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

International Life Sciences Institute
Washington, DC

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About ILSI

The International Life Sciences Institute (ILSI) is a nonprofit, worldwide foundation established in 1978 to advance the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment, and the environment. ILSI also works to provide the science base for global harmonization in these areas.

By bringing together scientists from academia, government, industry, and the public sector, ILSI seeks a balanced approach to solving problems of common concern for the well-being of the general public.

ILSI is headquartered in Washington, DC. ILSI branches include Argentina, Brazil, Europe, India, Japan, Korea, Mexico, North Africa and Gulf Region, North America, North Andean, South Africa, South Andean, Southeast Asia Region, the Focal Point in China, and the ILSI Health and Environmental Sciences Institute. ILSI also accomplishes its work through the ILSI Research Foundation (composed of the ILSI Human Nutrition Institute, the ILSI Risk Science Institute, and the ILSI Center for Health Promotion). ILSI receives financial support from industry, government, and foundations.

Table of Contents

Foreword v

Chapter 1: Introduction 1

Chapter 2: Production, Handling, Storage, and Processing of Crops 7
Appendix 2-1 10
Appendix 2-2 12

Chapter 3: Sampling and Analysis of Harvested and Processed Crop Material 25
Appendix 3-1 36
Appendix 3-2 42

Part I: Trait Groups

Chapter 4: Proteins and Amino Acids 45

Chapter 5: Carbohydrates 57

Chapter 6: Lipids and Fatty Acids 67

Chapter 7: Vitamins and Antioxidants 81

Chapter 8: Minerals 97

Chapter 9: Enzymes 109

Chapter 10: Antinutrients 123

Part II: Protocols for Evaluating Feedstuffs With Genetically Modified Output Traits

Chapter 11: Poultry Meat Production 137

Chapter 12: Poultry Egg Production 145

Chapter 13: Swine 151

Chapter 14: Lactating Ruminants 155

Chapter 15: Growing and Finishing Ruminants 165

Chapter 16: Aquaculture 177

Chapter 17: Statistical Analysis and Interpretation of Results 189

Foreword

For centuries, humans have selected, sowed, and harvested crops that produce food products to sustain them. Plant breeders using traditional methods have developed tools to identify and move new traits into plants. Traditional plant breeding can take 10 to 12 years to develop a plant with desired traits. In this process thousands of genes, both intended and unintended, are moved into the new plant. If any deleterious effects are observed, the relevant genes must be bred out of the plant before the new plant will be commercialized. Now, modern plant breeders using tools of biotechnology can select specific traits and move only the genes of interest into the target plant. The first wave of commercialized products using biotechnology were those that contain "input" traits (traits that provide an agronomic benefit such as herbicide tolerance or insect protection with no change in the crop's composition except for very small amounts of transgenic DNA and transgenic protein). Use of this technology has raised the level of interest in the safety of food and feed produced from genetically modified (GM) plants. Feed and livestock groups, allied industries, and others wanted the biotechnology trait providers and independent scientists to verify that animal performance and health of livestock, poultry, and fish fed GM crops and/or their coproducts was similar to that of animals fed conventional crops. The International Life Science Institute (ILSI), in collaboration with the Federation of Animal Science Societies (FASS), recognized the need for a globally harmonized approach for conducting these studies with the utmost scientific rigor and sensitivity. As a result, ILSI published *Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits* in 2003. Now, a second generation of traits ("output traits") is being developed for the enhancement of animal feed. In crops with output traits, nutrient composition is intentionally changed.

The rationale for the current project was to provide a platform for the scientific evaluation of crops containing output traits when fed to animals and to promote international harmonization of methods. In addition, the document is intended to provide direction to: 1) a person interested in assessing the scope of studies that should be considered for the evaluation of of animal performance for a particular trait, or 2) a person who wants to evaluate a particular crop containing a nutrient output trait when targeted to a particular species. This document aims to 1) recommend scientifically sound guidelines for the production, harvest, sampling, and analysis of GM crops containing "output" traits (i.e., enhanced protein or amino acids, lipid or fatty acids, carbohydrates, minerals, vitamins and antioxidants, enzymes, and reduced anti-nutrients); 2) describe the potential advantages of modifying specific output traits in plants; 3) provide a systematic approach to determine what studies should be considered; and 4) provide guidance on the experimental design, conduct, and analysis of poultry, livestock and aquaculture experiments using these crops and their coproducts. The safety of GM crops and the products derived from animals fed GM crops are reviewed elsewhere. This publication should be a valuable reference for animal, poultry, and aquaculture scientists worldwide in academia, industry, and government who desire to conduct bioavailability, performance, processing, and animal-derived food product studies with nutrient-enhanced crops and their coproducts.

In preparing this document, scientists with expertise in areas such as animal nutrition (various farm animal species, poultry, and aquaculture), health, feed chemistry, food science, statistics, and other relevant disciplines developed a process whereby best practices were developed under ILSI's leadership in collaboration with FASS. Individuals who are internationally recognized for their diverse perspectives and technical expertise in the respective nutrient and animal species areas have reviewed this document. However, it must be emphasized that this document's content is the authors' responsibility and not the reviewers', and it does not represent an endorsement by the reviewers' institutions. The authors and task force members would like to thank the following individuals for participating in the review process and for providing many constructive comments and suggestions:

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Chapter 1

Introduction

Biotechnology, including the use of microorganisms such as bacteria and yeasts or biological substances such as enzymes to perform specific industrial or manufacturing processes, has been used for thousands of years to produce foods such as beer, wine, cheese, and yogurt. In addition, genetic selection has been used for thousands of years to improve and create new plants for enhanced crop performance, yield, and nutritional value (i.e., corn from teosinte [Fedoroff 2003], tomatoes, canola, tangerines). Since the late 1970s, modern agricultural biotechnology involving the application of cellular and molecular techniques to transfer DNA that encodes a desired trait to crops has been successfully employed to improve plant products beneficial to livestock and humans. The first genetically modified (GM) plant was produced in 1983 and was followed in 1988 by transgenic production of chymosin, the enzyme used in curdling milk in cheese production, which has predominantly replaced the chymosin (rennet) isolated from calf stomachs (http://www.ncbe.reading.ac.uk/NCBE/GMFOOD/chymosin.html [accessed 2006 Mar 7]). The first commercial genetically modified crop was introduced in 1996. Since then, global acreage of biotech/GM crops has sustained annual double-digit growth with an estimated 102 million hectares grown in 22 countries by over 10 million farmers (90% were resource-poor farmers from developing countries) in 2006 (James 2006).

As the adoption of GM crops grew, the animal production industry and commercial groups began receiving questions about the performance and safety of farm animals fed GM crops. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) advocate the "substantial equivalence" concept 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). This approach assumes that "if a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner 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 evaluation of the gene and expressed protein, molecular characterization, agronomic traits, nutritional and anti-nutritional assessment, and toxicology assessment is conducted before any crop is deemed safe for use. Government and international scientific organizations including the FAO/WHO (FAO/WHO 1991), US Food and Drug Administration (US FDA 1992), Organization for Economic Co-operation and Development (OECD 1993), French Academy of Science (ADSF 2002), Society of Toxicology (SOT 2003) and others concluded that plant biotechnology does not pose any unique risk compared with other production methods. As with human food safety assessment, the safety assessment of a livestock feed derived from a GM crop looks at the compositional and nutritional characteristics of the GM feed compared with its conventional counterpart. This assessment includes the gene source; molecular characterization of the inserted DNA; history and safe use of the expressed protein and its function, concentration, toxicology, and mode of action; crop agronomic characteristics; and nutritional composition.

Global interest in conducting livestock, poultry, and aquaculture feeding studies with GM crops and their products has increased. To draw valid conclusions from research studies, the GM crop or derived products must be characterized, and each study must be designed and conducted in a scientifically valid and rigorous manner using internationally recognized best practices (VICH GL9 [GCP] 2000). Best practices have been developed for the conduct of animal studies to evaluate GM crops containing input traits in order to provide a globally harmonized approach (ILSI 2003).

The next generation of products (nutritionally enhanced or output traits) is being developed with the intent to provide benefits for humans and animals. Owens and Soderlund (2000) and Owens et al. (2002) provide an overview of the specialty grains containing agronomic traits and nutritionally enhanced traits for ruminants. Hartnell (2004) and Hartnell et al. (2005) provide a glimpse of a new generation of GM products that are targeted for the poultry, livestock, and aquaculture industries. Precise gene insertion through biotechnology now enables one to regulate specific metabolic pathways or insert new pathways in plants resulting in enhanced protein quality and/or quantity, modified amino acid levels (e.g., lysine-enhanced maize [Taylor et al. 2004], high methionine lupin [Ravindran et al. 2002]), modified lipid or carbohydrate composition and content, reduced lignin, reduced indigestible oligosaccharide content (Parsons et al. 2000, Sauber 2000), increased levels of oligofructans, enhanced

