions. J Agric Food Chem 42:2726-2731 Lehrfeld J (1989) High-performance liquid chromatography analysis of phytic acid on a pH-stable, macroporous polymer column. Cereal Chem 66:510-515

Trypsin Inhibitor

American Oil Chemists' Society (1997) Official methods and recommended practices of the American Oil Chemists' Society, 5th ed. Method Ba 12-75. American Oil Chemists' Society, Champaign, IL

CARBOHYDRATES

U.S. Department of Agriculture (1973) Energy Value of Foods, Agriculture Handbook No. 74. U.S. Government Printing Office, Washington, DC

Arabinose, Xylose, Mannose, Galactose

Brower HE, Jeffrey JE, Folsom MW (1966) Gas chromatographic sugar analysis in hydrolysates of wood constituents. Anal Chem 38;362-364

Sugar Profile

AOAC Official Method* 994.13, The Alditol Acetate Method for Determination of Dietary Fiber as Neutral Sugars. This method is the most widely used, and hence established, method for measuring all monosaccharides including rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, and glucose.

Scott RW (1979) Calorimetric determination of hexuronic acids in plant materials. Anal Chem 51:936-941 (Acidic sugars such as uronic acids)

Mason BS, Slover HT (1971) A gas chromatographic method for the determination of sugars in foods. J Agric Food Chem 19:551-554

Brobst KM (1972) Gas-liquid chromatography of trimethylsilyl derivatives. In Whistler RL, BeMiller JN (eds), Methods in Carbohydrate Chemistry, vol 6. Academic Press, New York

*All AOAC Official Methods are published in the Official Methods of Analysis, 17th ed.(2000) AOAC INTERNATIONAL, Gaithersburg, MD

Starch

AOAC Official Method 920.40, Starch in animal feed

AOAC Official Method 996.11, The Megazyme Kit method

Dry Matter

AOAC Official Method 930.15, Dry matter on oven drying for feeds (135 °C for 2 h)

AOAC Official Method 991.01, Moisture in forage, near-infrared reflectance spectroscopy

AOAC Official Method 925.04, Moisture in animal feed distillation with toluene

AOAC Official Method 934.01, Dry matter on oven drying at 95-100 °C for feeds

ENZYMATIC REACTIONS

Urease Activity

American Oil Chemists' Society (1997) Official methods and recommended practices of the American Oil Chemists' Society, 5th ed. Method Ba 9-58. American Oil Chemists' Society, Champaign, IL.

FAT

Acid Hydrolysis

AOAC Official Method 920.39, Fat (crude) or ether extract in animal feeds AOAC Official Method 954.02, Fat (crude) or ether extract in pet food, gravimetric method

Soxhlet Extraction

AOAC Official Method 960.39, Fat (crude) or ether extract in meat

Fatty Acids

American Oil Chemists' Society (1997) Official methods and recommended practices of the American Oil Chemists' Society, 5th ed. American Oil Chemists' Society, Champaign, IL

Sukhija PS, Palmquist DL (1988) Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J Agric Food Chem 36:1202-1206

Cyclopropenoid Fatty Acids

Wood R (1986) High performance liquid chromatography analysis of cyclopropene fatty acids. Biochem Arch 2:63-71

FIBER

Acid Detergent Fiber

ANKOM A200 Filter Bag Technique (FBT), reagent solutions same as described in AOAC Official Method 973.18, Fiber (acid crude) and lignin (H_2SO_4) in animal feed

AOAC Official Method 989.03, Fiber (acid detergent) and protein (crude) in forages, near-infrared reflectance spectroscopic method

U.S. Department of Agriculture (1970) Forage fiber analyses, Agriculture Handbook No.379. U.S. Government Printing Office, Washington, DC

Van Soest PJ, Robertson JB, LewisBA (1991) Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74:3583-3597

Neutral Detergent Fiber

ANKOM A200 Filter Bag Technique (FBT), reagent solutions same as described by Van Soest et al in J Dairy Sci 74:3583-3597

AOAC Official Method 962.09, Fiber (crude) in animal feed and pet food, ceramic fiber filter method

American Association of Cereal Chemists (1998) Approved methods of the American Association of Cereal Chemists, 9th ed. Method 32.20. AACC, St. Paul, MN

U.S. Department of Agriculture (1970) Forage fiber analyses, Agriculture Handbook No.379, U.S. Government Printing Office, Washington, DC

Van Soest PJ, Robertson JB, Lewis BA (1991) Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74:3583-3597

AOAC Official Method 2002.04, Amylase-treated neutral detergent fiber

Lignin

ANKOM A200 Filter Bag Technique (FBT), Solutions same as described in AOAC Official Method 973.18, Fiber (acid crude) and lignin (H2 SO4) in animal feed

AOAC Official Method 973.18, Fiber (acid crude) and lignin (H2 SO4) in animal feed

NIRS-NDF as in crude protein NIRS: AOAC Official Method 989.03, Fiber (acid detergent) and protein (crude) in forages, near-infrared reflectance spectroscopic method

In-vitro true digestibility: ANKOM DAISY filter bag technique (FBT)

Total Dietary Fiber AOAC Official Method 985.29, Soluble dietary fiber in food and food products, enzymatic-gravimetric method (phosphorus buffer) Van Soest PJ, Robertson JB, Lewis BA (1991) Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74:3583-3597

INORGANIC SALTS

Chlorides

AOAC Official Method 969.10, Chlorine (Soluble) in Animal Feed

MINERALS

Dahlquist RL, Knoll JW (1978) Inductively coupled plasma-atomic emission spectrometry: analysis of biological materials and soils for major, trace, and ultra trace elements. Appl Spectroscopy 32:1-29

AOAC Official Method 968.08, Minerals in animal feed and pet food

Ash

AOAC Official Method 942.05, Ash of animal feed

Selenium

AOAC Official Method 996.16, Selenium in feeds and premixes

Watkinson JH (1966) Fluorometric determination of selenium in biological material with 2,3-diaminonaphthalene. Anal Chem 38:92-7

Haddad PR, Smythe LE (1974) A critical evaluation of fluorometric methods for determination of selenium in plant materials with 2,3-diaminonaphthalene. Talanta 21:859-865

Bayfield RF, Romalis LF (1985) pH control in the fluorometric assay for selenium with 2,3-diaminonaphthalene. Anal Biochem 144:569-576

Sulfur

(1965) Soil Society America Proc 29:71-72

NATURAL TOXINS

Mycotoxins: General

USDA-GIPSA (1999) Grain fungal diseases & mycotoxin reference. United States Department of Agriculture-Grain Inspection, Packers and Stockyards Administration, Technical Services Division, Kansas City, MO. Available from http://www.usda.gov/gipsa/pubs/mycobook.pdf

Mycotoxins: Aflatoxins

AOAC Official Method 991.31, Aflatoxins in corn, raw peanuts, and peanut butter, immunoaffinity column (aflatest) method

AOAC Official Method 990.33, Aflatoxins in corn and peanut butter, liquid chromatographic method

Gossypol

American Oil Chemists' Society (1997) Official methods and recommended practices of the American Oil Chemists' Society, 5th ed. Methods Ba 7-58 and Ba 8-78. American Oil Chemists' Society, Champaign, IL

NITRATES

Hach method, Hack Company, Loveland, CO

- Plant tissue and SAP Analysis Manual. Literature Code #3118

- Extraction: pp. 130-131, n°Charcoal, shake 0.200 g in 100 mL water for 1 hour
- Analysis: pp. 132-133, Nitra Ver 5 substituted by Nitra Ver 6 and 3 (HPLC analysis).
- Cadmium reduction reaction using chromotropic acid followed by colorimetric analysis using spectrometer.

NONPROTEIN NITROGEN

AOAC Official Method 941.04, Urea and ammonical nitrogen in animal feed, urease method

AOAC Official Method 967.07, Urea in animal feed, colorimetric method

PROTEIN

Crude Protein

AOAC Official Method 954.01, Protein (crude) in animal feed and pet food, Kjeldahl method

AOAC Official Method 968.06, Protein (crude) in animal feed, Dumas method

Bradstreet RB (1965) The Kjeldahl method for organic nitrogen, Academic Press: New York

Kalthoff IM, Sandell EB (1948) Quantitative inorganic analysis. MacMillan, New York

AOAC Official Method 984.13, Protein (crude) in animal feed and pet food, copper catalyst Kjeldahl method

AOAC Official Method 976.06, Protein (crude) in animal feed and pet food, semiautomated methods

AOAC Official Method 989.03, Fiber (acid detergent) and protein (crude) in forages, near-infrared reflectance spectroscopic method

Amino Acid Composition

AOAC Official Method 994.12, Amino Acids in Feeds AOAC Official Method 982.30, Protein Efficiency Ratio, Calculation Method

Degradable Protein

Roe MB, Sniffen CJ, Chase LE (1990) Techniques for measuring protein fractions in feedstuffs. Proceedings 1990 Cornell Nutrition Conference for Feed Manufacturers, October 21-15, 1990, Holiday Inn-Genesee Plaza, Rochester, NY, pp 81-88

Coblentz WK, Abdelgadir IEO, Cochran RC, et al (1999) Degradability of forage proteins by in situ and in vitro enzymatic methods. J Dairy Sci 82:343-354

AOAC Official Method 989.03, Fiber (acid detergent) and protein (crude) in forages, near-infrared reflectance spectroscopic method

Soluble Protein

Roe MB, Sniffen CJ, Chase LE (1990) Techniques for measuring protein fractions in feedstuffs. Proceedings 1990 Cornell Nutrition Conference for Feed Manufacturers, October 21-15, 1990, Holiday Inn-Genesee Plaza, Rochester, NY, pp 81-88

AOAC Official Method 989.03, Fiber (acid detergent) and protein (crude) in forages, near-infrared reflectance spectroscopic method

ORGANOPHOSPHATES AND CHLORINATED INSECTICIDES

U.S. Food and Drug Administration (1999) Pesticide analytical manual, vol 1, Multiresidue methods, 3rd ed. [cited 2002 July 5]. Available from http://vm.cfsan.fda.gov/~frf/pami3.html

Griffitt R, Craun JC (1974) Gel permeation chromatographic system: an evaluation. J Assoc Off Anal Chem 57:168-172

Hopper ML, Griffitt KR (1987) Evaluation of an automated permeation cleanup and evaporation systems for determining pesticides residues in fatty samples. J Assoc Off Anal Chem 70:724-726

Watts RR, Storherr RW (1965) Rapid extraction method for crops. J Assoc Off Agric Chem 48:1158-1160

Erney DR (1974) A feasibility study of miniature florisil columns for the separation of some chlorinated pesticides. Bull Environ Contamin Toxicol 12:717-720

Griffitt KR, Hampton DC, Sisk RL (1983) Miniaturized florisil column cleanup of chlorinated and organophosphate eluates in total diet samples. Lab Information Bull 2722

Pesticide Profile

U.S. Food and Drug Administration (1999) Pesticide analytical manual, vol 1, Multiresidue methods, 3rd ed, Chapter 3, Multiclass multiresidue methods: 304 methods for fatty foods [cited 2002 July 5]. Available from http://vm.cfsan.fda.gov/~frf/pami3.html

VITAMINS

Folic Acid

AOAC Official Method 960.46, Vitamin assays, microbiological methods Infant Formula Council (1973) Methods of analysis for infant formulas, Section C-2. Infant Formula Council, Atlanta, GA

Vitamin A

AOAC Official Method 974.29, Vitamin A in mixed feeds, premixes, and human and pet foods, colorimetric method

Thompson JN, Duval S (1989) Determination of vitamin A in milk and infant formula by HPLC. J Micronutrient Anal 6(2):147-159

Vitamin B1 (Thiamin)

AOAC Official Method 942.23, Thiamine (vitamin B1) in human and pet foods, fluorometric method

AOAC Official Method 953.17, Thiamine (vitamin B1) in grain products, fluorometric (rapid) method

AOAC Official Method 957.17, Thiamine (vitamin B1) in bread, fluorometric method

Vitamin B2 (Riboflavin)

AOAC Official Method 940.33, Riboflavin (vitamin B2) in vitamin preparations, microbiological methods

Vitamin B6

AOAC Official Method 961.15, Vitamin B6 (pyridoxine, pyridoxal, pyridoxamine) in food extracts, microbiological methods

Vitamin C

AOAC Official Method 967.22, Vitamin C (total) in vitamin preparations, microfluorometric method

Vitamin E

Cort WM, Vincente TS, Waysek EH, Williams BD (1983) Vitamin E content of feedstuffs determined by high-performance liquid chromatographic fluorescence. J Agric Food Chem 31:1330-1333

Speek AJ, Schijver J, Schreurs WHP (1985) Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorometric quantitation. J Food Sci 50:121-124

McMurray CH, Blanchflower WJ, Rice DA (1980) Influence of extraction techniques on determination of a-tocopherol in animal feedstuffs. J Assoc Off Anal Chem 63:1258-1261