Box 1-1
Examples of Nutritionally Improved Crops

- Taylor et al. (2004) designed studies to evaluate lysine-enhanced maize in broilers. They reported no unexpected effects and no difference in the bioefficacy of the increased levels of lysine in GM maize as compared to conventional maize supplemented with crystalline lysine when fed to broilers for 42 days.
- Lupin has been genetically modified through the introduction of a chimeric gene encoding the sulfur-containing, amino acid-rich, sunflower seed albumin (Molvig et al. 1997). White et al. (2001) reported an 8% increase in wool growth and 7% increase in body weight gain in merino sheep when fed the methionine-enriched lupin.
- Drexler et al. (2003) extensively reviewed research on fatty acid modifications in oilseed crops for improved nutritional and processing characteristics. Of greatest interest are shifts in 18-carbon fatty acids and stearic acid (16:0) in both soybeans and canola.
- Researchers in Australia (Commonwealth Scientific and Industrial Research Organisation) and the US (Noble Foundation) are identifying genes and regulatory elements in the condensed tannin biosynthetic pathway to insert into white clover and lucerne (Spencer et al. 2000, Xie et al. 2003). Benefits from increased condensed tannins in forages include bloat-safe lucerne and better silage quality, as well as positive environmental effects from the reduced methane production (a greenhouse gas) and decreased release of nitrogen into the environment through improved nitrogen utilization.
- Guo et al. (2001) at the Noble Foundation have altered the lignin pathway in alfalfa by decreasing the expression of the COMT and CCOMT genes, which are both methyl transferases involved in the biosynthesis of coniferyl and sinapyl alcohol, the main building blocks of lignin. The result was lucerne plants with lower lignin content and improved digestibility.
- Barley, wheat, maize, and soybeans contain β-1, 4-glucan and xylan in their hulls that lead to increased viscosity of intestinal contents resulting in decreased digestibility and reduced animal performance. Baah et al. (2002) reported improved feed conversion and decreased digesta viscosity in broiler chickens when fed a barley-based diet containing transgenic potato that expressed 1,3-1,4 β-D-glucan 4-glucano hydrolase from *Fibrobacter succinogenes*.
- Phosphorus is organically bound in the seed as phytate phosphorus. Nonruminant animals, such as poultry and swine, do not have the necessary digestive enzymes to release the phosphorus, so it ends up in animal waste. The waste is applied to the fields where leaching and run-off can occur. Zhang et al. (2000a, 2000b) evaluated the effect of feeding phytase derived from *Aspergillus ficuum* when expressed in either *A. niger* or canola seed to broilers and young pigs, respectively. In both the chicken and the pig, no difference in animal performance was observed when fed the microbially expressed phytase as compared to the plant containing phytase.

vitamin and mineral content, and enzymes. Box 1-1 lists specific examples of nutritionally improved crops.

Spencer et al. (2000) also introduced the gene for sunflower seed albumin into subterranean clover resulting in an accumulation of this protein that represents 0.3–1.3% of the total leaf protein. Ravindran et al. (2002) determined the apparent metabolizable energy (AME) and ileal digestibility of the methionine-enriched lupins as compared to the conventional lupins when fed to chickens. The GM lupins were higher in AME than the conventional lupins, which may have been due to the lower non-starch polysaccharide content in the GM lupins. Apparent ileal digestibility of individual amino acids and performance of birds (gain, feed intake, and feed:gain) were not different in birds fed conventional or GM lupins. The authors concluded that the energy and amino acids in methionine-enriched lupins developed through biotechnology are as available to the bird as those in conventional lupins.

Halpin et al. (1995) reviewed transgenic approaches for altering starch biosynthesis, sucrose accumulation, fructan biosynthesis, and seed oil content in crops. The amount and type of starch is very important for driving rumen fermentation in ruminants and meeting the energy needs of nonruminants. Increasing the oil proportion in corn grain is important in increasing the grain's energy density. Changing the fatty acid profiles of grains and oilseeds to contain more omega-3 fatty acids may lead to improved nutritional value for humans who consume animal products. These non-

structural carbohydrate components are all important for enhancing value to animals and humans.

Lucerne cell walls contain xylans and cellulose, which have different digestibilities (Hatfield and Weimer 1995). The xylans in lucerne stems (20-25% of dry weight) have a slow rate and low extent of digestion. Replacing at least part of this cell wall fraction with a more digestible polysaccharide could have major impacts on total fiber digestion.

Future opportunities associated with biotechnology could lead to production of meat, milk, and egg products containing antioxidants and modified fatty acids that will have a health benefit to consumers.

With the increased demand for carotenoids, an interest in developing new methods for their biological production exists. Ausich (1997) provides a review of the previous and current chemical and biological methods for producing carotenoids and the benefits of using recombinant DNA technology. He states that using recombinant DNA technology is the tool that will most likely lead to increased carotenoid production in biological systems. Temple (2004) provides an excellent summary of the use and benefits of canthaxanthin in aquaculture.

Biotechnology is a valuable tool to plant scientists to reduce or eliminate undesirable components of feed ingredients such as erucic acid and glucosinolates in rapeseeds; trypsin inhibitor, lectin, raffinose and stachyose in soybean; tannin in sorghum; gossypol in cotton; phytate in corn; and protease inhibitors, phytohemagglutinins, and cyanogens and bloat compounds in legumes in a timely fashion. In the future, these crops may contain enzymes, such as beta glucanase and xylanase, to increase digestibility of fibrous ingredients contained in animal feeds. To be useful, the enzymes must be active following feed processing and must have activity in the targeted gut segment(s).

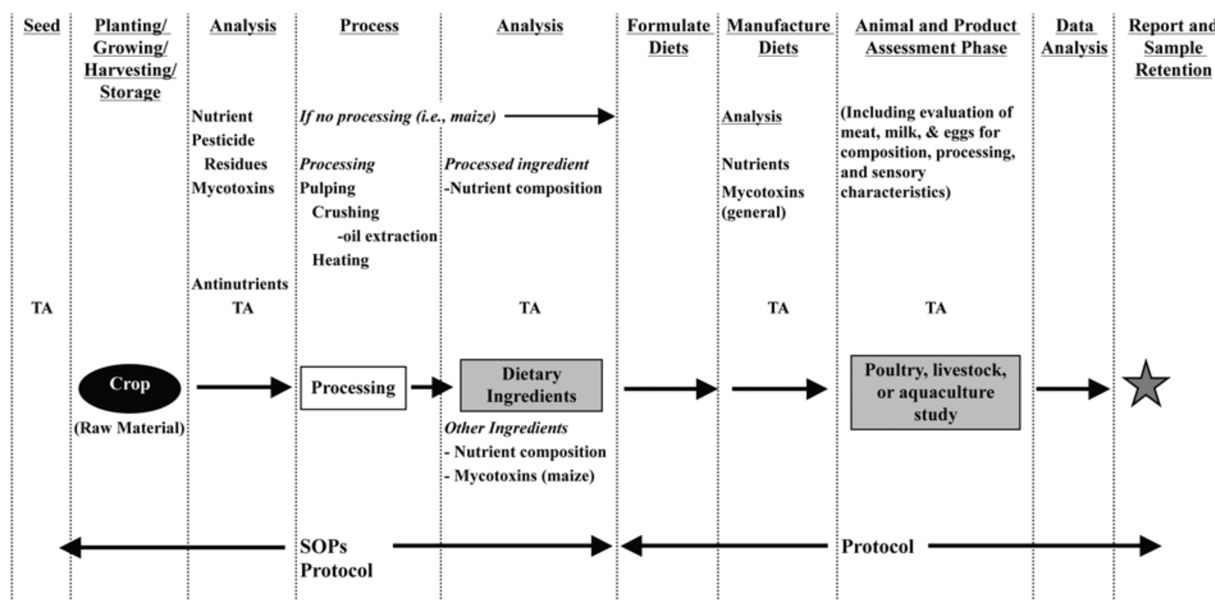
Livestock producers will continue to face issues regarding waste management including waste disposal, excessive phosphorus and nitrogen excretion, methane production, and odor. Increasing digestibility of nutrients in animal feeds will be a major factor in reducing waste problems. In the future, forages will be bioengineered to contain more digestible fiber components and/or fiber digestive enzymes. The plant may contain cellulose and hemicellulose that are more easily digested or cellulase, hemicellulase, and lignase enzymes may be bioengineered into plants or rumen microbes that enhance the use of these energy sources. Low lignin maize has already been developed via conventional plant breeding. The brown midrib mutant gene was identified and bred into certain corn hybrids. This product contains about 40% less lignin than its parental variety. Enzymes could be genetically engineered to convert roughages to substrates that can be efficiently used by nonruminants.

There is now global interest in the conduct of livestock, poultry, and aquaculture feeding studies with these types of GM crops and their products (Flachowsky and Aulrich 2001, OECD 2003, EFSA 2004, ILSI 2004, Flachowsky and Böhme 2005, Flachowsky et al. 2005). Flachowsky and Böhme (2005) and Flachowsky et al. (2005) recently proposed methods for evaluating this next generation of traits in crops for animal feed. However, researchers need to use a globally harmonized, internationally accepted approach to determine the necessary studies for assessing the impact of feeding GM crops to target animal species and the resulting animal-derived food products (ILSI 2004). To draw valid conclusions, the GM crop must be characterized, the proper comparator(s) must be selected, and each study must be designed and conducted in a scientifically valid and rigorous manner.

The purpose of this document is to provide best practices to scientists on how to: (a) produce, handle, store, and process GM crops containing the genetically enhanced trait(s); (b) sample and analyze the harvested and processed crop; (c) determine what studies to consider in the evaluation of specific genetically modified traits such as protein (amino acids), lipid, carbohydrates, vitamins and antioxidants, enzymes, minerals, and antinutrients in specific crops when fed to target animal species; (d) design and conduct livestock, poultry, aquaculture studies including proper comparator selection, animal-product processing effects, and sensory evaluation of the end-product; and (d) analyze and interpret the results. This publication focuses on "output" traits (i.e., traits such as those that provide added value to animal feed). GM traits in plants that are intended for animal health claims are outside the scope of this document. To complete this document, the authors decided to include updated versions of the "Production, Handling, Storage and Processing of Crops"; "Sampling and Analysis of Harvested and Processed Crop Material"; and "Statistical Analysis and Interpretation of Results" chapters, which were previously published in the ILSI document, *Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits* (2003).

An overall flow diagram from seed production 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[1]



TA = biotech Trait Analysis
SOP = Standard Operating Procedure
[1]Product quality studies may be desirable on a case by case basis, after the animal phase.