Appendix 3-2. Microbiological Methods

Coliforms

Hitchins AD, Feng P, Watkins WD, et al (1998) Escherichia coli and the coliform bacteria. In U.S. Food and Drug Administration, Bacteriological analytical manual, 8th ed., 4.01-4.26. [cited 2003 May 23]. Available from http://vm.cfsan.fda.gov/~ebam/bam-toc.html

Hitchins AD, Hartman PA, Todd ECD (1992) Coliforms - Escherichia coli and its toxins. In Vanderzant C, Splittstiesser DF (eds), Compendium of methods for the microbiological examination of foods, 3rd ed. American Public Health Association, Washington, DC, pp 325-370

Christen GL, Davidson PM, McAllister JS, Roth LA (1992) Coliform and other indicator bacteria. In Marshall RT (ed), Standard methods for the examination of dairy products, 16th ed. American Public Health Association, Washington, DC, pp 247-270

Escherichia coli

Hitchins AD, Feng P, Watkins WD, et al (1998) Escherichia coli and the coliform bacteria. In U.S. Food and Drug Administration, Bacteriological analytical manual, 8th ed, 4.01-4.26. [cited 2003 May 23]. Available from http://vm.cfsan.fda.gov/~ebam/bam-toc.html

Salmonella

Andrews WH, June GA, Sherrod PS, et al (1998) Salmonella. In U.S. Food and Drug Administration, Bacteriological analytical manual, 8th ed, 5.01-5.20. [cited 2003 May 23]. Available from http://vm.cfsan.fda.gov/~ebam/bam-toc.html

Standard Plate Count

Maturin LJ, Peeler JT (1998) Aerobic plate count. In U.S. Food and Drug Administration, Bacteriological analytical manual, 8th ed, 3.01-3.10. [cited 2003 May 23]. Available from http://vm.cfsan.fda.gov/~ebam/bam-toc.html

Swanson KMJ, Busta FF, Peterson EH, Johnson MG (1992) Colony count methods. In Vanderzant C, Splittstiesser DF (eds), Compendium of methods for the microbiological examination of foods, 3rd ed. American Public Health Association, Washington, DC

Houghtby GA, Maturin LJ, Koenig EK (1992) Microbiological count methods. In Marshall RT (ed), Standard methods for the examination of dairy products, 16th ed. American Public Health Association, Washington, DC, pp 213-246

Yeast and Mold Count

Stack ME, Mislivec PB, Koch HA, Bandler R (1998) In U.S. Food and Drug Administration, Bacteriological analytical manual, 8th ed, 18.01-18.10. [cited 2003 May 23]. Available from http://vm.cfsan.fda.gov/~ebam/bam-toc.html

Chapter 4: Protocols for Evaluating Feedstuffs with Genetically Modified Input Traits: Poultry Meat Production

This chapter focuses on guidelines for conducting nutritional evaluations of genetically modified (GM) crops and their byproducts containing input traits as measured by performance of broiler chickens for meat production. Researchers interested in digestibility studies should refer to the publication by Fuller (1991).

Experiments conducted under outdoor conditions (e.g., open-front buildings, free-range conditions) should include a daily report of the climatic conditions. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health. In addition, unapproved GM crops and animals fed such crops should be handled and disposed of in accordance with each country's regulations.

Evaluation of Cereal Grains in Broiler Experiments

This protocol is a guide on how to determine the nutritional value of GM cereal grains for broiler chickens from 1 to 3 days posthatch to 5 weeks of age or older. It is essential that one specific control treatment—a near-isogenic counterpart that lacks the particular input trait under investigation—be included as well as commercial conventional varieties when possible.

Maize

When conducting a study to assess the nutritional value of a GM maize for poultry, a near-isogenic conventional maize variety should be included as control treatment. In addition, two to four other treatments consisting of different sources of nontransgenic maize varieties typically produced in the test region should also be included.

Production, handling, storage, and processing of the maize will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the maize for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Broilers. Healthy male and female broilers of defined genetic background will be used in the study. Equal numbers of chicks of the same sex will be penned by sex, and the pen will serve as the experimental unit. Generally there will be 9 to 12 birds per pen but more birds per pen can be used if necessary. Floor pens will usually be used and pen space per bird will be 800 to 950 cm^2 in accordance with approved guidelines as described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999) or accepted local guidelines. Constant lighting (24 hours/day) is recommended. Birds will be weighed at the beginning and end of each experimental period. Records of bird weights, feed disappearance, bird health, and other data will be kept.

Design and allotment. The goal is to ensure that the number of replications (number of pens per treatment) will be adequate to detect, at $P < 0.05$, a 5% difference from the mean using an alpha level of 0.05 and a beta level of 0.20 for a coefficient of variation of 4% to 5%. In most cases a randomized complete block design will be used. Birds will be blocked by sex and randomly assigned to 10 to 12 pens per treatment holding 9 to 12 birds of the same sex per pen.

Diets. Balanced diets should be formulated according to National Research Council (NRC 1994) requirements or accepted local nutrient requirements with most of the energy requirement of the test species being met by the inclusion of maize. Diets will be based on maize and soybean meal (or other protein source commonly used for poultry in the region) and supplemented with phosphorus, calcium, salt, trace minerals, and vitamins as necessary.

Diets should be formulated on the basis of total or digestible amino acids. The amount of maize in all of the treatment diets must be the same. Likewise, the amount of soybean meal in the diets should be approximately the same. Other high-protein meals may be used in addition to or in place of soybean meal, but their levels should be the same for each treatment diet. Crystalline amino acids may be included in the diets as appropriate to ensure that all diets meet or slightly exceed the amino acid requirements of broilers. In countries where commercial diets normally contain added fat, all diets should contain 3% to 5% added fat with adjustments made so that all diets are isoenergetic. All dietary ingredients should be mixed before delivery to birds and any sorting and rejection of specific fractions should be monitored and recorded.

The experiment will be divided into two or more feeding phases based on bird age. Bird weights will be measured and recorded at the end of each phase. Diets will be reformulated at the beginning of each phase as described above.

The maize should be ground to a consistent geometric mean particle size and the processing should be documented. The diets should be processed to a physical form (meal, pellets, crumbles, etc.) that is common to local stan-

dard practices and the processing should be documented; all diets should be fed in the same form. Diets may include growth promoters, coccidiostats, and enzymes at the discretion of the investigator and according to local best practice and regulations. The inclusion rate for additives should be the same for each treatment diet.

Removal of birds. Mortality will be recorded daily and dead birds will be removed. Weight of dead birds should be recorded. A qualified veterinarian should perform or supervise a diagnostic necropsy on all dead birds. The final growth data should not include data from any birds removed from the experiment. Adjusted feed conversion should be calculated by dividing the total feed consumed by the total weight gain of surviving and dead birds per pen.

Termination of experiment. The experiment will be terminated when birds reach a desired market weight (at 5 weeks of age or older). Carcass data should be collected if possible.

Statistical analysis of data. Performance data (gain, feed intake, gain-feed or feed-gain ratio) will be summarized and statistically analyzed as a randomized complete block using appropriate analysis of variance methodology. The pen will be considered the experimental unit for all traits. See detailed protocols in Chapter 9, "Statistical Analysis and Interpretation of Results."

Other Cereal Grains

Procedures for other cereal grains (e.g., wheat, rice, and barley) will be similar to those described for maize. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient and the two to four commercially available varieties. When wheat is studied, it should be the only cereal grain included in the diet. Xylanases may be added to wheat-based diets if deemed necessary, but the use of enzymes should be consistent in all diets. Similarly, a typical amount of beta-glucanase or other digestive enzymes may be added to barley-based diets provided that the use of enzymes is consistent in all diets.

Evaluation of Crop Protein Supplements in Broiler Experiments

This protocol will be used to evaluate the nutritional value of GM soybean meals for broiler chickens from 1 to 3 days posthatch to 5 weeks of age or older. It is essential that one specific control treatment-a near-isogenic counterpart that lacks the particular input trait under investigation-be included as well as commercial conventional varieties when possible.

Soybean Meal

Studies will assess soybean meal from GM soybeans and soybean meal from a near-isogenic, conventional (control) soybean. In addition, two to four other treatments consisting of different sources of nontransgenic soybean varieties typically produced in the region of the investigator should also be included. All soybean meals compared in the same experiment must be produced by the same oil extraction process (i.e., solvent extraction or expeller extraction).

Production, handling, storage, and processing of the soybeans and soybean meals will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the soybeans and soybean meals for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Broilers. Same as described in the maize section.

Design and allotment. Same as described in the maize section.

Diets. Balanced diets should be formulated according to NRC (1994) requirements or accepted local nutrient requirements with most of the energy requirement of the test species being met by the inclusion of maize. Diets will be based on maize and soybean meal and supplemented with phosphorus, calcium, salt, trace minerals, and vitamins as necessary. Other cereal grains or grain byproducts may be used in addition to or in place of maize but their levels should be about the same for each treatment diet.

Diets should be formulated on the basis of total or digestible amino acids. The amount of soybean meal in all of the treatment diets must be the same. Likewise, the amount of maize (or alternate cereal grain) in the diets should be approximately the same. Crystalline amino acids may be included in the diets as appropriate to ensure that all diets meet or slightly exceed the amino acid requirements of broilers. In countries where commercial diets normally contain added fat, all diets should contain 3% to 5% added fat with adjustments made so that all diets are isoenergetic. All dietary ingredients should be mixed before delivery to birds and any sorting and rejection of specific fraction should be monitored and recorded.

The experiment will be divided into two or more feed-

ing phases based on bird age. Diets will be formulated to be isoenergetic and isonitrogenous within each phase. At the beginning of each subsequent phase, the dietary amino acid concentration will be changed by adjusting the amounts of soybean meal, cereal grain, and other ingredients.

The diets should be processed to a physical form (meal, pellets, crumbles, etc.) that is common to local standard practices and the processing should be documented; all diets should be fed in the same form. Diets may include growth promoters, coccidiostats, and enzymes at the discretion of the investigator and according to local best practice and regulations. The inclusion rate for additives should be the same for each treatment diet.

Removal of birds. Same as described in the maize section.

Termination of experiment. Same as described in the maize section.

Statistical analysis of data. Same as described in the maize section.

Other Crop Protein Supplements

Procedures to evaluate other protein supplements (e.g., cottonseed meal, canola [rapeseed] meal, sunflower meal, lentils, peas, faba beans, heated full-fat soybeans) will be similar to those described for soybean meal. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient. The amounts of protein supplements in the diets should conform to local industry practice. Soybean meal (or other protein source used in common practice in the region) should constitute the rest of the supplemental protein. Cottonseed meal should constitute no more than one-half of the supplemental protein source and should be tested for free gossypol content. The amount of free gossypol in the diet generally should be 0.01% (100 ppm) or less. However, the diet may contain up to 0.04% (400 ppm) free gossypol if additional ferrous sulfate is included to provide a 1:1 weight ratio of iron to free gossypol. Canola meal should be tested for glucosinolates and be within the limits for canola meal. In studies with full-fat soybeans, the soybeans should be properly heated by extrusion, infrared heaters, gas heaters, or other acceptable heating mechanisms. Proper heating for destruction of trypsin inhibitors should be evaluated by subjecting the beans to urease analysis using standard methods. Whole beans should be ground after heating. If the GM and conventional soybeans differ in fat content, additional supplemental fat (preferably soybean oil) should be included in the diet containing the soybeans with the lowest fat content to make the diets isoenergetic.

References

FASS (Federation of Animal Science Societies) (1999) Guide for the care and use of agricultural animals in agricultural research and teaching. FASS, Savoy, IL

Fuller MF (ed) (1991) In vitro digestion for pigs and poultry. CAB International, Wallingford, United Kingdom

NRC (National Research Council) (1994) Nutrient requirements of poultry, 9th ed. National Academy Press, Washingon, DC

Chapter 5: Protocols for Evaluating Feedstuffs with Genetically Modified Input Traits: Poultry Egg Production

This chapter focuses on guidelines for conducting nutritional evaluations of genetically modified (GM) crops and their byproducts containing input traits as measured by performance of laying hens. Researchers interested in digestibility studies should refer to the publication by Fuller (1991).

Experiments conducted under outdoor conditions (e.g., open-front buildings, free-range conditions) should include a daily report of the climatic conditions. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health. In addition, unapproved GM crops and animals fed such crops should be handled and disposed of in accordance with each country's regulations.

Evaluation of Cereal Grains in Layer Experiments

This protocol is a guide on how to evaluate the nutritional value of GM cereal grains for layers from approximately 18 to 40 weeks of age and possibly throughout the entire laying cycle. It is essential that one specific control treatment-a near-isogenic counterpart that lacks the particular input trait under investigation-be included as well as commercial conventional varieties when possible.

Maize

The studies will assess a GM maize and a near-isogenic, conventional (control) maize. Each study will evaluate a minimum of two treatments. In addition, two to four other treatments consisting of different sources of nontransgenic maize varieties typically produced in the region of the investigator should also be included.