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Zhang ZB, Kornegay ET, Radcliffe JS, Wilson JH, Veit HP (2000b) Comparison of genetically engineered microbial and plant phytase for young pigs. J Anim Sci. 78:2868–2878.

Chapter 2

Production, Handling, Storage, and Processing of Crops

This chapter is an updated version of Chapter 2 published in the ILSI document, *Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits* (ILSI 2003).

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

Planting of Crops

Complete records of the seed planted should be maintained including source, variety, line, event, seed population, seed type, and planting dates. Examples of information to record and sample forms to capture data are given in Appendix 2-1. Specific location and country guidelines for 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 conventional and GM crops. See Table 2-1 for examples of isolation distances for GM 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 represents 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 GM and its near isogenic counterpart must be produced at similar, if not identical, environmental locations (e.g., irrigation vs. dryland, etc.). 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 used for producing test material may be planted in replicates depending on the location and amount of material needed. The production plot should be sufficiently large so 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.

Table 2-1. Example of isolation distances for GM crops[a]

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 conventional 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 conventional cotton to act as a pollen sink for insect pollinators (this material must be 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

[a]Adapted from the US Environmental Protection Agency (1999)

Growing Season

Careful record keeping should continue throughout the growing of the test material. Records should include dates of pesticide treatment, visual observations related 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.

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 GM 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 GM 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.

It is important to consider adequate and timely moisture for normal plant growth and development throughout the test. All typical 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 to ensure that material has been thoroughly purged from commercial equipment. Whenever possible, all equipment should be used first for conventional crops and then for GM crops. Harvesting equipment should be thoroughly cleaned between conventional and GM 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 forage should be cleaned 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, conventional crops should be transported before GM crops.

Maintenance of Grain and Forage Storage Locations

Storage locations should be carefully cleaned and visually inspected before storing the GM crop material. Legs, pits, conveyors, augers, and all other grain- or forage-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 forage.

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.

Moisture and temperature are the main determinants of how well grain keeps in storage.

Table 2-2. Maximum recommended storage moisture contents for oilseed and aerated grain		
Crop	Maximum recommended storage moisture, %	
	Storage ≤ 1 y	Storage ≥ 1 y
Maize and sorghum	14	13
Soybean	12	11
Cottonseed	9	9
Canola	8	8
Small grain (wheat and barley)	13	13

Aeration will help keep the grain at the desired moisture and temperature. Stored grain should be inspected every two weeks to verify grain temperature and to assess whether insect control is necessary.

Lot Removal from Storage

A lot is the stated portion of the consignment to be tested. A sample is one or more items (or a portion of material) selected in some manner from a lot; it is intended to provide information representative of the lot and to possibly serve as the basis for decision on the lot (ISO definition). Before samples are removed from storage, identity preservation procedures should be reviewed and all equipment should be inspected. Various analytical methods can be used to verify the test material's identity 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, conventional crops should be processed before GM 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.

References

ILSI (2003) Best practices for the conduct of animal studies to evaluate crops genetically modified for input traits. International Life Sciences Institute, Washington, DC.

US EPA (1999) Isolation Standards per 7 CFR 201.76: for regulated GM crops. US Environmental Protection Agency, Washington, DC.

Appendix 2-1

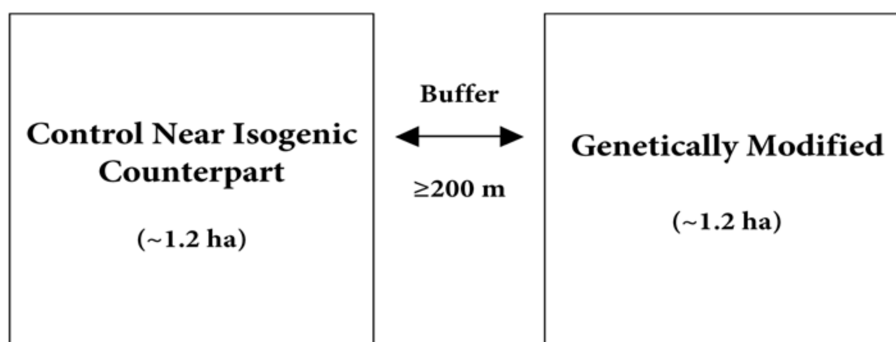
Example of Plot and Planting Information for an Experiment with Maize Planting Design

Available online at http://www.ilsi.org/file/Bestpractices.pdf

Planting Design

1. Each entry will be planted to an area of approximately 1.2 hectares per entry (Figure 2-A1).
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 of 200 m (660 feet USDA/Animal Plant Health and Inspection Service [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.

Figure 2-A1. Example trial design



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).

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, kernels, 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) ______________________________

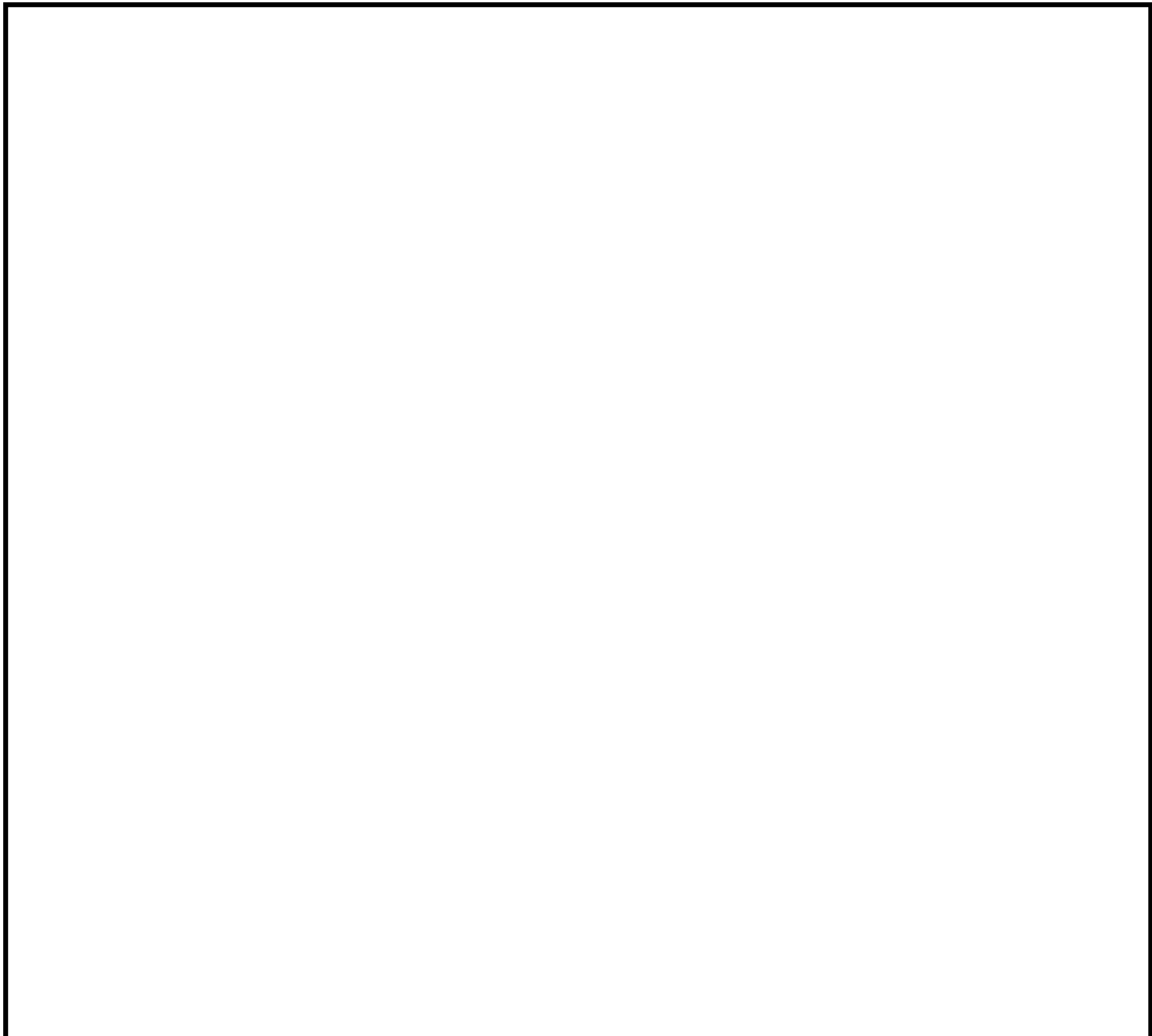
Distance and direction to nearest town: ______________________________

____

Initials:______________________ 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.