Production, handling, storage, and processing of the maize will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the maize for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Layers. Healthy pullets (16 weeks of age) of defined genetic background will be used in the study. From hatch to age 16 weeks, all birds will be fed the same diets formulated to meet the nutritional needs of developing pullets. At approximately 16 to 18 weeks of age, birds will be randomly placed in cages. Generally there will be three to five hens per cage but fewer or more birds per cage can be used if necessary. Birds will be allowed space in accordance with approved guidelines as described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999) or local regulations. Feed consumption will be measured at the end of every feeding phase and egg production will be determined daily. Hens will be weighed at the beginning of the experiment and at the beginning and end of every feeding phase. Records of bird weights, egg production (saleable and nonsaleable), feed consumption, hen health, egg weight, and other data will be kept as appropriate for good management practices.

Design and allotment. A complete randomized block design will be used. Twelve to 15 cages holding three to five layers per cage will be randomly assigned to treatments. Cages will be randomly assigned within the research facility to eliminate any bias due to location in the building.

Number of replications. The number of replications (number of cages per treatment) should be adequate to detect, at $P < 0.05$, a 5% difference from the mean using an alpha level of 0.05 and a beta level of 0.20. For a coefficient of variation of 4% to 5%, 12 to 15 replications per treatment will likely be required.

Diets. Balanced diets should be formulated according to National Research Council requirements (NRC 1994) or accepted local nutrient requirements with the energy requirement of the test species being met by the inclusion of maize. Diets will be based on maize and soybean meal (or other protein source used in common practice in the region) and supplemented with phosphorus, calcium, salt, trace minerals, and vitamins as necessary.

Diets should be formulated on the basis of total or digestible amino acids. The amount of maize in all of the treatment diets must be the same. Likewise, the amount of soybean meal in the diets should be approximately the same. Other high-protein meals may be used in addition to or in place of soybean meal but their levels should be the same for each treatment diet. Crystalline amino acids may be included in the diets as appropriate to ensure that all diets meet or slightly exceed the amino acid requirements of layers. In countries where commercial diets normally contain added fat, all diets should contain 3% to 5% added fat with adjustments made so that all diets are isoenergetic. All dietary ingredients should be mixed before delivery to birds and any sorting and rejection of specific fraction should be monitored and recorded.

The experiment will be divided into a minimum of three 28-day phases based on stage of egg production. Diets will be formulated to be isoenergetic and isonitrogenous within each phase. At the beginning of each subsequent phase, the dietary amino acid concentration will be changed by adjusting the amounts of maize, soybean meal, and other feed ingredients. Dietary energy concentration will be changed by altering the amount of fat in the diets.

The maize should be ground to a consistent geometric mean particle size and the processing should be documented. The diets should be processed to a physical form (meal, pellets, crumbles, etc.) that is common to local standard practices and the processing should be documented; all diets should be fed in the same form. Diets may include growth promoters, coccidiostats, and enzymes at the discretion of the investigator and according to local regulations and best practice and regulations. The inclusion rate for additives should be the same for each treatment diet.

Removal of birds. Mortality will be recorded daily; the weight of dead birds should be recorded. A qualified veterinarian should perform or supervise a diagnostic necropsy on all dead birds.

Termination of experiment. The experiment will be terminated after peak production (minimum of 32 weeks of age). Egg quality data (i.e., egg weight, eggshell quality, albumin quality, and yolk quality) should be collected.

Statistical analysis of data. Performance data (egg production, feed intake, body weight changes, egg quality traits, and feed conversion [expressed as kg feed/ kg eggs produced]) will be summarized from the start of the experiment to the end of the various phases and for the entire experiment. These and other performance and egg traits will be statistically analyzed as a completely randomized or randomized block design using appropriate analysis of variance methodology. The cage will be considered the experimental unit for all traits. See detailed protocols in Chapter 9, "Statistical Analysis and Interpretation of Results."

Other Cereal Grains

Procedures for other cereal grains (e.g., wheat, rice, and barley) will be similar to those described for maize. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient and the two to four commercially available varieties. When wheat is studied, it should be the only cereal grain included in the diet. Xylanases may be added to wheat-based diets if deemed necessary, but the use of enzymes should be consistent in all diets. Similarly, a typical amount of beta-glucanase or other digestive enzymes may be added to barley-based diets, provided that the use of enzymes is consistent in all diets.

Evaluation of Crop Protein Supplements in Layer Experiments

This protocol will be used to evaluate the nutritional value of GM oilseed meals for layers from approximately 18 to 40 weeks of age and possibly throughout the entire laying cycle. It is essential that one specific control treatment-a near-isogenic counterpart that lacks the particular input trait under investigation-be included as well as commercial conventional varieties when possible.

Soybean Meal

The studies will assess soybean meal from GM soybeans and soybean meal from a near-isogenic, conventional (control) soybean. In addition, two to four other treatments consisting of different sources of nontransgenic soybean varieties typically produced in the region of the investigator should also be included. All soybean meals compared in the same experiment must be produced by the same oil extraction process (i.e., solvent extraction or expeller extraction).

Production, handling, storage, and processing of the soybeans and soybean meals will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the soybeans and soybean meals for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Layers. Same as described in the maize section.

Design and allotment. Same as described in the maize section.

Number of replications. Same as described in the maize section.

Diets. Balanced diets should be formulated according to National Research Council (NRC 1994) requirements or accepted local nutrient requirements, with the protein requirement of the test species being met by the inclusion of soybean meal. Diets will be based on cereal grain and soybean meal and supplemented with phosphorus, calcium, salt, trace minerals, and vitamins as necessary.

Diets should be formulated on the basis of total or digestible amino acids. The amount of soybean meal in all of the treatment diets must be the same. Likewise, the

amount of maize in the diets should be approximately the same. Other cereal grains or grain byproducts may be used in addition to or in place of maize, but their levels should be the same for each treatment diet. Crystalline amino acids may be included in the diets as appropriate to ensure that all diets meet or slightly exceed the amino acid requirements of layers. In countries where commercial diets normally contain added fat, all diets should contain 3% to 5% added fat with adjustments made so that all diets are isoenergetic. All dietary ingredients should be mixed before delivery to birds and any sorting and rejection of specific fraction should be monitored and recorded.

The experiment will be divided into a minimum of three 28-day phases based on stage of egg production. Diets will be formulated to be isoenergetic and isonitrogenous within each phase. At the beginning of each subsequent phase, the dietary amino acid concentration will be changed by adjusting the amounts of maize, soybean meal, and other feed ingredients. Dietary energy concentration will be changed by altering the amount of fat in the diets.

The diets should be processed to a physical form (meal, pellets, crumbles, etc.) that is common to local standard practices and the processing should be documented; all diets should be fed in the same form. Diets may include growth promoters, coccidiostats, and enzymes at the discretion of the investigator and according to local best practice and regulations. The inclusion rate for additives should be the same for each treatment diet.

Removal of birds. Same as described in the maize section.

Termination of experiment. Same as described in the maize section.

Statistical analysis of data. Same as described in the maize section.

Other Crop Protein Supplements

Procedures to evaluate other protein supplements (e.g., cottonseed meal, canola [rapeseed] meal, sunflower meal, lentils, peas, faba beans, lupins, heated full-fat soybeans) will be similar to those described for soybean meals. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient. When oilseed meals are evaluated, the GM and isogenic control oilseed meals should be produced by the same oil extraction process. The amounts of protein supplements in the diets should conform to local industry practice. In many cases the vegetable protein may not be able to constitute the entire supplemental protein source because of adverse effects on feed intake and growth performance. Soybean meal (or other protein source used in common practice in the region) should constitute the rest of the supplemental protein. Cottonseed meal should constitute no more than one-half of the supplemental protein source and should be tested for free gossypol content (see Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material"). The amount of free gossypol in the diet generally should be ≤0.01% (100 ppm). However, the diet may contain up to 0.04% (400 ppm) free gossypol if additional ferrous sulfate is included to provide a 1:1 weight ratio of iron to free gossypol. Canola meal should be tested for glucosinolates and be within the limits for canola meal (see Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material"). In studies with full-fat soybeans, the soybeans should be properly heated by extrusion, infrared heaters, gas heaters, or other acceptable heating mechanisms. Proper heating for destruction of trypsin inhibitors should be evaluated by subjecting the beans to urease analysis using standard methods (see Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material"). Whole beans should be ground after heating. If the GM and conventional soybeans differ in fat content, additional supplemental fat (preferably soybean oil) should be included in the diet containing the soybeans with the lowest fat content to make the diets isoenergetic.

References

FASS (Federation of Animal Science Societies) (1999) Guide for the care and use of agricultural animals in agricultural research and teaching. FASS, Savoy, IL

Fuller MF (ed) (1991) In vitro digestion for pigs and poultry. CAB International, Wallingford, United Kingdom

NRC (National Research Council) (1994) Nutrient requirements of poultry, 9th ed. National Academy Press, Washington, DC

Chapter 6: Protocols for Evaluating Feedstuffs with Genetically Modified Input Traits: Swine

This chapter focuses on guidelines for conducting nutritional evaluations of genetically modified (GM) crops and their byproducts containing input traits as measured by performance of swine. Researchers interested in digestibility studies should refer to publications by Fuller (1991), Adeola (2001), and Gabert et al. (2001).

Experiments conducted under outdoor conditions (e.g., open-front buildings, pastures, dry lots) should include a daily report of the climatic conditions. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health. In addition, unapproved GM crops and animals fed such crops should be handled and disposed of in accordance with each country's regulations.

Evaluation of Cereal Grains in Starter or Grower Pig Experiments

Short-term experiments are sometimes conducted with weanling pigs over a constant time period during the postweaning "starter" period of growth. Typically, these experiments will involve weaned pigs with an initial average age of 3 to 5 weeks of age (7 to 12 kg body weight) and will last 4 to 6 weeks. "Grower" experiments will generally involve pigs with an initial average body weight of 15 to 25 kg and will last 6 to 8 weeks. The protocols will be similar to those described for growing-finishing pigs. However, pigs will be weighed and feed consumption will be determined weekly during the experiment. The more frequent measurements in the shorter trials with younger pigs enable closer monitoring of pig performance and accurate adjustments of performance data if a pig dies or is removed from the experiment.

Evaluation of Cereal Grains in Growing-Finishing Pig Experiments

This protocol will be used to evaluate the nutritional value of GM cereal grain for growing-finishing pigs over the body weight range of 20 to 120 kg (or other final weight depending on local practice). In this protocol maize is used as the example. It is essential that an appropriate control-a near-isogenic cereal grain (in this case, a near-isogenic maize) that lacks the particular input trait under investigation-as well as commercial conventional varieties be included when possible.

Maize

The studies will assess a GM maize and a near-isogenic, conventional (control) maize. Each study will evaluate these two treatments and, if possible, other treatments that involve one or more other types of conventional maize typically produced in the region of the investigator.

Production, handling, storage, and processing of the maize will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the maize for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Pigs. Healthy pigs of similar genetic background that have been fed a common diet for at least 1 week before assignment to experimental treatments will be used in the study. Females (gilts) and either castrates (barrows) or intact males (boars) may be used in the study. All pigs will be individually identified by ear notches, ear tags, or another method. Generally, there will be four to eight pigs per pen but fewer or more pigs per pen can be used if necessary. Alternatively, pigs may be housed in individual pens, in which case the experimental unit will be the individual pig. Pen space per pig (or pen size of individually housed pigs) will be in accordance with approved guidelines as described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999) or a similar set of guidelines.

All pigs will be individually weighed before assignment and then again at the start of the experiment unless the assignment and start are on the same day. In addition, pigs will be weighed and feed consumption will be determined at 2- or 3-week intervals during the experiment. Whenever possible, pigs should be weighed at a similar time of the day to reduce differences in gut fill and other sources of variation. In most experiments pigs should be allowed free access to diets throughout the experiment to examine whether treatment affects voluntary feed intake. Care must be taken to ensure that feed wastage is keep to a minimum so that feed disappearance can be equated to feed consumption. Periodic weighing and feed intake measurements rather than only initial and final measurements enables pig performance to be monitored more closely and performance data to be adjusted more accurately if a pig dies or is removed from the experiment. Records of pig weights, feed disappearance, animal health, and other data will be kept as appropriate for general standards of good animal management practices.

Design and allotment. In most cases a randomized complete block design is recommended. Pigs will be randomly assigned to treatments and outcome groups based on their initial weight and sex. For example, in a study with two treatments, the first outcome group may be the two heaviest males randomly assigned to two pens in the first block, the second outcome group is the next two heaviest males, etc. In this example, the two pens in the first block (each consisting of four to eight outcome groups) will be randomly allotted to the two treatments. Blocks may consist of the same sex or of mixed sexes but the sex ratio must be constant within each block. Having the same sex within blocks makes it possible to identify sex and sex × (treatment effects; these effects cannot be evaluated in blocks of mixed sexes. Pens will be assigned to blocks within the research facility to eliminate bias due to location in the building. Similar allotment guidelines should be followed in experiments where pigs are housed individually (i.e., same sex within blocks and blocks based on body weight and position in the research facility).

Number of replications. The number of replications (or blocks) per treatment should be adequate to detect, at $P < 0.05$, a 10% difference between treatment means 80% of the time (see Chapter 9, "Statistical Analysis and Interpretation of Results"). For a coefficient of variation of 5% to 7% (typical for growth rate and feed efficiency in group-penned pigs), six to nine replications per treatment are required. More replications are required for individually penned pigs or pens with fewer than four pigs, which typically have a higher coefficient of variation.

Diets. Diets will consist of maize and soybean meal (or other protein source commonly used for swine in the region) fortified with a highly bioavailable source of phosphorus, calcium, salt, trace minerals, and vitamins to meet or exceed nutrient requirements as specified by the National Research Council (NRC 1998) or a similar set of standards. Care should be taken to ensure that the bioavailable phosphorus requirement is met.

Diets should be formulated on the basis of total or digestible amino acids. The amount of maize in all of the treatment diets must be the same. Likewise, the amount of soybean meal in the diets should be approximately the same. Other high-protein meal sources (e.g., fish meal, flash dried blood meal, and milk powder) may be used in addition to or in place of soybean meal but their levels should be the same for each treatment diet. Diets should be formulated to meet 105% of the lysine requirement. Crystalline lysine (L-lysine·HCl), threonine, tryptophan, and methionine may be included to ensure that the total and digestible lysine, threonine, tryptophan, and methionine plus cystine contents are nutritionally adequate in all diets. In countries where commercial diets normally contain added fat, all diets should contain 1% to 3% added fat with adjustments made so that all diets are isoenergetic.

The experiment will be divided into two or three phases based on mean body weights of the pigs (e.g., 20 to 50 kg, 50 to 90 kg, 90 to 120 kg). At the beginning of each subsequent phase, the dietary essential amino acid concentrations will be reduced by adjusting the amounts of maize and soybean meal. All diet changes will be made at the same time within each replication.

The maize should be ground to a consistent geometric mean particle size (600 to 900 μm) and the processing should be documented. The diets should be processed to a physical form (meal, pellets, crumbles, etc.) that is common to local standard practices and the processing should be documented; all diets should be fed in the same form. Diets should not include added enzymes but may contain antimicrobial growth promoters at the discretion of the investigator and according to local best practice and regulations. The inclusion rate for additives should be the same for each treatment diet.

Removal of pigs. Any pig that loses body weight during a weigh period or gains very little body weight for two consecutive weigh periods should be removed from the experiment. Appropriate adjustments in pen feed consumption should be made based on the estimated feed intake of the removed pig (the pig's gain multiplied by the pen feed-gain ratio). The final growth data should not include data for any pigs removed from the experiment. A qualified veterinarian should perform or supervise a diagnostic necropsy on all pigs that are removed or die during the experiment.

Termination of experiment. The experiment will be terminated on a replication (block) basis when the average pig weight within a block reaches 120 kg or another targeted final body weight. Experiments are expected to last approximately 16 weeks. When possible, carcass data (such as carcass lean, lean-fat ratio, etc.) that are indicators of economically important traits should be obtained. Examples of such data in the United States could include carcass yield (100 × hot carcass weight/final live body weight), 10^{th} rib backfat, 10^{th} rib longissimus muscle area, and estimated carcass lean percentage using the National Pork Producers Council equation for fat-free lean (NPPC

2000). Experiments conducted in other countries may use locally accepted measures of carcass leanness or fat quality evaluation.

Statistical analysis of data. Performance data (daily gain, daily feed intake, gain-feed or feed-gain ratio) will be summarized from the start of the experiment to the end of the various phases and for the entire experiment. These and other performance and carcass traits will be statistically analyzed as a randomized complete block using approved analysis of variance methodology (see Chapter 9, "Statistical Analysis and Interpretation of Results"). Either the general linear model (GLM) or MIXED procedure of the Statistical Analysis System (SAS Institute, Cary, NC; http://www.sas.com) or an equivalent procedure in GenStat (VSN International Ltd., Hemel Hempstead, Herts HP1 1ES, United Kingdom; http://www.vsn-intl.com/genstat/) is recommended. The pen will be considered the experimental unit for all traits. In some instances (e.g., carcass traits), it may be desirable to use covariance procedures to adjust for differences in final body weight.

Other Cereal Grains

Procedures for evaluating other cereal grains (wheat, sorghum, rice, and barley) will be similar to those described for maize. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient. Diets should contain the maximum amount of grain possible but the amount should be in line with normal feeding practices. If barley is evaluated, the beta-glucan content should be determined if possible. Cereal grains should be ground to a consistent geometric mean particle size and the processing should be documented.

Evaluation of Crop Protein Supplements in Starter or Grower Pig Experiments

Short-term experiments are sometimes conducted with weanling pigs over a constant time period during the postweaning starter period of growth. Typically, these experiments will involve weaned pigs with an initial average age of 3 to 5 weeks (7 to 12 kg body weight) and will last 4 to 6 weeks. Grower experiments will generally involve pigs with an initial average body weight of 15 to 25 kg and will last 6 to 8 weeks. The protocols will be similar to those described for growing-finishing pigs. However pigs will be weighed and feed consumption will be determined at weekly intervals during the experiment. The more frequent measurements in the shorter trials with younger pigs enable closer monitoring of pig performance and accurate adjustments of performance data if a pig dies or is removed from the experiment.

Evaluation of Crop protein Supplements in Growing-Finishing Pig Experiments

This protocol will be used to evaluate the nutritional value of GM oilseed meals for growing-finishing pigs over the body weight range of 20 to 120 kg (or other final weight depending on local practice). In this protocol soybean meal is used as the example. It is essential that an appropriate control—a near-isogenic oilseed (in this case, a near-isogenic soybean meal) that lacks the particular input trait under investigation—as well as commercial conventional varieties be included when possible.

Soybean Meal

The studies will assess soybean meal from GM soybeans and near-isogenic conventional (control) soybeans. Each study will evaluate a minimum of these two treatments and, if possible, other treatments that will involve one or more other types of conventional soybean meal typically produced in the region of the investigator. All soybean meals compared in the same experiment must be produced by the same oil extraction process (i.e., solvent extraction or expeller extraction).

Production, handling, storage, and processing of the soybeans and soybean meals will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the soybeans and soybean meals for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Pigs. Healthy pigs of similar genetic background that have been fed a common diet for at least 1 week before assignment to experimental treatments will be used in the study. Females (gilts) and either castrates (barrows) or intact males (boars) may be used in the study. All pigs will be individually identified by ear notches, ear tags, or another method. Generally, each pen will house four to eight pigs but fewer or more pigs per pen can be used if necessary. Alternatively, pigs may be housed in individual pens, in which case the experimental unit will be the individual pig. Pen space per pig (or pen size of individually housed pigs) will be in accordance with approved guidelines as described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999) or a similar set of guidelines.

All pigs will be individually weighed before assignment and then again at the start of the experiment unless the assignment and start are on the same day. In addition, pigs will be weighed and feed consumption will be determined at 2- or 3-week intervals during the experiment. In most experiments, pigs should be allowed free access to diets throughout the experiment to examine whether treatment affects voluntary feed intake. Care must be taken to ensure that feed wastage is keep to a minimum so that feed disappearance can be equated to feed consumption. Periodic weighing and feed intake measurements rather than only initial and final measurements enables pig performance to be monitored more closely and performance data to be adjusted more accurately if a pig dies or is removed from the experiment. Records of pig weights, feed disappearance, animal health, and other data will be kept as appropriate for general standards of good animal management practices.

Design and allotment. In most cases a randomized complete block design is recommended. Pigs will be randomly assigned to treatments and outcome groups based on their initial weight and sex. For example, in a study with two treatments, the first outcome group may be the two heaviest males, randomly assigned to two pens in the first block, the second outcome group is the next two heaviest males, etc. In this example, the two pens in the first block (each consisting of four to eight outcome groups) will be randomly allotted to the two treatments. Blocks may consist of the same sex or of mixed sexes, but the sex ratio must be constant within each block. Having the same sex within blocks makes it possible to identify sex and sex × treatment effects; these effects cannot be evaluated in blocks of mixed sexes. Pens will be assigned to blocks within the research facility to eliminate any bias due to location in the building. Similar allotment guidelines should be followed in experiments where pigs are housed individually (i.e., same sex within blocks and blocks based on body weight and position in the research facility).

Number of replications. The number of replications (or blocks) per treatment should be adequate to detect, at $P < 0.05$, a 10% difference between treatment means 80% of the time (see Chapter 9, "Statistical Analysis and Interpretation of Results"). For a coefficient of variation of 5% to 7% (typical for growth rate and feed efficiency in group-penned pigs), six to nine replications per treatment are required. More replications are required for individually penned pigs or pens with fewer than four pigs, which typically have a higher coefficient of variation.

Diets. Diets will consist of maize (or other cereal grain commonly used for swine in the region) and soybean meal fortified with a highly bioavailable source of phosphorus, calcium, salt, trace minerals, and vitamins to meet or exceed nutrient requirements as specified by NRC (1998) or a similar set of standards. Care should be taken to ensure that the requirement for bioavailable phosphorus is met.

Diets should be formulated on the basis of total or digestible amino acids. The amount of soybean meal in all treatment diets must be the same. Likewise, the amount of maize in the diets should be approximately the same. Other cereal grains or byproducts may be used in addition to or in place of maize but their levels should be the same for each treatment diet. Diets should be formulated to meet 105% of the lysine requirement. Crystalline lysine (L-lysine·HCl), threonine, tryptophan, and methionine may be included to ensure that the total and digestible lysine, threonine, tryptophan, and methionine plus cystine contents are nutritionally adequate in all diets. In countries where commercial diets normally contain added fat, all diets should contain 1% to 3% added fat with adjustments made so that all diets are isoenergetic.

The experiment will be typically divided into two, three, or more phases based on mean body weights of the pigs (e.g., 20 to 50 kg, 50 to 90 kg, 90 to 120 kg). Phases are based on diet changes. After the initiation phase the dietary essential amino acid concentrations will be reduced during each subsequent phase by adjusting the amounts of maize and soybean meal. All diet changes will be made at the same time within each replication.

The maize should be ground to a consistent geometric mean particle size and the processing should be documented. The diets should be processed to a physical form (meal, pellets, crumbles, etc.) that is common to local standard practices and the processing should be documented; all diets should be fed in the same form. Diets should not include added enzymes but may contain antimicrobial growth promoters at the discretion of the investigator and according to local best practice and regulations. The inclusion rate for additives should be the same for each treatment diet.

Removal of pigs. Any pig that loses body weight during a weigh period or gains very little body weight for two consecutive weigh periods should be removed from the experiment. Appropriate adjustments in pen feed consumption should be made based on the estimated feed intake of the removed pig (the pig's gain multiplied by the

pen feed-gain ratio). The final growth data should not include any pigs removed from the experiment. A qualified veterinarian should perform or supervise a diagnostic necropsy on all pigs that are removed or die during the experiment.

Termination of experiment. The experiment will be terminated on a replication (block) basis when the average pig weight within a block reaches 120 kg body weight or another targeted final body weight. Duration of the experiment is expected to be approximately 16 weeks. When possible, carcass data (such as carcass lean, lean-fat ratio, etc.) that are indicators of economically important traits should be obtained. Examples of such data in the United States could include carcass yield (100 × hot carcass weight/final live body weight), 10^{th} rib backfat, 10^{th} rib longissimus muscle area, and estimated carcass lean percentage using the National Pork Producers Council equation for fat-free lean (NPPC 2000). Experiments conducted in other countries may use locally accepted measures of carcass leanness or fat quality evaluation.

Statistical analysis of data. Performance data (daily gain, daily feed intake, gain-feed or feed-gain ratio) will be summarized from the start of the experiment to the end of the various phases and for the entire experiment. These and other performance and carcass traits will be statistically analyzed as a randomized complete block using approved analysis of variance methodology (see Chapter 9, "Statistical Analysis and Interpretation of Results"). Either the GLM or MIXED procedure of SAS or an equivalent procedure in GenStat is recommended. The pen will be considered the experimental unit for all traits. In some instances (e.g., carcass traits) it may be desirable to use covariance procedures to adjust for differences in final body weight.