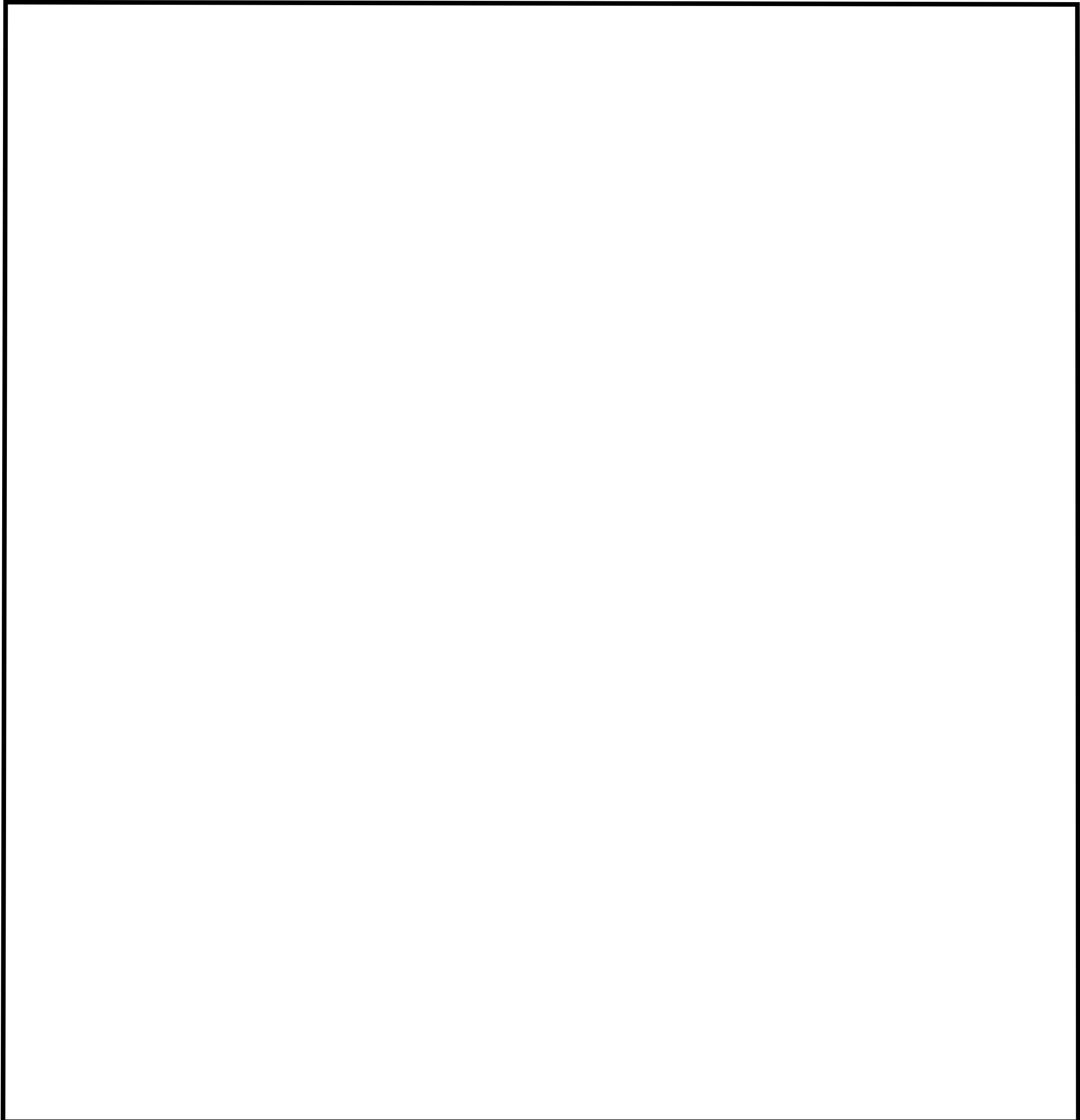


Initials:______________________ Date: ________

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.



Initials:______________________ 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	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	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		HCD
Source of information:					

c. **Herbicide Application** (this season, before planting)

Product(s) / Formulation	Active 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	15Dec99	*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 unit 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 d after silking)
Scale = height (cm) to the base of the primary ear of 10 individual plants per plot.

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.	

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: _____________________________
(e.g., Truck, FedEx, Air)

Condition of Samples: ____
(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., -80°C, -20°C, ambient)

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

Sample Receipt Date: __________________________

Received and Checked by: ______________________________________
Signature

Condition 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

This chapter is an updated version of Chapter 3 published in the ILSI document, *Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits* (ILSI 2003).

It is important to determine the nutrient and antinutrient content of genetically modified (GM) crops as well as to confirm whether the forage, grain, or other feed products contain the intended genetic modification. Verifying that the sample contains the intended t ransgenic trait or the effects derived from the trait can follow different formats. If the output trait can be measured directly in the feed sample (e.g., increased quantity or quality of protein), then this should be measured and confirmed prior to initiating the feeding experiment. It may also be important to confirm the presence in the sample of the modified gene or its protein product by molecular techniques such as event-specific polymerase chain reaction (PCR, Lipp et al. 2005) or enzyme-linked immunosorbent assay (ELISA, Reen 1994), respectively. Accurate feed analyses are needed to formulate diets properly.

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.

Basic principles of obtaining a representative sample include: collecting several samples from different areas of the lot; combining these samples to form a single representative 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 as well as proper sample handling. The following information about sampling principles and practices is provided in a book edited by Pfost (1976), the AOAC International manual (2000), Bell (1997), Herman (2001), Potter (2000), Schneider and Sedivec (1993) and the USDA (2001).

Sampling of the Lot

A testing program's validity rests on obtaining a representative 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 (Trotter and Johnson 2007, Barnhart 2004). It is most important to consider uniformity when identifying a lot. All forage from the same lot should be similar in terms of plant type, field (soil type), cutting date, maturity, variety, weed contamination, harvest equipment type, growth and harvest weather conditions, 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 uses 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 either 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 and come in standard sizes (1.5–4.0 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.

Table 3-1. Probe Sampling

Carriers and 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-unloading (hopper-bottom) trucks	1.8 2.4 3.0	12 16 20
Sacks and bags	1.5	11
Other containers	Use grain probes that will reach the bottom of the container.	

Sampling Canvas or Trough

Sampling canvases, which are usually made out of flat duck cloth or a 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 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 (0.5–1 kg) and the samples should be mixed thoroughly. A representative sample (0.5–1 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. With the slots closed and facing upward, the probe is pushed diagonally through the sack until its end touches the opposite bottom corner. The probe is then opened, two up-and-down motions are made, and the probe is closed and removed. The probe's contents are emptied into a clean, dry container or onto a canvas. This procedure is repeated with the other randomly selected sacks.

If all the probe samples have 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

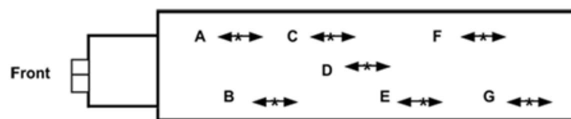
Ideally, if a bin can be accessed from the top, a grain probe should be used to obtain at least three samples of grain. If the bin cannot be accessed from the top, 12 to 20 random samples are collected when the grain is discharged (see tailgate sampling above) and combined in a clean plastic bucket. 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 0.5–1 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 its slots 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 and for objectionable odor, insect infestation, large stones, pieces of metal or glass, and any other potentially harmful contaminants.

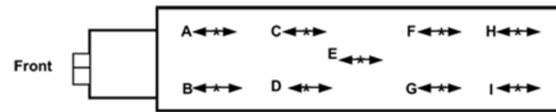
Figures 3-1 through 3-3 present examples of three different types of carriers and suggested locations to insert the probes. In all cases, the probe should be inserted at a 10-degree angle in the direction of the arrow.

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



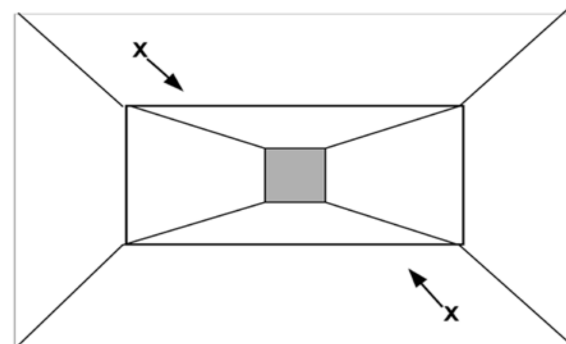
Site A: Approximately 60 cm from the front and side. Site B: The opposite side of site A, approximately halfway between the front and center of the carrier and approximately 60 cm from the side. Site C: The same side as site A, approximately 75% of the distance between the front and center of the carrier 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 such as pellets, cubes, small two-wire or string bales, small three-wire bales, large square bales (900 kg fresh weight), 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 coring device must have an inside diameter between 0.95– 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 hay flakes 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 its face. 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 two to four 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 two to four 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

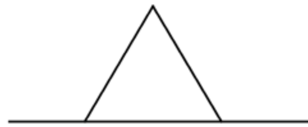
Green chopped forage should be sampled as it goes into the silo. If green chopped forage is fed directly without ensiling, it should be sampled as it is delivered to the animals. One handful is taken from every fourth or fifth wagon or truckload and placed in a clean plastic bucket or bag. The container 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.

Silage

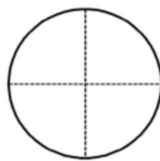
Silage samples should represent several locations within the silo (upright, horizontal, pit clamp, stack, or silo bag) to ensure adequate representation of the silage. The sample must be tightly packed in a plastic bag with excess air removed and sealed tightly. The samples can be shipped cold to the laboratory or frozen and then

Figure 3-4. Quartering (subsampling) procedure

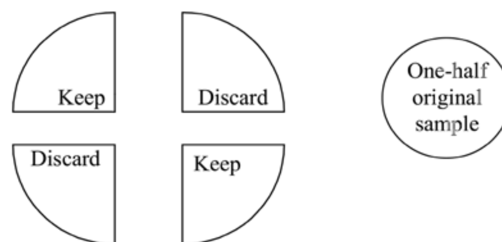
1) Make a conical pile of the chopped forage (side view).



2) Divide pile into four or six pieces (top view).



3) Randomly choose one section and the opposite section and discard the remainder (top view).



4) Repeat this procedure until 1 to 2 L of material remain. Transfer all material to a 2 L plastic bag, remove excess air, seal tightly, and store frozen.

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 15–20 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 can be difficult as fertility gradients and moisture differences, even within a single paddock (a defined grazing area), add to the random variation that must be accounted for in the analyses. Traits of interest include dry matter yield, plant morphology (leaf appearance rate, leaf area index, the area of leaf per hectare), and feed quality. There are a number of ways to sample pasture, depending on the trait most interested in and/or the uniformity of the sward being sampled, including random sampling, transect sampling, and cage sampling. For detailed information on these methods, please see the *Sward Measurement Handbook* (Davies et al. 1993).