Other Crop Protein Supplements

Procedures to evaluate other protein supplements (e.g., cottonseed meal, canola [rapeseed] meal, sunflower meal, lentils, peas, faba beans, lupins, and heated full-fat soybeans) will be similar to those described for soybean meals. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient. When oilseed meals are evaluated, the GM and the isogenic control oilseed meals should be produced by the same oil extraction process. The amounts of protein supplements in the diets should conform to local industry practice. In many cases the vegetable protein may not be able to constitute the entire supplemental protein source because of adverse effects on feed intake and growth performance. Soybean meal (or other protein source used in common practice in the region) should constitute the rest of the supplemental protein. Cottonseed meal should constitute no more than one-half of the supplemental protein source and should be tested for free gossypol content (see Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material"). The amount of free gossypol in the diet generally should be 0.01% (100 ppm) or less. However, the diet may contain up to 0.04% (400 ppm) free gossypol if additional ferrous sulfate is included to provide a 1:1 weight ratio of iron to free gossypol. Canola meal should be tested for glucosinolates and be within the limits for canola meal (see Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material"). In studies with full-fat soybeans, the soybeans should be properly heated by extrusion, infrared heaters, gas heaters, or other acceptable heating mechanisms. Proper heating for destruction of trypsin inhibitors should be evaluated by subjecting the beans to urease analysis using standard methods (see Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material"). Whole beans should be ground after heating. If the GM and conventional soybeans differ in fat content, additional supplemental fat (preferably soybean oil) should be included in the diet containing the soybeans with the lowest fat content to make the diets isoenergetic.

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Chapter 7: Protocols for Evaluating Feedstuffs with Genetically Modified Input Traits: Lactating Dairy Cows

This chapter focuses on guidelines for conducting nutritional evaluations of genetically modified (GM) crops and their byproducts containing input traits as measured by performance of lactating dairy cattle.

Experiments conducted under outdoor conditions (e.g., open-front buildings, pastures, dry lots, freestall housing) should include a daily report of the climatic conditions. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health. In addition, unapproved GM crops and animals fed such crops should be handled and disposed of in accordance with each country's regulations.

Evaluation of Cereal Grains, Cereal Silage, and Forages in Lactating Dairy Cow Experiments

This protocol will be used to evaluate the nutritional value of GM cereal grain and silage and harvested forages containing input traits for lactating dairy cows in postpeak lactation when crossover, switchback, or Latin square designs are used. These designs with 28-day periods should be appropriate to address significant unintended effects on intake and milk yield and composition. Because GM products containing input traits are nutritionally equivalent to their non-GM counterparts and the expressed transgenic protein is rapidly degraded in the gut, there is no scientific basis for expecting animal performance to be affected. In these designs, all cows receive all treatments thus allowing the researcher to use fewer animals (i.e., for four treatments, 12 to 16 cows total) to obtain the desired power of the test. However, if the researcher prefers a randomized block design, lactating dairy cows in prepeak lactation may be used as well. In this scenario, individual cows receive only one treatment and a minimum of 20 to 25 cows are needed per treatment (80 to 100 cows total for study containing 4 treatments) to achieve the same power. It is essential that an appropriate control—a near-isogenic cereal grain and silage that lacks the particular input trait under investigationas—well as commercial conventional varieties be included when possible.

Maize

The studies will assess a GM maize and a near-isogenic conventional (control) maize. Each study will evaluate a minimum of these two treatments and, if possible, other treatments that involve one or more other types of conventional maize typically produced in the region of the investigator. Production, handling, storage, and processing of the maize will be conducted as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the maize for mycotoxins and chemical components will be performed as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Cows. Healthy cows of similar genetic background that were fed a common diet for at least 2 weeks before start of the trial will be used. Animals will be fed in accordance with guidelines described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999). Multiparous cows should usually be used, although in some studies primiparous cows may be preferred. An equal number of multiparous and primiparous cows per treatment should be in postpeak lactation for crossover, switch-back, or Latin square designs or in prepeak lactation for randomized complete block designs; the breed should represent a major breed in the region where the study is conducted.

Design. In most cases when two treatments are used, a crossover design with two 28-day periods will be used. A Latin square design with 28-day periods generally would be used in studies evaluating more than two treatments. If a completely randomized block design is used, a 2-week pretreatment period should be used as a covariate for data analysis. Cows would be blocked by parity and stage of lactation when cows are prepeak. When feasible, milk yield and quality from a 2- to 3-week preexperimental period may also be used in assigning cows to blocks or as a covariate in the analysis of data. Ideally, cows would be housed in individual stalls to allow measurement of individual feed intakes. Using gates that detect sensors placed on individual cows (such as those manufactured by Calan) are another way to obtain individual measurements of feed intake of cows housed in groups.

Number of replications. The number of replications should be adequate to detect, at $P < 0.05$, a 5% to 10% difference between treatment means 80% of the time. Usually 10 to 12 animals per treatment are needed in a two-treatment crossover design, 12 to 16 animals per treatment (three to four replications of four cows in a square) are needed in a four-treatment Latin square design, and 20 to 25 animals per treatment are needed in a completely randomized block design.

Diets. Diets should meet or slightly exceed NRC (2001) requirements or accepted local nutrient requirements. Maize grain should be the primary or sole grain source and maize silage should be the primary source of forage. The amount of maize in all of the treatment diets must be the same. Likewise, the amounts of maize silage and any protein supplements should be approximately the same in each diet. All diets should be fortified with calcium, phosphorus, magnesium, salt, trace minerals, and vitamins as needed to meet the animals' requirements. Each dietary component (maize, maize silage, soybean meal, etc.) should be prepared in the same way (ground, rolled, chopped, etc.) for each of the dietary treatments so that all diets are similar in particle size, forage content, etc. Other locally available good-quality roughage may replace maize silage as the primary source of forage, and other protein sources available to the investigator may replace soybean meal. Diets should be isoenergetic and isonitrogenous.

Endpoint measurements. Dry matter intake, milk yield, fat-corrected milk yield, milk composition (i.e., fat, protein, and lactose), body weight, body condition score, somatic cell counts in milk, and observational measurements such as health should be recorded.

Statistical analysis of data. Data (daily milk yield, daily feed intake, body weight, body condition score, milk composition, somatic cell count, etc.) will be summarized for each experimental period and for the entire study. Data will be statistically analyzed using accepted analysis of variance methodology. Either the general linear model (GLM) or MIXED procedure of the Statistical Analysis System (SAS Institute, Cary, NC; http://www.sas.com) or an equivalent procedure in GenStat (VSN International Ltd., Hemel Hempstead, Herts HP1 1ES, United Kingdom; http://www.vsn-intl.com/genstat/) is recommended. When feed is fed to individual cows, the cow will be considered the experimental unit; when cows are fed as a group, the pen will be the experimental unit. See detailed guidelines in Chapter 9, "Statistical Analysis and Interpretation of Results."

Other Cereal Grain

Procedures will be similar to those described for maize. It is important that the GM feed ingredient under investigation be included in the diets at the same level as the near-isogenic control ingredient. When wheat is studied, care should be taken not to feed too much finely ground wheat, which could result in digestive upsets. To avoid problems when feeding wheat, gradually increase the level of wheat in the grain mixture to 20% over 2 weeks, after which wheat may be increased to 35% to 40% of the grain mixture.

Forages or Forage Products

These studies will compare nutritive value of a GM forage or forage product with its nearest available near-isogenic conventional (control) forage or forage product. Each study that is conducted will include a minimum of these two treatments and all other dietary ingredients will be held constant. Additional treatments, which may include one or more types of conventional forage or forage product of the same genus typically produced or used in the region, also may be fed so that comparisons with more diverse hybrids or strains can be drawn.

A sponsoring organization will provide the investigator with the two types of forage seed (GM and control) for some studies and the two types of forage or forage product (GM and control) for other studies. The investigator may process both types of forages separately under identical processing conditions, such as chopping and ensiling, depending on the objectives of the experiment.

If seed is supplied, the GM forage will be grown in an area sufficiently isolated from other crops to prevent cross pollination. Commonly accepted agronomic practices for the region will be used. The control forage will be grown in the same area with soil type and agronomic practices as similar as practical to those for the GM grain. Any differences in agronomic practices (fertilization, weed or insect control, irrigation) will be recorded and reported. Differences in insect damage or disease presence between the GM and control plants, quantified at several stages of plant growth by a qualified plant physiologist or disease specialist, will be recorded and reported. The GM and control forage will be harvested, handled, stored, and processed similarly but separately and held until the feeding trial begins. Harvest will be at a similar stage of maturity or moisture for both the GM and the control forage. Yield difference between the GM and control crops (fresh and dry matter basis) will be recorded. Care must be taken to identify clearly each forage or forage product and prevent cross mixing of forages or forage products of different type. If the forage or forage residue is to be grazed, subdivisions that will form paddocks around small groups of animals will be installed.

GM and control forage or forage products supplied for the feeding trial will be stored in separate but similar storage facilities and properly identified. Samples of the

forage taken at harvest and before feeding or grazing should be retained in case genetic verification of identity is required.

Forage or forage product analysis. If the forage is to be grazed, the quantity of available forage will be determined before the animal experiment begins and at 2-week intervals during the trial. A representative sample of each forage or forage product will be obtained at the start, midpoint, and end of the study using appropriate forage sampling procedures. Esophageal samples of grazed forage may be obtained. Whether forage or forage products are grazed or harvested for feeding, representative samples will be analyzed for dry matter, crude protein, crude fat, acid and neutral detergent fiber, and ash in a laboratory known to produce high-quality, consistent results. For pre-ensiled forage, fermentation quality predictors such as water-soluble carbohydrates and pH should also be measured. For ensiled forage, additional measurements to estimate recovery of dry matter after fermentation (100 (weight of silage × dry matter of silage/weight of forage harvested × dry matter of forage harvested) and silage quality (lactic and volatile fatty acids, ethanol, pH, ammonia, water-soluble protein, aerobic stability) should be taken.

Evaluation of Crop Protein Supplements in Lactating Dairy Cow Experiments

This protocol will be used to evaluate the nutritional value of GM crop protein supplements for lactating dairy cows in postpeak lactation (crossover, switch-back, and Latin square designs). In this protocol, soybean meal and soybeans are used as the example. It is essential that an appropriate control-near-isogenic soybean meal or soybeans-that lacks the particular input trait under investigation as well as commercial conventional varieties be included when possible.

Soybean Meal and Raw or Roasted Soybeans

The studies will assess GM soybeans and soybean meal from GM soybeans, and near-isogenic conventional soybeans and soybean meal from near-isogenic conventional (control) soybeans and commercial conventional soybean varieties. Production, handling, storage, and processing of the soybeans and soybean meals will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the soybeans and soybean meals for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Cows. See maize section.

Design. See maize section.

Number of replications. See maize section.

Diets. Diets should meet or slightly exceed NRC (2001) requirements or accepted local nutrient requirements. Soybean meal should be the primary or sole protein source. Roasted or raw soybeans could be added as an additional source of protein and energy. The amount of fat in the ration should be 6% or less of total diet dry matter.

Endpoint measurements. See maize section.

Statistical analysis of data. See maize section.

Other Crop Protein Supplements

Procedures for other crop protein supplements (e.g., cottonseed meal, whole cottonseed, canola [rapeseed] meal, sunflower meal, lentils, peas, faba beans, and lupins) will be similar to those described for soybean meal and soybeans. It is important that the GM crop protein supplement under investigation be included in the diet at the same level as the near-isogenic control feed ingredient. When possible, cows should be fed a diet containing the crop to be evaluated during a preexperimental period to allow them to become adapted to the ingredient. Whole cottonseed should not exceed 3.5 kg per cow per day. Canola meal should be tested for glucosinolate content. In studies with sunflower meal, lentils, peas, etc., the amounts of these protein supplements in the diets should conform to local industry practice.

References

FASS (Federation of Animal Science Societies) (1999) Guide for the care and use of agricultural animals in agricultural research and teaching. FASS, Savoy, IL

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Chapter 8: Protocols for Evaluating Feedstuffs with Genetically Modified Input Traits: Growing and Finishing Ruminants

This chapter focuses on guidelines for conducting nutritional evaluations of genetically modified (GM) crops and their byproducts containing input traits as measured by performance of growing and finishing ruminants.

Experiments conducted under outdoor conditions (e.g., open-front buildings, pastures, dry lots) should include a daily report of the climatic conditions. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health. In addition, unapproved GM crops and animals fed such crops should be handled and disposed of in accordance with each country's regulations.

This chapter focuses on the use of finishing ruminants to evaluate GM grains and grain and grain products and the use of growing and finishing ruminants to evaluate GM protein supplements. Finishing ruminants are ruminants during the last 90 to 120 days of feeding before they are taken to market. Finishing ruminants are used to evaluate grains because the energy requirement and the inclusion rate of grain products is the highest during this feeding period. Growing and finishing ruminants are used to evaluate protein supplements because the protein requirements and subsequent inclusion rates of protein sources are the highest during the growing phase and are reduced during the finishing phase.

This chapter also addresses the use of growing ruminants to evaluate GM forages or forage products and crop residues from GM forages.

Evaluation of Grains and Grain Products in Experiments with Finishing Ruminants

This protocol will be used to evaluate the nutritional value of GM grains (e.g., barley, maize, sorghum grain, millet, oats, rice, triticale, wheat) or products produced by extraction or processing of these grains (e.g., wet or dry milled products, fermentation residues) for finishing ruminants (beef cattle and sheep). In all experiments, an appropriate control grain or product—preferably the near-isogenic cultivar of the same hybrid that lacks the input trait being studied—must be included. Other controls may be included as specified below.

The studies will assess a GM grain and a near-isogenic conventional (control) grain. Each study will evaluate a minimum of these two treatments. Other treatments involving one or more other types of conventional grain typically produced in the region of the investigator will be included if possible.

Production, handling, storage, and processing of the grain will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the grain for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Test animals. Male, castrates or female ruminants of similar breed can be used; steers (bull castrates) will be fed from 300 kg or more until finished, heifers will be fed from 270 kg or more until finished, and lambs will be fed from 20 kg or more until finished. All cattle or sheep used should be healthy, free of parasites, and have a similar genetic history. Each animal will be individually identified with an ear tag, ear notch, or brand. Animals may be fed individually or in groups in accordance with guidelines described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999). Each animal will be weighed individually at the start of the trial and at 2- to 4-week intervals until the end of the feeding trial. Standardized procedures for weighing animals at the start and end of the trial should be used. These procedures can include weighing on two consecutive days with feed intake being restricted during the interval or weighing after an overnight period when animals have no access to feed or water. Weights can be taken at interim dates without limiting access to feed or water. Animal weights, feed delivery and refusals, dry matter and nutrient content of delivered and refused feed, and other animal and feeding data will be recorded and maintained as appropriate following good management practices.

Design and allotment. A design appropriate for statistical testing of effects will be used. For growth or performance measurements (i.e., when the animals will be fed the GM or the control grain for the full trial) the design typically will be a randomized complete block (preferably with blocking by initial body weight, breed, or sex as well as pen location). Different sexes will be placed in different blocks or balanced within pen among test diets. If sex is balanced within pen among test diets, sex and sex × treatment effects cannot be tested. For intake or digestibility measurements, crossover or Latin square experiments that provide increased statistical power can be used. Different blocks can be in different locations or buildings but the

environment within each block must be similar to avoid bias. Treatments will be randomly assigned to pens or animals within a block.

Number of replications. The number of replications (number of pens per treatment or, for animals fed individually, the number of animals per treatment) will be adequate to detect, at

$P < 0.05$, a 10% difference between treatment means 80% of the time. With a coefficient of variation of 5.0% to 7.5%, 6 to 10 replications per treatment will be required. The number of replicates required increases as the number of animals per pen decreases or the coefficient of variation increases. An estimated minimum would be four to six pens per treatment with six to eight animals per pen for group-fed animals.

Diets. Test grains will be harvested and processed using the same equipment; see Chapter 2, "Production, Handling, Storage, and Processing of Crops." Particle size of processed grains will be measured and recorded. If grain is harvested and stored as high-moisture grain, both GM and control grain must be harvested at the same kernel moisture content. Inclusion of the maximum feasible amount of the test ingredient into the diet will increase the power of the test. Therefore, diets will consist of 60% or more of the diet dry matter as the test grain (55% or more for lambs) with addition of appropriate amounts of protein, roughage, mineral, vitamin, and feed additives so that nutrient requirements specified by the National Research Council (NRC 1985, 2000) or accepted local requirements for the species used for testing are supplied and so that tolerance limits are not exceeded. If grain products (e.g., distillers or brewers grain, hominy feed, maize gluten meal, or maize gluten feed) are being tested, the maximum feasible dietary percentage of these products should be included based on their composition and potential effects on animal health. For example, feeding ground or rolled wheat may lead to acute indigestion. Thus, wheat should not exceed 25% of the dry matter in the diet for beef cattle. The amount of grain or grain products in each dietary treatment should be the same throughout the trial. Animals should be fed a single, nutritionally adequate diet for at least 14 days before assignment to treatments. During the adaptation to high-concentrate diets, extra roughage can be included in the diet. The concentration of roughage will be sequentially decreased for all dietary treatments at the same time. All dietary ingredients will be mixed before delivery to livestock with any sorting and rejection of specific fractions being monitored and recorded.

Removal of test animals. Any animal that exhibits morbidity or loses weight or gains little weight during two consecutive periods will be removed from the experiment and the reasons for the removal will be documented. Feed-gain ratio should be calculated for the overall study in two ways:

1) by dividing total feed consumption in a pen by the total weight gain of the surviving animals and the weight gain of the animals that died and were removed, and

2) by subtracting the assumed feed consumption of the dead or removed animals from total feed consumption and then dividing by the total growth (weight gain) of the surviving animals at the end of the study.

Adjustments for feed consumption should be based on estimated net energy intake for the animal removed relative to the calculated net energy value of the diet based on feed intake and performance of all animals in the pen. Final performance data should not include information from animals removed from the experiment. A qualified veterinarian should perform or supervise a diagnostic necropsy on animals that die during the experiment; body weight at and date and cause of death should be recorded.

Termination of the experiment. The experiment will be terminated on a block basis when a block of pens of animals (mean of all pens in the block) reaches the projected market weight. Trial duration must be at least 56 days for cattle and at least 28 days for lambs, with preferred lengths being 100 and 50 days, respectively. If carcass data are obtained, the same number of animals per pen within a block will be harvested at the same location on the same date. Data for cattle should include hot carcass weight, dressing percentage ($100 \times$ carcass weight/final live weight), incidence and severity of liver abscesses, longissimus muscle area, fat thickness over the rib, marbling score, kidney-heart-pelvic fat percentage, yield grade (preliminary, adjusted, and calculated), and quality grade to the nearest one-third of a grade. Data for lambs should include hot carcass weight, dressing percentage ($100 \times$ carcass weight/final live weight), incidence and severity of liver abscesses, longissimus muscle area, fat thickness over the rib, flank streaking, maturity, yield grade (preliminary, adjusted, and calculated), body wall thickness, and quality grade to the nearest one-third of a grade.

Statistical analysis of data. Performance data (mean daily gain, dry matter intake, feed-gain or gain-feed ratios)

will be summarized from the start to the end of various phases as well as to the end of the experiment. Health, performance, and carcass data will be analyzed as appropriate for the experimental design, with variance due to blocking being removed. Either the general linear model (GLM) or MIXED procedure of the Statistical Analysis System (SAS Institute, Cary, NC; http://www.sas.com) or an equivalent procedure in GenStat (VSN International Ltd., Hemel Hempstead, Herts HP1 1ES, United Kingdom; http://www.vsn-intl.com/genstat/) is recommended. The pen is used as the experimental unit for all analyses. Covariance adjustment for carcass weight may be used for evaluating carcass traits. See detailed protocols in Chapter 9, "Statistical Analysis and Interpretation of Results."

Evaluation of Crop Protein Supplements in Experiments with Growing and Finishing Ruminants

This protocol will be used to evaluate the nutritional value of GM crop protein supplements (e.g., soybeans, canola [rapeseed], cottonseed, sunflower, safflower, lentils, or lupins or meals produced from these crops) for growing and finishing ruminants (beef and dairy cattle, sheep and goats) grown from weaning to the end of the growing period (growing) and from the end of the growing period to market weight (finishing). In all experiments, an appropriate control crop or product (preferably the near-isogenic variety of the same variety that lacks the input trait being studied) must be included. Other controls may be included as specified below.

These studies will assess a GM oilseed or oilseed product and its nearest available near-isogenic conventional (control) oilseed or oilseed product. Each study will include a minimum of these two treatments. Additional treatments, which may include one or more types of conventional oilseed or oilseed product of the same genus typically produced or used in the region, also may be fed so that comparisons with more diverse varieties can be drawn.

Production, handling, storage, and processing of the oilseeds or oilseeds products will be as described in Chapter 2, "Production, Handling, Storage, and Processing of Crops." Sampling and analysis of the oilseeds or oilseeds products for mycotoxins and chemical components will be as described in Chapter 3, "Sampling and Analysis of Harvested and Processed Crop Material."

Test animals. Male, castrates or female ruminants can be used. In the growing phase the maximum final weights for bulls, steers, and heifers will be approximately 270 kg and the maximum final weight for lambs and goats will be approximately 20 kg. In the finishing phase, steers will be fed from 300 kg or more until finished, heifers will be fed from 270 kg or more until finished, and lambs will be fed from 20 kg or more until finished. All animals used should be healthy with similar genetic history. Each animal will be individually identified with an ear tag, ear notch, or brand. Animals may be fed individually or in groups in accordance with guidelines described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999). Each animal will be weighed individually at the start of the trial and at 2- to 4-week intervals during the feeding trial. Standardized procedures for weighing animals at the start and end of the trial should be used. These procedures can include weighing on two consecutive days with feed intake being restricted during the interval or weighing after an overnight period when animals have no access to feed or water. Weights can be taken at interim dates without limiting access to feed or water. Animal weights, feed delivery and refusals, dry matter and nutrient content of delivered and refused feed, and other animal and feeding data will be recorded and maintained as appropriate following good management practices.

Design and allotment. See grain section.

Number of replications. See grain section.

Diets. Including the maximum feasible amount of the test ingredient in the diet will increase the power of the test. To provide maximum levels if extracted oil is fed, diets will contain at least 3% added oil from the test materials; if intact or ground oilseed is fed, the added oilseed will be fed at a level to add at least 3% oil to the diet; if extracted oilseed meal is fed, the oilseed protein should add at least 3% protein to the diet. Diets containing control and GM oilseeds or oilseed products should be isonitrogenous and isoenergetic. Diets should have appropriate amounts of protein, energy, minerals, vitamins, and feed additives along with roughage so that nutrient requirements specified by NRC (1985, 2000) or locally accepted standards for the species being used are supplied and that tolerance limits are not exceeded. The amount of oilseed or oilseed product and other ingredients in each treatment diets should be the same. During adaptation to high-concentrate diets, a larger percentage of roughage can be included in the diet with the percentage sequentially decreased for all dietary treatments at the same time. All

dietary ingredients will be mixed before delivery to livestock with any sorting and rejection of specific fractions being monitored and recorded. If some ingredients (e.g., roughages) are fed free choice separately from the test feed, the ratio of roughage to supplement may vary among animals or groups; this difference prevents interpretation of data.

Removal of test animals. See grain section.

Termination of the experiment. Experiments with growing animals will be terminated on a block basis when animals in the block (mean of all pens in the block) reach an assigned weight or the end of their growing period. Trial duration must be ≥56 days for growing cattle and ≥28 days for lambs and sheep, with preferred lengths being 100 and 50 days, respectively.

Experiments with finishing animals will be terminated on a block basis when a block of pens of animals (mean of all pens in the block) reaches the projected market weight. Trial duration must be at least 56 days for cattle and at least 28 days for lambs, with preferred lengths being 100 and 50 days, respectively. If carcass data are obtained, the same number of animals per pen within a block will be harvested at the same location on the same date. Data for cattle should include hot carcass weight, dressing percentage (100 × carcass weight/final live weight), incidence and severity of liver abscesses, longissimus muscle area, fat thickness over the rib, marbling score, kidney-heart-pelvic fat percentage, yield grade (preliminary, adjusted, and calculated), and quality grade to the nearest one-third of a grade. Data for lambs should include hot carcass weight, dressing percentage (100 × carcass weight/final live weight), incidence and severity of liver abscesses, longissimus muscle area, fat thickness over the rib, flank streaking, maturity, yield grade (preliminary, adjusted, and calculated), body wall thickness, and quality grade to the nearest one-third of a grade.

Statistical analysis of data. See grain section.

Evaluation of Forages or Forage Products with Growing Ruminants

This protocol will be used to evaluate the nutritional value of GM forages (e.g., maize silage, sugar or fodder beets, legumes, grasses) or specific components (e.g., maize stover or fodder, beet tops, leaf meal, or protein) produced from such forages when fed after harvest with or without storage or when grazed by growing ruminants (growing beef and dairy cattle, growing water buffalo, growing sheep and goats). In all experiments, an appropriate control forage or forage product—preferably forage or the product from the near-isogenic cultivar of the same hybrid that lacks the input trait being studied—must be included in similar physical form. Other controls may be included as specified below.

These studies will compare the nutritive value of a GM forage or forage product to its nearest available near-isogenic conventional (control) forage or forage product. Each study that is conducted will include a minimum of these two treatments and all other dietary ingredients will be held constant. Additional treatments, which may include one or more types of conventional forage or forage product of the same genus typically produced or used in the region, also may be fed so that comparisons with more diverse hybrids or strains can be drawn.

A sponsoring organization will provide the investigator with the two types of forage seed (GM and control) for some studies and the two types of forage or forage product (GM and control) for other studies. The investigator may process both the types of forages separately under identical processing conditions, such as chopping and ensiling, depending on the objectives of the experiment.