Sampling for Dry Matter Yield

When dry matter (DM) yield is of primary interest, sampling involves cutting quadrants of pasture from randomly chosen areas within the paddock. Destructive methods involve cutting the sward to ground level, while non-destructive methods cut the pastures to a predefined cutting height (e.g. 40 mm). The number of quadrants required depends on the area allocation and the individual quadrant's size. For example, Roche (1995) described a method whereby four areas measuring 7 to 10 m^2 each were cut to 40 mm within 0.5 ha using a finger-bar power 1.0 m in width. In comparison, Roche et al. (2005) described an alternative method whereby 10 pasture samples from the treatment area (approx. 0.1 ha) were cut to ground level within rectangular quadrants (0.125 m^2) placed randomly. Both are equally effective methods of determining pasture DM yield.

Irrespective of sampling method, 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 to 100°C for 16–24 h (until dry) to determine the dry matter. The remaining sample, if being used for quality assessment, is dried at a lower temperature to ensure protein is not degraded.

Sampling to Determine Morphological Changes

Sampling pasture to measure changes in leaf appearance rate, tiller number, leaf area index, or other morphological changes of interest can be undertaken either randomly or along a predefined transect. Random sampling involves criss-crossing the defined area, sampling pasture randomly at regular intervals (Roche et al. 2005). This method is useful in uniform pastures. Alternatively, a transect line can be established, with sampling occurring at regular predefined points along the transect (Davies et al. 1993). This method is useful in less uniform swards and removes the potential for sampler bias. In both measurements, the entire plant (or plants within a quadrant) is removed.

Sampling to Determine Quality Differences

When sampling to determine differences in pasture quality, one is always trying to emulate the animal's selection preferences. For example, Dalley et al. (1999) reported that irrespective of herbage allowance, cows selected a diet higher in *in vitro* dry matter digestibility and crude protein and lower in neutral detergent fiber than that in the herbage on offer. Therefore, sampling aims to select pasture similar to what the animal would consume. This can be accomplished through:

1. Random sampling of pasture prior to defoliation, having evaluated the previous defoliation severity of the herd or flock (Roche et al. 2005).
2. Placing cages in the paddock prior to grazing and subsequently cutting the forage within the cages to the estimated grazing height in the surrounding paddock (Dalley et al. 2001).
3. Using esophageal fistulated animals (Dillon et al. 1998)

All these methods have their advantages and disadvantages. Random sampling is quick, but one assumes that the pasture selected is similar to what the animal would have selected; it is most useful in relatively uniform pastures. The cage sampling method is less subjective in determining cow preference, but is very labor intensive; it is most useful in less uniform pastures. In comparison, esophageal fistulated animals are neither subjective nor labor intensive, but there are obvious ethical issues that must be accommodated.

Sampling Methods

Regardless of the sampling method chosen, the samples must not be collected from areas that are overgrown or not being grazed. If the entire pasture area is used, sample locations should be distributed evenly, either through regular interval sampling or by using pre-located transects. Forage can be harvested using either a lawn mower or finger-bar mower for large quadrant areas, or using hand shears (either manual or electric) for smaller area quadrants. Samples from all sites are mixed and quartered as described to reduce sample size and yet ensure a representative sample is obtained. 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. Drying temperatures will vary depending on the trait being investigated.

Mixed Diet Sampling

Mixed diets are difficult to sample because they are seldom homogeneous even when well mixed. When it is unlikely that a sampling method can produce a representative sample of the diet, 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; 12–20 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 0.5–1-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 using 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; allowing animals 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.

If a GM byproduct or other feed ingredient derived from a GM crop is being tested in feeds as a feed supplement or is otherwise a minor constituent of the feed (e.g., an enzyme that is produced in a GM crop and added to the feed at low inclusion rates), the homogeneity of the GM feedstuff in the final feed preparations should be assessed. This should be accomplished by removing many small (about 100 g) samples from widely dispersed locations in the bulk feed. The trait being tested should be measured at least two times in each sample, and the data from all measurements should be statistically analyzed to determine the coefficient of variation. A homogeneous mixture of the GM feedstuff in the feed would be indicated by a coefficient of variation of less than 10%.

Handling of Sample

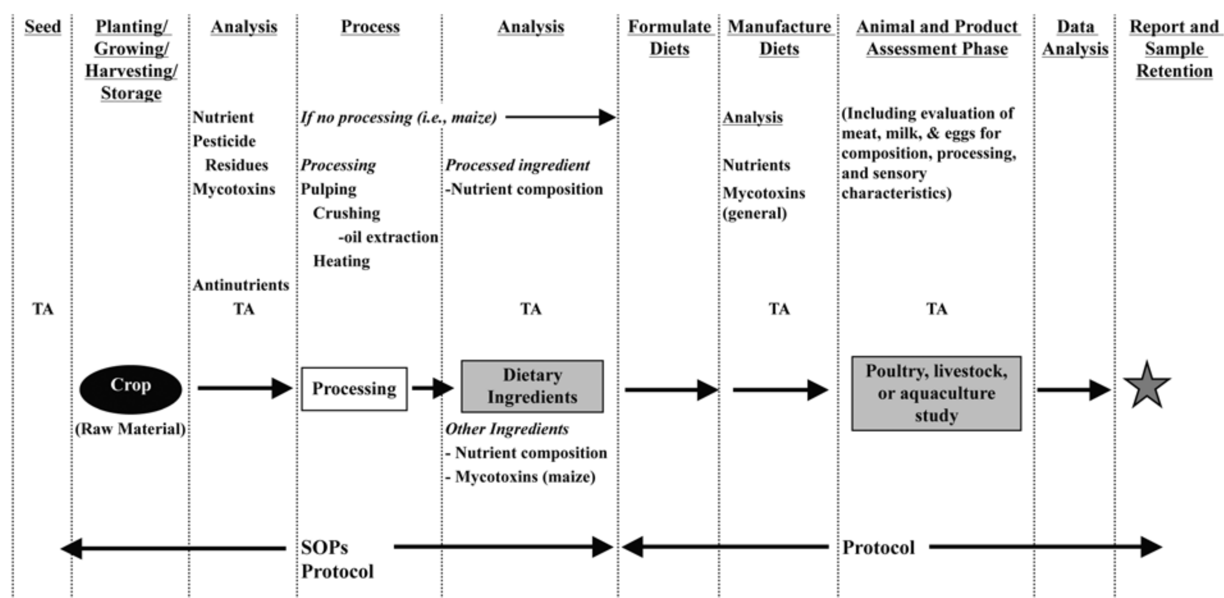
Proper sample handling between the farm or research facility and laboratory ensures that a result will be representative of the lot. It is good practice to divide the sample in half and send one half for analysis and retain the other. Each sample should be 0.5–1 kg and should be labeled with the date, 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 GM and control material.

In many animal feeding experiments, the test

Figure 3-5. Project Flow Diagram for Animal Studies[1]



TA = biotech Trait Analysis
SOP = Standard Operating Procedure

[1]Product quality studies may be desirable on a case by case basis, after the animal phase.

feed may be prepared and analyzed before the start of the experiment and stored for use throughout the experiment. In cases where the prepared feeds, including the test feed and all control feeds, will be stored for periods of time prior to use, it is important to monitor the trait's stability in the feeds under conditions simulating those by which the sample will be stored during, and at the conclusion of, the animal feeding experiment. This will provide valuable information on the stability of the trait in the feed for the duration of the experiment. 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 reliable source to ensure its identity. 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 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 procedures such as enzyme-linked immunosorbent assays (Reen 1994, Tijssen 1985) or radioimmuno assays (Vazques et al. 1996).

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	

Pesticide, Mycotoxin, Nutrient, and Antinutrient analysis

After harvest and storage and before processing and expected use, the crop should be checked for pesticide residues, mycotoxins, and nutrient and antinutrient content. Pesticides that were sprayed on the crop during the growing season determine which pesticide residues

to evaluate. If pesticide residues exceed the locally accepted tolerance levels, the feedstuff is not suitable for animal studies.

The crop 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. Toxins 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 other factors (CAST 2003). Analytical methods are listed in Appendices 3-1 and 3-2.

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

Table 3-3. Examples of Antinutrients in Crops

Crop or Product	Antinutrient
Soybeans, soybean meal	Trypsin inhibitors, lectins
Canola, canola meal, rapeseed, rapeseed 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 and microbial contamination.

Nutrient content should be analyzed after harvest, before and after processing for oilseeds and sugar beets, and after feed manufacture. The nutrients to be analyzed are those that are important for meeting the recipient

Table 3-4. Recommendations for Nutrient Analysis

Crops/Grains/Coproducts	Livestock Type	Analyte[a]
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

[a]ADF, acid detergent fiber; ADIN, acid detergent insoluble nitrogen; ADL, acid detergent lignin; ADICP, acid detergent insoluble crude protein; CP, crude protein; DM, dry matter; DNDF, digestible neutral detergent fiber; EE, ether extract (crude fat); NDF, neutral detergent fiber; NDICP, neutral detergent insoluble protein; NDIN, neutral detergent insoluble nitrogen; NPN, nonprotein nitrogen.

livestock and poultry species' requirements. 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. Two other components of proximate analysis (crude fiber and nitrogen-free extract) are not included. Crude fiber analysis 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. Additional nutrient analysis may be needed if the Cornell Net Carbohydrate and Protein System is utilized (Sniffen et al. 1992). Additional compositional analysis is warranted if a GM crop was designed to alter the content of a particular nutrient such as a vitamin, amino acid, oil, or fatty acid. References for analytical methods are provided in Appendix 3-1.