If seed is supplied, the GM forage will be grown in an area sufficiently isolated from other crops to prevent cross pollination. Commonly accepted agronomic practices for the region will be used. The control forage will be grown in the same area with soil type and agronomic practices as similar as practical to the GM grain. Any differences in agronomic practices (fertilization, weed or insect control, irrigation) will be recorded and reported. Differences in insect damage or disease presence between the GM and control plants, quantified at several stages of plant growth by a qualified plant physiologist or disease specialist, will be recorded and reported. If the forage is to be harvested with or without processing before feeding, the GM and control forage will be harvested, handled, stored, and processed similarly but separately and held until the feeding trial begins. Harvest will be at a similar stage of maturity or moisture for both the GM and the control forage. Yield difference between the GM and control crops (fresh and dry matter basis) will be recorded. If crop residues are harvested to avoid an effect of a difference in the amount of dropped ears from GM and control grain, harvest of the crop residues should be at the same time after grain harvest and bales should be wrapped in plastic or ensiled to

avoid mold development. Care must be taken to identify clearly each forage or forage product and prevent cross-mixing of forages or forage products of different type. If the forage or forage residue is to be grazed, subdivisions that will form paddocks around small groups of animals will be installed. For experiments designed to evaluate growing forage, a "put and take" system that adds or removes animals depending on the amount of available forage mass is preferred.

If forage or forage product is supplied for the feeding trial, the GM and control forage or forage products will be stored in separate but similar storage facilities and properly identified. Samples of the forage taken at harvest and before feeding or grazing should be retained in case genetic verification of identity is required.

Analysis. If the forage is to be grazed, the amount of available forage will be quantified before the animal experiment begins and at 2-week intervals during the trial. A representative sample of each forage or forage product will be obtained at the start, midpoint, and end of the study using appropriate forage sampling procedures. Esophageal samples of grazed forage may be obtained. Whether forage or forage products are grazed or harvested for feeding, representative samples will be analyzed for dry matter, crude protein, crude fat, acid and neutral detergent fiber, and ash in a laboratory known to produce high-quality and consistent results. For preensiled forage, fermentation quality predictors such as water-soluble carbohydrates and pH should be measured in addition to the proximate analyses. For ensiled forage, additional measurements to estimate recovery of dry matter after fermentation (100 × weight of silage (dry matter of silage/weight of forage harvested × dry matter of forage harvested) and silage quality (lactic and volatile fatty acids, ethanol, pH, ammonia, water-soluble protein, aerobic stability) should be taken.

Test animals. Male, castrate or female ruminants after weaning can be used with maximum final weights for growing bulls, steers, and heifers being approximately 270 kg and for lambs and goats being approximately 20 kg. Healthy ruminants with similar genetic and nutritional history will be fed a single, nutritionally adequate diet (preferably containing the control forage or silage) for at least 14 days before assignment to treatments or paddocks. Animals will be blocked by sex or sex will be balanced within pen or paddock among test diets. Each animal will be individually identified with ear tag, ear notch, or brand. Animals may be fed a harvested forage or may graze individually (in a separate paddock or tethered in a group) or as a member of a group in accordance with guidelines described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999). Each animal will be weighed individually at the start of the trial and at 2- to 4-week intervals during the trial. The protocol may include weighing on two consecutive days with feed intake being restricted during the interval or weighing after an overnight period when animals have no access to feed or water. Animal weights and other animal health and feeding data will be recorded and maintained as appropriate following good management practices.

Design and allotment. A design appropriate for testing effects will be used. For growth or performance measurements, when the animals will graze or be fed the GM or the control forage or forage product for the full trial, the design typically will be a randomized complete block. Animals should be assigned to blocks on the basis of initial weight, breed, and sex, and paddocks within the block should have similar agronomic and environmental properties. For intake or digestibility measurements, indigestible markers (e.g., acid-insoluble ash, n-alkanes, chromic oxide) can be fed with a supplement. To increase statistical power when obtaining ruminal samples from animals fed GM or control forage, animals can be rotated among paddocks in crossover or Latin square experiments. An adjustment period should be used and should be long enough for transition of the ruminant microbial population, which depends on the degree of change in dietary ingredients. Different blocks can be in different locations but the environment within each block must be similar to avoid bias. Treatments will be randomly assigned to paddocks within a block.

Number of replications. The number of replications (number of paddocks per treatment or, for animals fed individually, the number of animals per treatment) will be adequate to detect, at $P < 0.05$, a 10% difference between treatment means 80% of the time. With a coefficient of variation of 5.0% to 7.5%, 6 to 10 replications per treatment will be required. The number of replicates required increases as the number of animals per pen decreases or the coefficient of variation increases. An estimated minimum would be four to six paddocks per treatment with six to eight animals per paddock for animals grazing trials.

Diets. Based on forage analysis, supplements will be supplied so that appropriate amounts of protein, roughage, mineral, vitamin, and feed additives are provided.

Nutrient intakes should meet or exceed nutrient requirements specified by the National Research Council (NRC 1985, 2000) or accepted local nutrient requirements for growing ruminants of the species of interest. In addition, tolerance limits should not be exceeded. Composition and quantity of supplement provided per animal should be equal for animals receiving GM and control forage or forage product. Supplements shall be analyzed for the same nutrients as the forage.

Removal of test animals. Any animal that exhibits morbidity, loses weight, or gains little weight during two consecutive periods will be removed from the experiment. Final performance data should not include information from animals removed from the experiment. A qualified veterinarian should perform or supervise a diagnostic necropsy on animals that die during the experiment; cause of death should be recorded.

Termination of the experiment. The experiment will be terminated on a block basis when a block of animals reaches the end of the growing period or the forage supply is exhausted. Trial duration must be at least 56 days for cattle and at least 28 days for lambs, with preferred lengths being 100 and 50 days, respectively.

Statistical analysis of data. Performance data for grazing animals (mean daily gain) or animals fed harvested forage (daily gain, dry matter intake, feed-gain or gain-feed ratios) will be summarized from the start to the end of various phases as well as to the end of the experiment. Health and performance data will be analyzed as appropriate for the experimental design with variance due to blocking being removed. Either the general linear model (GLM) or MIXED procedure of the Statistical Analysis System (SAS Institute, Cary, NC; http://www.sas.com) or an equivalent procedure in GenStat (VSN International Ltd., Hemel Hempstead, Herts HP1 1ES, United Kingdom; http://www.vsn-intl.com/genstat/) is recommended. The mean for all animals in a paddock is used as the experimental unit for all analysis.

Evaluation of Crop Residues from Genetically Modified Forages with Growing Ruminants

This protocol will be used to evaluate the nutritional value of residues from GM forages (e.g., maize silage, sugar or fodder beets, legumes) when grazed by growing or mature ruminants (beef and dairy cattle, growing water buffalo, growing sheep and goats). In all experiments an appropriate crop residue—preferably from the near-isogenic cultivar of the same hybrid that lacks the input trait being studied—must be included. Other controls may be included as specified below.

These studies will assess nutritional value of residues from a GM crop relative to the residue from its nearest available near-isogenic conventional (control) crop. Each study will include a minimum of these two treatments and all other dietary ingredients and supplements will be held constant. Additional treatments, which may include one or more types of conventional forage or forage product of the same genus typically produced or used in the region, also may be fed so that comparisons with more diverse hybrids or strains can be drawn. Alternatively, various amounts of supplemental feed or forage can be supplied so that animals grazing the two crop residues maintain similar rates of performance (weight maintenance or gain). In a supplementation study the amount of supplemental feed or forage used to maintain equal rates of production must be monitored.

The GM crop must be grown in an area sufficiently isolated from other grain to prevent cross pollination. Commonly accepted agronomic practices for the region will be used. The control forage will be grown in the same area with soil type and agronomic practices as similar as practical to those for the GM grain. Any differences in agronomic practices (fertilization, weed or insect control, irrigation) will be recorded and reported. Differences in insect damage or disease presence between the GM and control plants, quantified at several stages of plant growth by a qualified plant physiologist or disease specialist, will be recorded and reported. Harvest time and methods for the GM and control crops must be similar and the same harvesting equipment should be used. Yield differences should be recorded. Subdivisions that will form paddocks around small groups of animals will be installed so that animals remain in their assigned paddock. Care must be taken to identify clearly each paddock used for grazing and the animal group assigned to each paddock.

Analysis. Before the animal experiment begins and at 2-week intervals during the trial, the amount of standing forage will be quantified and amounts of various components will be determined. For forage crops, the amount and composition of live and dead plant material must be measured. For grain crop residue, the amounts of stalk, leaf, cob, and grain available for consumption by grazing animals must be measured. If different amounts of specific crop residues (e.g., cob and grain) remain after harvest of the GM and control crop, the supply of energy available for grazing livestock will be different. Such differences make studies of

preference based on the amount of time that free-ranging cattle spend grazing residues from GM and control crops meaningless. A representative sample of each fraction will be obtained at the start, midpoint, and end of the study using appropriate forage sampling procedures and for verification of GM trait identity if necessary. In addition, esophageal samples of grazed forage may be obtained. Crop residue samples will be analyzed for dry matter, starch, crude protein, crude fat, and acid and neutral detergent fiber in a laboratory known to produce high quality and consistent results. Laboratory procedures will be as described by AOAC INTERNATIONAL (2000). The crop residues will be screened for presence of fungi and mycotoxins by a laboratory specializing in this technology.

Test animals. Growing male, castrate or female ruminants after weaning can be used or cows that are pregnant or not pregnant with or without calves can be used. Only healthy ruminants with similar genetic and nutritional history should be used. If pregnant animals are used, maintenance of reproductive status can be monitored and birth weights and health status of calves can be recorded and reported. Animals should be fed a single, nutritionally adequate diet for at least 14 days before assignment to paddocks (treatments within a block). Animals will be blocked by sex or sex will be balanced within pen or paddock among test diets. Each animal will be individually identified with ear tag, ear notch, or brand. Animals will graze in groups in accordance with guidelines described in *Guide For the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999). Each animal will be weighed individually at the start of the trial and at 2- to 4-week intervals during the feeding trial. Standardized procedures should be used for weighing animals at the start and end of the trial and may include weighing on two consecutive days with feed intake being restricted during the interval or weighing after animals have no access overnight to feed or water. Visual or automated systems to record grazing time and activity or exercise may be used. Animal weights and other animal health and feeding data will be recorded and maintained as appropriate following good management practices.

Design and allotment. A design appropriate for testing effects will be used. For growth or performance measurements when the crop residue from a paddock will be grazed for the full trial, the design typically will be a randomized complete block (preferably with blocking by initial weight, breed, or sex) with the several paddocks (GM and control crop residues) in each block having similar area or animal units, agronomic properties, and environmental properties. For intake or digestibility measurements, indigestible external markers can be fed in a supplement. Because of the possibility of soil consumption, acid insoluble ash cannot be used as an intake or digestion marker. To increase statistical power, animals can be weighed and rotated at specified times among paddocks (treatments) within a block. When ruminal samples are obtained from animals fed GM or control crop residues to study their effect on rumen fermentation, animals can be rotated among paddocks in crossover or Latin square experiments to increase statistical power. Different blocks can be in different locations, but the environment within all blocks must be similar to avoid bias. Treatments will be randomly assigned to paddocks within a block.

Number of replications. The number of replications (number of paddocks per treatment) will be adequate to detect, at $P < 0.05$, a 10% difference between treatment means 80% of the time. With a coefficient of variation of 5.0% to 7.5%, 6 to 10 replications per treatment will be required. The number of replicates required increases as the number of animals per pen decreases or the coefficient of variation increases. An estimated minimum would be four to six paddocks per treatment with six to eight animals per paddock.

Diets. On the basis of forage analysis, supplements will be provided so that adequate amounts of protein, roughage, mineral, vitamin, and feed additives are consumed by the animals. Intakes should meet or exceed nutrient requirements specified by NRC (1985, 2000) or accepted local nutrient requirements for growing or adult ruminants of the species being used, but tolerance limits should not be exceeded. Composition and quantity of supplement provided per animal should be equal for animals receiving GM and control crop residues. Supplements should be analyzed for the same nutrients as the crop residues.

Removal of test animals. Any animal that exhibits morbidity, loses an excessive amount of weight, or gains much less weight than other animals in the paddock during two consecutive periods will be removed from the experiment. Final performance data should not include information from animals removed from the experiment. A qualified veterinarian should perform or supervise a diagnostic necropsy on animals that die during the experiment; cause of death should be recorded.

Termination of the experiment. The experiment will be terminated on a block basis when a block of animals reaches the specified weight, at the end of a prespecified

grazing period, or when crop residue supply is exhausted. Trial duration must be at least 56 days for cattle and at least 28 days for lambs, with preferred lengths being 100 and 50 days, respectively. If adverse weather or snow cover prevents animals from grazing, equal amounts of supplemental forage or grain should be supplied to animals within each paddock of a block. If a crossover design is used, animals should have access to residue from the GM and the control crop for the same number of days.

Statistical analysis of data. Performance data for grazing animals (mean daily gain) or for animals fed supplemental harvested forage to maintain weight or gain (daily gain, dry matter intake, feed-gain or gain-feed ratios) will be summarized from the start to the end of various phases as well as to the end of the experiment. Health and performance data will be analyzed as appropriate for the experimental design with variance due to blocking being removed. Either the GLM or MIXED procedure of SAS or an equivalent procedure in GenStat is recommended. The mean for all animals in a paddock is used as the experimental unit for all analysis.