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Appendix 3-1

Analytical Methods and Chemical Analysis

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 glucosinolates content—Part 1: Method using high-performance liquid chromatography. ISO 9167-1:1992, Ed. 1, Current stage 90.92, TC 34/SC 2. ISO, Geneva, Switzerland.

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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.

International Organization for Standardization. Fruit and vegetable products—Determination of dry matter content by drying under reduced pressure and of water content by azeotropic distillation. ISO 1026:1982, Ed. 1, Current stage 90.93, TC 34/SC 3. ISO, Geneva, Switzerland.

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

Soxhlet Extraction

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International Organization for Standardization. Animal feeding stuffs—Determination of the content of fatty acids—Part 1: Preparation of methyl esters. ISO/TS 17764-1:2002, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

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FIBER

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International Organization for Standardization. Agricultural food products—Determination of crude fibre content—Modified Scharrer method. ISO 6541:1981, Ed. 1, Current stage 90.60, TC 34. ISO, Geneva, Switzerland.

Neutral Detergent Fiber

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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.

AOAC Official Method. 2002.04, Amylase-treated neutral detergent fiber.

International Organization for Standardization.Animal feeding stuffs—Determination of amylase-treated neutral detergent fibre content (aNDF). ISO 16472:2006, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

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Lignin

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AOAC Official Method 973.18, Fiber (acid crude) and lignin (H_2SO_4) in animal feed.

International Organization for Standardization. Animal feeding stuffs—Determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents. ISO/DIS 13906, Ed. 1, Current stage 40.60, TC 34/SC 10. ISO, Geneva, Switzerland.

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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.

Total Dietary Fiber

AOAC Official Method 985.29, Soluble dietary fiber in food and food products, enzymatic-gravimetric method (phosphorus buffer).

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INORGANIC SALTS

Chlorides

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

International Organization for Standardization. Animal feeding stuffs—Determination of water-soluble chlorides content. ISO 6495:1999, Ed. 2, Current stage 90.92, TC 34/SC 10. ISO, Geneva, Switzerland.

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

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International Organization for Standardization. Animal feeding stuffs—Determination of potassium and sodium contents—Methods using flame-emission spectrometry. ISO 7485:2000, Ed. 1, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

Ash

AOAC Official Method 942.05, Ash of animal feed.

International Organization for Standardization. Animal feeding stuffs—Determination of ash insoluble in hydrochloric acid. ISO 5985:2002, Ed. 2, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

Calcium

International Organization for Standardization. Animal feeding stuffs—Determination of calcium content—Part 1: Titrimetric method. ISO 6490-1:1985, Ed. 1, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

Phosphorus

International Organization for Standardization. Animal feeding stuffs—Determination of phosphorus content—Spectrometric method. ISO 6491:1998, Ed. 2, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

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AOAC Official Method 996.16, Selenium in feeds and premixes.

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Mycotoxins: General

Codex Alimentarius (2003) Code of Practice for the Prevention and Reduction of Mycotoxin Contamination in Cereals, including Annexes on Ochratoxin A, Zearalenone, Fumonisins and tricothecenes. VOLUME 1A Reference CAC/RCP 51.

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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.

International Organization for Standardization. Animal feeding stuffs—Determination of aflatoxin B_1. ISO 17375:2006, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

International Organization for Standardization. Animal feeding stuffs—Semi-quantitative determination of aflatoxin B_1—Thin-layer chromatographic methods. ISO 6651:2001, Ed. 3, Current stage 90.60, TC 34/SC 10. ISO, Geneva, Switzerland.

International Organization for Standardization. Animal feeding stuffs—Determination of aflatoxin B_1 content of mixed feeding stuffs—Method using high-performance liquid chromatography. ISO 14718:1998, Ed. 1, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

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.

International Organization for Standardization. Animal feeding stuffs—Determination of free and total gossypol. ISO 6866:1985, Ed. 1, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

NITRATES

Hach method, Hach Company, Loveland, CO

- Plant tissue and SAP Analysis Manual. Literature

Code #3118
- Extraction: pp. 130–131, no 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 chromatopic 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.

PROTEINS

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.

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.

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International Organization for Standardization. Animal feeding stuffs—Determination of nitrogen content and calculation of crude protein content—Part 1: Kjeldahl method. ISO 5983-1:2005, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

International Organization for Standardization. Animal feeding stuffs—Determination of nitrogen content and calculation of crude protein content—Part 2: Block digestion/steam distillation method. ISO 5983-2:2005, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

International Organization for Standardization. Food products—General guidelines for the determination of nitrogen by the Kjeldahl method. ISO/DIS 1871, Ed. 2, Current stage 40.20, TC 34. ISO, Geneva, Switzerland.

International Organization for Standardization. Animal feeding stuffs—Determination of soluble nitrogen content after treatment with pepsin in dilute hydrochloric acid. ISO 6655:1997, Ed. 1, Current stage 90.92, TC 34/SC 10. ISO, Geneva, Switzerland.

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Amino Acid Composition

AOAC Official Method 994.12, Amino Acids in Feeds.

AOAC Official Method 982.30, Protein Efficiency Ratio, Calculation Method.

International Organization for Standardization. Animal feeding stuffs—Determination of amino acids content. ISO 13903:2005, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

International Organization for Standardization. Animal feeding stuffs—Determination of tryptophan content. ISO 13904:2005, Ed. 1, Current stage 60.60, TC 34/SC 10. ISO, Geneva, Switzerland.

International Organization for Standardization. Animal feeding stuffs—Determination of available lysine. ISO 5510:1984, Ed. 1, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

Degradable Protein

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

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Soluble Protein

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

ORGANOPHOSPHATES AND CHLORINATED INSECTICIDES

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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.

International Organization for Standardization. Animal feeding stuffs—Determination of vitamin A content—Method using high-performance liquid chromatography. ISO 14565:2000, Ed. 1, Current stage 90.93, TC 34/SC 10. ISO, Geneva, Switzerland.

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

Vitamin B_1 (Thiamin)

AOAC Official Method 942.23, Thiamine (vitamin B_1) in human and pet foods, fluorometric method.

AOAC Official Method 953.17, Thiamine (vitamin B_1) in grain products, fluorometric (rapid) method.

AOAC Official Method 957.17, Thiamine (vitamin B_1) in bread, fluorometric method.

Vitamin B_2 (Riboflavin)

AOAC Official Method 940.33, Riboflavin (vitamin B_2) in vitamin preparations, microbiological methods.

Vitamin B_6

AOAC Official Method 961.15, Vitamin B_6 (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

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Appendix 3-2

Microbiological Methods

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Carcass sampling for microbiological analysis. ISO 17604:2003, Ed. 1, Current stage 60.60, TC 34/SC 9. ISO, Geneva, Switzerland.

Coliforms

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

Hitchins AD, Feng P, Watkins WD, et al (1998) *Escherichia coli* and the coliform bacteria. In US 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.

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International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of coliforms—Colony-count technique. ISO 4832:2006, Ed. 3, Current stage 60.60, TC 34/SC 9. ISO, Geneva, Switzerland.

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of coliforms—Most probable number technique. ISO 4831:2006, Ed. 3, Current stage 60.60, TC 34/SC 9. ISO, Geneva, Switzerland.

Escherichia coli

Hitchins AD, Feng P, Watkins WD, et al (1998) *Escherichia coli* and the coliform bacteria. In US 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.

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*—Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. ISO 16649-1:2001, Ed. 1, Current stage 90.93, TC 34/SC 9. ISO, Geneva, Switzerland.

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*—Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-lucuronide. ISO 16649-2:2001, Ed. 1, Current stage 90.93, TC 34/SC 9. ISO, Geneva, Switzerland.

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*—Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide. ISO/TS 16649-3:2005, Ed. 1, Current stage 60.60, TC 34/SC 9. ISO, Geneva, Switzerland.

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the detection of *Escherichia coli* O157. ISO 16654:2001, Ed. 1, Current stage 90.60, TC 34/SC 9. ISO, Geneva, Switzerland.

International Organization for Standardization. Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of presumptive *Escherichia coli*—Most probable number technique. ISO 7251:2005, Ed. 3, Current stage 60.60, TC 34/SC 9. ISO, Geneva, Switzerland.

Salmonella

Andrews WH, June GA, Sherrod PS, et al (1998) Salmonella. In US 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

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

Maturin LJ, Peeler JT (1998) Aerobic plate count. In US 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 Compendium of methods for the microbiological examination of foods, 3rd ed. American Public Health Association, Washington, DC.

Yeast and Mold Count

Stack ME, Mislivec PB, Koch HA, Bandler R (1998) In US 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

Proteins and Amino Acids

The Concept of Protein Quantity and Quality

Genetic modifications to improve the protein component of crops and crop products can generally be classified into one of three categories: an increase in the amount of protein, an increase in the quality of protein, or an improvement in the biological availability of the protein. To understand the potential benefits to animals, a brief explanation of the three categories is necessary.

Protein Quantity

Although there is clear evidence that animals do not require proteins per se but only their constituent amino acids, the total protein content of animal feedstuffs remains an important element in any discussion of feedstuff quality. There are a number of reasons for this. Some are historical (e.g., crude protein is part of the traditional proximate analysis [Weende system; AOAC 2003] of feedstuff analysis), some are legal (e.g., labeling requirements), and some are practical (e.g., for many species, amino acid requirements are not well defined and protein "requirements" are a reasonable substitute; protein analysis is much simpler than amino acid analysis). Therefore, the amount of protein in a feedstuff remains important.