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Chapter 9: Statistical Analysis and Interpretation of Results

Good science is only as good as the process of conducting properly designed experiments, accurately collecting data, subjecting the data to appropriate statistical analysis, and interpreting the results correctly. Statistical design of experiments refers to the process of planning the experiment so that appropriate data that can be statistically analyzed will be collected, resulting in valid and objective conclusions (Montgomery 2001). According to Aaron and Hays (2001), statistical techniques should be considered as research tools that can produce meaningful, reliable, and unbiased results when properly applied to situations for which they are designed. No statistical technique can protect against poor planning, inaccuracies in the data, unsound analysis, or incorrect interpretation of the data. High-quality research requires proper planning and careful execution of experiments, correct application of statistical techniques, and interpretation of results by researchers who understand not only the statistical techniques, but also the field to which the results are applied.

Proper design of experiments is paramount to any research endeavor that seeks to discover new information. Experiments must be designed to obtain unbiased estimates of treatment effects, treatment differences, and experimental error. In addition, experiments should be designed and replicated in such a way that treatment effects will be estimated with adequate precision to detect differences, if they truly exist, at the desired probability level.

Before an experiment is conducted, important questions should be addressed by the researcher:

- What is the hypothesis to be tested and what is to be accomplished by the experiment? The basic objectives of the research should be clear and obtainable.
- What treatments should be included? The success of the experiment depends on careful selection of treatments that will fulfill the initial objectives. A control or reference treatment should always be included in experiments.
- What will be the experimental unit—an individual animal or a pen of animals? The experimental unit is the smallest unit to which a given treatment is applied. If animals are penned in groups and all the animals in the pen share the same feed source, then the experimental unit is the pen, not the individual animal. This is important because it is the variation among experimental units treated alike that gives the unbiased estimate of error used to evaluate treatment effects.
- What measurements will be taken (how, where, when, by whom, etc.)? These decisions must be made during the planning stage so that unintentional bias is not introduced into the results.
- What will be the experimental design? The method of assignment of animals to treatments determines the experimental design. The proper design for the conditions of the experiment will help to minimize experimental error and will help researchers draw valid conclusions from the results.
- How many replications are needed per treatment? The number of replications must be large enough to estimate treatment effects with the precision necessary to detect differences, if they truly exist, at the desired probability level.
- Can the experimental design be analyzed properly and the desired treatment comparisons be made? Obviously, this is probably the most important question of all. Sources of variation and appropriate degrees of freedom along with planned treatment comparisons should be described before the experiment is started to make sure the experiment will satisfy the original objectives.

It is not possible to discuss in detail all factors that should be considered when designing an experiment, collecting the data, statistically analyzing the data, and interpreting the results. However some of the more important concepts that apply to research on genetically modified (GM) crops are addressed. For additional information, readers are referred to other publications such as Montgomery (2001), Aaron and Hays (2001), Morris (1999), Hinkelmann and Kempthorne (1994), Lentner and Bishop (1993), Damon and Harvey (1987), Steel and Torrie (1980), Snedecor and Cochran (1980), Gill (1978a,b), and Cochran and Cox (1957).

Important Concepts Involving Research with Genetically Modified Crops

Treatments

In general, it is best to keep the number of treatments to a minimum. For example, an experiment might be designed to compare two treatments—GM maize and control maize. In this instance, it would be best if the control maize is genetically similar, or near isogenic, to the GM maize except for the specific GM trait. In addition, the control maize should have been produced under environmental and agronomic conditions that are as similar as possible to the GM maize. Diets should be the same except for the feedstuffs under evaluation, in this case the two maizes.

For input traits it is desirable to include one or more commercial reference lines to help put the data into perspective. Some statistically significant differences between the GM and near-isogenic line may occur by chance and may not be biologically relevant. Reference lines help to delineate the range of values typical of the crop type.

Randomization

According to Montgomery (2001), randomization is the cornerstone underlying the use of statistical methods in experimental design. Animals should be assigned to treatments using proper randomization. The randomization may be from within groups that have been formed on the basis of body weight, gender, genetic background, or other such factors. If animals of the same gender are penned together, it is important to have the same gender distribution across treatments within a replication to eliminate bias. The same applies to breed and other factors that could introduce bias.

Experimental Design

Two of the most common designs in animal experiments are the completely randomized design and the randomized complete block design. If the population of animals is extremely uniform and the environment in the building or field where the experiment is to be conducted is uniform, a completely randomized design may be the best choice. In this instance animals are randomly assigned to pens and pens are randomly allotted to treatments. However, in most cases animals are not uniform and neither is the environment within buildings or fields in which they are kept. Thus, a randomized complete block design is more commonly used.

Blocking is a technique used to improve the precision with which comparisons among factors of interest are made. In this design, animals are blocked on factors such as their initial weight, gender, breed, egg production, milk yield, and milk composition and randomly assigned to treatments within blocks (i.e., groups). Pens are often blocked in the building depending on ventilation, lighting, and other environmental factors. Pastures are usually blocked to adjust for environmental effects such as prevailing winds. The objective is to remove the effects of the blocking factors (building location, initial weight, gender, environmental temperature, etc.) from the experimental error.

Unfortunately, confounding factors and bias are sometimes introduced into experiments because they seem to make the experiment easier to conduct. Examples include having one treatment in one building and a second treatment in another building, placing one treatment at one end of a building and the other treatment on the opposite end of the building, and feeding males one treatment and females another treatment. Obviously, these arrangements introduce bias. Confounding treatment effects with environmental factors, gender, etc. usually leads to results that have little scientific value. This type of confounding should obviously be avoided.

A Latin square design is sometimes used when animal numbers, quantity of test material, or experimental facilities are not sufficient to accommodate more conventional experimental designs. These designs are more complicated, and using the same animals for several treatments can introduce confounding effects in rapidly growing animals when their body weight increases appreciably during an experimental period. This design should usually be avoided if a treatment effect has the potential of being carried over into another period. Typically, modifications of a Latin square design, such as a crossover or switchback design (a 2×2 Latin square), are used with lactating dairy cows after peak milk yield in their lactation has been reached.

Experimental Unit and Experimental Error

An experimental unit is the smallest unit to which a treatment is applied given that two such units could receive different treatments. If animals are penned individually and each is fed an experimental diet from a feeder in an individual pen, the animal is the experimental unit. If animals are penned in groups and all animals in the pen share the same feed source, the pen is the experimental unit. The

individual animals in the pens, even if measurements are taken on those individual animals, represent the sampling unit, not the experimental unit.

A clear understanding of what constitutes the experimental unit is important because the variation among experimental units is the experimental error—the proper error term to use in testing treatment effects. Some researchers erroneously use the sampling error (the variation of animals within pens) as the error term with which to test treatments. This choice is usually made because of lack of understanding of statistical principles or because it increases the degrees of freedom in the error term, making it easier to obtain significant differences. However, the sampling error is not the correct error term and using it can result in errors in interpretation of results.

Numbers of Replications

The precision or sensitivity of an experiment refers to its ability to detect true differences at a given level of statistical significance. Generally, the smaller the experimental error, the more precise the experiment will be in detecting treatment differences. Also, as the number of replications increases, the precision increases.

The number of replications needed depends on the size of the difference to be detected, the desired precision, and the variability of the trait being measured. For a specific situation, the number of replications needed can be estimated using procedures described by Cochran and Cox (1957) or Berndtson (1991). Table 9-1 gives estimates of the number of replications needed to detect differences of various sizes at several levels of variability (expressed as coefficient of variation) and a significance level of $P < 0.05$. In this table, estimates are based on an 80% chance of obtaining a significant result in a randomized complete block experiment with two dietary treatments.

Treatment Comparisons

A decision on the specific treatments to compare should be made during the planning process. This decision is simple if there are only two treatments but the choice is more complicated when there are several treatments. Preplanned orthogonal (independent) comparisons are the best and most accurate with the least chance of drawing erroneous conclusions. Nonorthogonal comparisons are acceptable if they were initially planned and if the comparisons are not simply based on the outcome of the experiment. An example of preplanned nonorthogonal contrasts is comparison of a single control treatment with each of several other treatments. Generally, Dunnett's t-like test is the one to use for this but Fisher's least significant difference (LSD) test is also acceptable.

If treatments are dose related, such as levels of some factor, then linear and curvilinear contrasts (linear, quadratic, cubic, etc.) are the most appropriate tests to make. If the treatment arrangement is factorial, such as a 2×2 factorial that has two levels of factor A and two levels of factor B, comparisons should be between the main effects of the two factors and the interaction. If the interaction is not significant, testing of the simple effects (level of factor A within each level of factor B or vice versa) is not necessary.

Many researchers fall into the trap of making all possible comparisons and present their data to show treatment differences with superscripts on each mean. An accompanying footnote indicates that means not bearing the same superscript letter are significantly different. However, comparisons such as this are not appropriate in most instances and can lead to erroneous conclusions. They often indicate that differences are real when they are not (type I error). Fixed-range, pairwise, multiple comparison tests are only appropriate when the treatments are unstructured or completely unrelated to each other. Examples of such tests are Fisher's LSD test and Tukey's honestly significant difference test (both are fixed-range tests) and Duncan's multiple-range test and Student-Newman-Kuel's test (both are multiple-range tests). Most statisticians recommend the LSD test as the procedure of choice for pairwise multiple comparisons. Carmer and Walker (1985) present an excellent review of the properties of these and other multiple-comparison tests.

Some statisticians believe that specific treatment comparisons should only be made if the overall treatment effect is significant at some level of probability, such as $P < 0.05$. This "protected LSD" procedure is a more conservative approach in that it is less likely to detect a treatment difference when one actually exists. In other words, the protected LSD procedure reduces the power of the test and increases the chances of a type II error (concluding that there are no differences when a difference actually exists).

Table 9-1. Estimated number of replications (blocks) needed to detect a treatment difference at *P* <0.05*

Coefficient of variation (%)	Expected difference (%)				
	5	10	15	20	25
2	4	3	2	—	—
3	7	3	3	2	—
4	12	4	3	3	2
5	17	6	4	3	3
6	24	7	4	3	3
7	32	9	5	4	3
8	42	12	6	4	3
9	52	14	7	5	4
10	63	17	9	6	4
12	91	24	12	7	5
14	124	32	15	9	7
16	161	42	19	12	8
18	204	52	24	14	10
20	252	63	29	17	12
25	393	99	45	26	17
30	566	142	63	37	24

*Adapted from Berndtson (1991). Assumes a randomized complete block design with two treatments, two-tailed test of significance at $P < 0.05$, and an 80% chance of detecting a significant difference (i.e., 80% power).

Covariance Procedures

Most data are analyzed by conventional variance procedures; however, covariance procedures are appropriate in some instances. Covariance adjusts for inherent differences among animals that could affect treatment effects. For example, covariance may be used to analyze data from dairy cattle experiments in which the cows' preexperimental milk yield is known. Covariance is often used to analyze carcass data in swine when the final carcass weight differs among treatment groups. In these cases, milk yield or carcass weight is included in the statistical model as a covariate and treatment means are adjusted accordingly. Generally, least squares means that are adjusted for the covariates in the model are calculated for the various treatments.

Software Programs for Statistical Analysis

Various software packages are available to assist researchers in statistical analysis of experimental data. One of the most popular is SAS (SAS Institute, Cary, NC; http://www.sas.com). This system accepts data from spreadsheets and does numerous types of statistical analyses quickly and efficiently. Either the GLM procedure or the MIXED procedure of SAS is generally used to analyze data. If repeated measures are important, the MIXED procedure can be used. Covariance analysis of data with generation of least squares (adjusted) means can also be accomplished using these procedures. An alternative statistical package also widely used in agricultural applications is GenStat (VSN International Ltd., Hemel Hempstead, Herts HP1 1ES, United Kingdom; http://www.vsn-intl.com/genstat/).

Interpretation of Experimental Results

Researchers should have background and training that will enable them to interpret the results of their studies, including the statistical results. Interpretations and conclusions should be made in light of results of other experiments conducted at their own research institute as well as at other research institutes around the world.

Summary

Sound statistical methods can greatly increase the efficiency of experimentation and will strengthen the conclusions obtained. Researchers should remember the following points about statistics (adapted from Montgomery, 2001):

- Nonstatistical knowledge of the problem should be incorporated. Most researchers are highly knowledgeable in their fields. In the field of animal nutrition, there is a large body of information on which to draw in explaining relationships between factors and responses. This type of nonstatistical knowledge is invaluable in choosing factors, determining factor levels, deciding how many replications to include, interpreting the results of the analysis, and so forth. Using statistics is no substitute for thinking about the problem.
- The design and analysis should be kept simple. Unnecessarily complex, sophisticated statistical techniques should be avoided. Relatively simple design and analysis methods are almost always best. If the design is simple, the statistics will likely give straightforward results. Even the most complex and elegant statistics cannot compensate for a complex design that is poorly conducted.
- The difference between statistical and practical significance is important. Just because two treatments are significantly different does not mean that the difference is large enough to have any biological importance or any practical significance.

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