It is fairly easy to measure the amount of protein present in a simple solution, but measurement in a complex matrix, such as a feedstuff, is difficult. Methods to determine protein content using near infrared analysis have been developed for specific crops and crop products, but these methods are not easily applied across a wide variety of feedstuffs. For these reasons, the usual approach for feedstuffs is to measure the nitrogen (N) content (either by traditional wet chemistry analysis [Kjeldahl procedure] or combustion N analysis) and multiply the value by 100/16 (6.25). This multiplication factor is used because the average N content of proteins is 16 g N/100 g protein. The value obtained when N content is multiplied by 6.25 is usually called *crude protein*.

The term "crude" is used because the calculation involves two assumptions that are not always valid. In evaluating crops with improved nutritional characteristics, it is important to keep these two assumptions in mind. The first assumption is that all proteins contain 16 g N/100 g protein. Although the assumption is untrue, animal diets contain mixtures of feedstuffs and the average N content is usually approximately 16 g N/100 g protein. Nevertheless, individual feedstuffs can vary considerably. For example, wheat (whole grain) contains 17.5 g N/100 g protein and a conversion factor of 5.70 is commonly used for wheat and wheat products. A list of various conversion factors is provided by AOAC (2003). The key point is to be clear about which conversion factor is being used.

The second assumption is that all the N present in feedstuffs is in the form of proteins. In fact, much of the N is present in compounds other than proteins (nonprotein nitrogen; NPN). According to Van Soest and Sniffen (1984), approximately 10% of the crude protein of maize and 13% of the crude protein of soybean meal is NPN. In silages, the proportion of crude protein that is NPN often exceeds 50%. Thus, crude protein comprises true protein and a variety of NPN compounds (e.g., free amino acids, ammonia and urea, nucleic acids, etc.). In ruminants, the NPN contributes to the rumen ammonia pool and has potential value to the animal. However, nonruminants make little use of NPN sources other than free amino acids. In terms of genetic improvement, it is important to recognize that an increase in crude protein may not be entirely attributable to an increase in true protein.

Protein Quality

The quality of a protein refers to its nutritional value for animals. Some proteins, such as those in eggs and milk, are high quality; others, such as the storage proteins in most cereal grains, are poor quality. There are three major components of protein quality: amino acid composition, protein digestibility, and amino acid availability. From a protein chemistry point of view, all of these components are actually determined by the protein's primary structure i.e., the sequence of amino acids that are linked together to make up the protein chain). The sequence of amino acids determines the number of amino acid "residues" that are present and, therefore, the relative proportions of the amino acids. The degree to which the proportions of the 10 or 11 essential amino acids (arginine, glycine [in poultry], histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) match the animal's dietary

Table 4-1. Amino acid concentrations in maize, wheat, and soybean meal compared with amino acid requirements of swine, poultry, and fish (values are on an "as-fed" basis)[a]

	Amino acid levels, %			Amino acid requirements, %		
Amino acid	**Maize**	**Wheat**	**Soybean meal, Dehulled**	**Pig, 35 kg**	**Chick, 3–6 wk**	**Trout, rainbow**
Arginine	0.37	0.60	3.48	0.35	1.10	1.50
Histidine	0.23	0.32	1.28	0.29	0.32	0.70
Isoleucine	0.28	0.41	2.16	1.49	0.73	0.90
Leucine	0.99	0.86	3.66	0.86	1.09	1.40
Lysine	0.26	0.34	3.02	0.90	1.00	1.80
Methionine + cystine	0.36	0.49	1.41	0.52	0.72	1.00
Phenylalanine + tyrosine	0.64	0.98	4.21	0.83	1.34	1.80
Threonine	0.29	0.37	1.85	0.59	0.74	0.80
Tryptophan	0.06	0.15	0.65	0.16	0.18	0.20
Valine	0.39	0.54	2.27	0.62	0.82	1.20
Glycine + serine	0.70	1.18	4.53	-	1.14	-

[a]NRC (1993, 1994, 1998)

amino acid requirements is the primary determinant of protein quality. However, proteins must be digested and the amino acids absorbed in a form that can be utilized. The digestibility of a protein is influenced by its susceptibility to digestive enzymes; this is determined by the secondary, tertiary, and quaternary structure of the protein. However, these levels of structure are determined in large part by the primary structure and therefore the sequence of amino acids in the protein determines all aspects of protein quality.

Biological Availability

A highly digestible protein is always desirable for nonruminant animals (e.g., pigs and poultry), but this is not always the case for ruminants (e.g., cattle and sheep). Proteins that are highly digestible may be rapidly degraded in the rumen, resulting in the production of ammonia that may be absorbed from the rumen and not utilized by the host animal. Thus, for ruminants, whether an increase in protein digestibility is desirable or not will depend on numerous factors, including the nature of the other components of the diet and the particular productive situation. Therefore, it is impossible to generalize the benefits of increased protein digestibility for ruminants. The development of crops with modified output traits offers the potential for partitioning protein digestibility between ruminal and small intestinal sites depending on specific situations.

The Value of Increased Protein Quantity and Quality

Quantity

Cereal grains (e.g., maize, wheat, barley, sorghum, oats, and rice) form the basis of the diet for most nonruminant animals raised in modern productions systems. This is because cereals provide the most economical way to meet the animal's energy needs. Although cereals also provide protein, the amounts are insufficient to meet the animal's needs and protein supplements (e.g., soybean meal, cottonseed meal, and fish meal) must be included in the diet. Table 4-1 compares the amino acid composition of maize, wheat, and soybean meal with the amino acid requirements of swine, poultry, and fish. Protein supplements are expensive, so increasing the amount of protein in either cereal grains or protein supplements reduces the amount of supplement that needs to be added, thereby reducing the diet's cost.

Diets of ruminant animals vary more than those of most nonruminants. In some systems of ruminant production, forages/roughages form the basis of the diet; whereas in other intensive systems for finishing ruminants and in systems designed to maximize milk production, a mixture of forages and cereal grains form the basis of the diet. However, protein contents are low in either case (except for some grazing systems

based on high quality forage), and protein supplements must be provided in most modern systems. Increasing the protein content of forages, grains, and protein supplements reduces the amount of protein supplement that needs to be added and therefore reduces diet cost.

Quality

The majority of protein in cereal grains and legumes is found in seed storage proteins. The biological function of storage proteins is to provide nutrients (nitrogen and sulfur) required during germination and establishment of a seedling. Consistent with this function, storage proteins are generally rich in asparagine, glutamine, arginine, and proline, but low in most essential amino acids (Higgins 1984). The primary storage proteins in cereals are prolamines and glutelins, whereas legume seed proteins are mostly albumins and globulins, with globulins being subdivided into glycinin and conglycinin (Clarke and Wiseman 2000). The prolamines of cereals are especially low in lysine, threonine, and tryptophan, and the globulins of legumes are low in the sulfur-containing amino acids, methionine, and cysteine (Mandal and Mandal 2000). Consequently, most plant proteins have a relatively poor protein quality (their amino acid composition [pattern or profile] differs from the amino acid pattern required by animals). An exception to this is the protein consumed by animals grazing high quality temperate grass/legume pastures. Animal diets are often supplemented with additional sources of protein to compensate, but this increases diet cost, especially when high-quality protein supplements, such as soybean meal and those derived from animal products (whey, poultry byproduct meal, fishmeal, etc.), are included. An alternate approach is the judicious supplementation of crystalline forms of the amino acids in shortest supply (generally lysine, methionine, threonine, or tryptophan), but this also increases diet cost. Obviously, production of plant-based feedstuffs with an improved protein quality has the potential to decrease animal feeding costs.

Recent concerns about environmental pollution by excess nitrogen in soil and groundwater derived from animal manure (Mallin and Cahoon 2003) have increased incentives to improve plant protein quality. On average, only about 20% of feed protein is converted into edible nutrients for humans (Chen 2001). The remaining proportion of feed nutrients is added to the environment in one manner or another. Increasing the protein quality of feedstuffs by matching their amino acid composition more closely with the animal's requirements is a key method to increase efficiency of protein utilization and decrease nitrogen output in animal manure (feces and urine).

In ruminants, discussions of protein quality are more complex because, in addition to meeting the amino acid needs of the host animal, the presence and activity of the rumen microflora and microfauna must be considered. Dietary nitrogen (both protein and NPN) must provide sources of nitrogen for rumen bacteria and protozoa, and must also provide adequate amounts of "ruminally undegraded intake protein" that escape degradation in the rumen and complement the amino acid pattern provided by the microbial protein. Thus, optimal utilization depends not only on the amino acid pattern, but also on the amount of metabolizable protein that reaches that small intestine. Modification of crop composition offers the potential for increasing or decreasing the degradation of proteins in the rumen.

Overall Benefit

In developed countries, genetic improvements in the protein components of feedstuffs are unlikely to have major effects on increasing growth rate, milk production, etc. The benefits of feedstuffs with improved traits are more likely to come from reducing feed costs by reducing the amount of grain or protein supplements needed in the diet or by reducing excretion of nutrients that have the potential to cause environmental pollution. In developing countries, sources of supplemental protein may be unavailable or severely limited. In these countries, genetic improvements in the protein components of cereals may lead to substantial increases in animal production.

Intended Compositional Changes

Changes in a feedstuff's amounts, quality, and biological availability (including rumen protection) of proteins can be achieved using either traditional plant breeding techniques (selection, crossbreeding, backcrossing, and propagation) or modern biotechnology methods. For the purposes of evaluating crops with improved nutritional traits intended for animal feeding, it generally makes little difference which method is used to achieve the improvement.

Increases in the amount of protein in several different cereal grains (e.g., wheat, barley, and oats) and legumes (e.g., soybeans) have been produced by traditional plant breeding methods. A classic approach is to screen collections of plant varieties for protein content and select those with the highest protein content. This approach has met with limited success because protein content is generally inversely related to yield (Brim and Burton 1979). Backcrossing, a traditional plant breeding technique, has been used to overcome

the negative effects on yield. For example, Wilcox and Cavins (1995) crossed a high-protein-low yield soybean variety (49.8% CP) with a standard variety (40.8% CP). After several backcross generations, lines were identified that had ≥ 47% CP and had yields similar to the standard variety. Of course, increases in protein may be at the expense of other components, such as oil in the case of soybeans.

Traditional methods have also been successful in improving protein quality (increasing the amounts of the most limiting amino acids). Probably the most well-known examples are the high-lysine maize varieties developed at Purdue University in the 1960s. Researchers screened a large collection of maize varieties and discovered several that contained about twice the normal levels (approximately 0.29% on a dry matter basis) of lysine (e.g., *opaque-2* in Mertz et al. 1964, *floury-2* in Nelson et al. 1965). The high lysine content was due to a change in the relative proportions of the storage proteins (a decrease in the prolamine, zein, which contains no lysine). Numerous feeding trials with various species of animals demonstrated that these varieties were nutritionally superior to conventional varieties. Similar screening programs led to the identification of high-lysine lines of barley (Munck et al. 1970) and grain sorghum (Singh and Axtell 1973).

During the past decade, the advent of modern biotechnology techniques has enabled new approaches to enhancing the content of specific essential amino acids and improving protein quality. The primary methods have been reviewed by Sun and Liu (2004) and are summarized below.

Manipulation of homologous protein expression. This technique involves overexpression of indigenous genes to enhance the synthesis and accumulation of desirable proteins that are inherent to the plant.

Modification of protein sequences. This involves site-directed mutagenesis or insertion of essential amino acid-rich sequences into native plant proteins.

Insertion and expression of heterologous proteins. A gene encoding a desirable protein is isolated from any source organism and is transferred into and expressed in a target plant.

Synthetic proteins. It is now possible to design and synthesize a gene that encodes a new protein with desirable essential amino acid composition and to insert the gene into a target plant.

Recent examples of transgenic approaches include the overexpression of a methionine-rich protein in maize (Lai and Messing 2002) and the development of a dominant mutant of maize that suppressed storage protein synthesis without affecting synthesis of proteins with a higher lysine content (Segal et al. 2003).

One approach that is unique to biotechnology, and has no traditional plant breeding equivalent, is the manipulation of plant biosynthetic and degradative pathways to result in the accumulation of free amino acids (Galili and Hofgen 2002, Galili et al. 2002). For example, two key enzymes in lysine biosynthesis are aspartokinase and dihydrodipicolinate synthase, which are feedback inhibited by lysine. By increasing the expression of mutant forms of these two enzymes that are insensitive to lysine feedback, lysine synthesis can be increased. Significant increases in the lysine content of maize have been achieved by these techniques (Mazur et al. 1999). The lysine is likely to be very bioavailable because it is not protein bound. On the other hand, free amino acids are vulnerable to leaching from plant tissues and to destruction during feed processing.

Although there do not seem to have been any direct attempts to increase amino acid bioavailability by either traditional plant breeding or biotechnology, changing either the amount or quality of protein may change bioavailability of the constituent amino acids. Similarly, changes in protein quantity and quality may affect degradability in the rumen and result in significant effects on animal performance. In this regard, attempts have been made to improve forage for ruminants. Sunflower seed albumin, which is resistant to *in vitro* degradation by rumen fluid (McNabb et al. 1994), has been inserted and expressed in forages. Unfortunately, the protein accumulation in both alfalfa (Tabe et al. 1995) and tall fescue (Wang et al. 2001) has been low.

Undesirable Effects

All genetic changes can potentially produce undesirable effects. This is true for both traditional and transgenic methods. Some undesirable changes are predictable, whereas others are not.

Plants and feedstuffs with improved protein traits often have undesirable agronomic or processing characteristics. "Yield drag" is one of the most frequent problems. Again, the early varieties of high-lysine maize serve as an excellent example. The initial lines of *opaque-2* maize had ears that were smaller than normal, and yields were 8–15% lower than traditional varieties. The grain was more susceptible to fungi and insects, both in the field and in storage, and it dried more slowly. During processing in hammer mills, *opaque-2* maize tended to produce a floury product, more prone to bridging in feed distribution systems. Subsequent work by several different research groups throughout the world (most notably at the Centro Internacional de Mejoramiento de Maíz y Trigo [CIMMYT] near Mexico City) resolved many of these problems and combined the nutritional excellence of *opaque-2* maize with the

kernel structure of conventional maize varieties. The new varieties were labeled "quality-protein maize" (NRC 1988).

Another example of an undesirable effect is the creation of amino acid antagonisms or imbalances as the result of genetic modifications. However, these effects are very rare in whole proteins and are generally created by inappropriate additions of crystalline amino acids to the diet.

Many undesirable effects that result from genetic changes are manifested by reductions in palatability of the feedstuff.

Evaluation Methods

The purpose of the evaluation methods is to establish whether the new (nutritionally improved) product is superior to the conventionally bred form of the crop.

Deciding What Experiments Are Needed

What experiments (if any) are needed should be decided in a systematic, science-based manner. The need for, and types of, experiments will depend on the changes in the amount or type of protein and/or amino acids that have been made to the crop or product. Because a wide variety of changes are possible, it is not possible to generalize the types of studies needed. Each new crop or product will need to be considered on a case-by-case basis. Nevertheless, there are some general guidelines that can be applied. A systematic approach to deciding what experiments to conduct is provided in Figures 4-1 through 4-5 (see pages 52–56).

Animal Performance

Diet formulation: Appropriate diet formulation is crucial. In general, improvements in protein/amino acid quantity or quality are unlikely to enable major increases in rate of weight gain or overall feed efficiency compared with an adequately formulated diet containing a conventional crop/product. The benefits are more likely to be an improvement in the efficiency of protein utilization (and therefore a decrease in N excretion) and a reduction in diet cost to achieve the same performance. Unlike the situation with improved agronomic traits, simple substitution of the nutritionally improved crop for a near-isogenic control crop in a standard diet formulation is not recommended because no benefit is likely to be observed. Diets should be formulated on an amino acid basis (total, digestible, available, or metabolizable) to take advantage of the nutritional improvement instead. One approach is to evaluate the response to three diets: 1) a diet formulated with the nutritionally improved crop/product that just meets or is slightly below the requirement for the first limiting amino acid (note: it is crucial that this diet does not exceed the requirement); 2) a diet in which a near-isogenic control crop/product is substituted on a weight basis (as fed or dry matter) for the nutritionally improved crop; and 3) a diet similar to diet 2 but with supplemental protein or crystalline amino acids so that the content of the limiting amino acid is the same as diet 1. In diet 3, care needs to be exercised to ensure that other amino acids do not become limiting. One way to address this issue is to take amino acid ratios (the "ideal protein" concept) into account, at least for the four or five amino acids most likely to become limiting. Additional diets could also be considered, but all experiments should contain at least these three treatments. It is also crucial that all three diets meet the requirements for all other nutrients.

Animal performance (feed intake, weight gain, feed efficiency, milk production, egg production, etc.): The experimental design will be similar to those for crops with improved agronomic traits (e.g., the importance of adequate replication, the identification of "pen" as the experimental unit [keeping in mind that a "pen" might be a pasture, an aquarium, or similar unit]). In some cases, differences among treatments, although important, are likely to be small. Therefore, calculation of the number of replications needed and proper statistical design is crucial. It is frequently preferable to have fewer treatments and more replications than vice versa. In addition to the standard measures, it would be desirable to assess the efficiency of protein utilization. The classic estimate of "Protein Efficiency Ratio," although a less than ideal measure, could be easily calculated in most experiments. In experiments with dairy cows, the index of milk protein efficiency (milk protein:diet protein ratio) may also be calculated.

Digestibility/bioavailability: Changes (either increases or decreases) in digestibility/bioavailability of proteins and amino acids are possible. In many cases, significant changes in bioavailability are unlikely and experiments would contribute little. Therefore, studies to determine bioavailability should be considered on a case-by-case basis. Knowledge about bioavailability enables more precise diet formulation and enables maximum advantage to be obtained from the nutritionally improved product (in theory). These types of studies may depend on the species. In swine, ileal digestibility of amino acids would be the usual measurement; whereas in poultry, growth assays of bioavailability with young broilers, digestibility studies with cecetomized cockerals, or measurements of ileal digestibility are the most common. Digestibility and utilization studies

enable calculation of N excretion and the potential for environmental pollution.

Rumen degradability: For ruminant animals, the rate of protein hydrolysis and amino acid catabolism in the rumen is an important component of protein quality. Proteins that are degraded (degraded intake protein) contribute to the rumen ammonia pool and can supply nitrogen for microbial protein synthesis, but do not contribute directly to the amino acid supply for the host animal. Proteins that are resistant to ruminal degradation (undegraded intake protein) contribute directly to the host and can be used to modify the amino acid pattern available for absorption. The proteins must be digested, and the amino acids absorbed, in the small intestine. A variety of methods (*in vitro*/ continuous culture, *in situ*/*in sacco*, and *in vivo*) have been developed to measure rumen degradability of proteins. One or more of these methods will need to be used to verify that intended modifications to protein degradability (or solubility) have been accomplished.

Product Quality

In general, improvements in protein quantity and quality are not expected to have major effects on the quality of products derived from animals. For example, feeding diets with a better amino acid pattern is unlikely to increase the protein content or improve the amino acids pattern of milk from dairy cows (although it could reduce milk urea N), or to have any substantial effects on the proportion of fat and lean in pigs or poultry. Therefore, the emphasis of experiments designed to assess product quality will be to measure whether there are any unexpected/unintended effects that either increase or decrease product quality.

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Figure 4-1. Initial steps

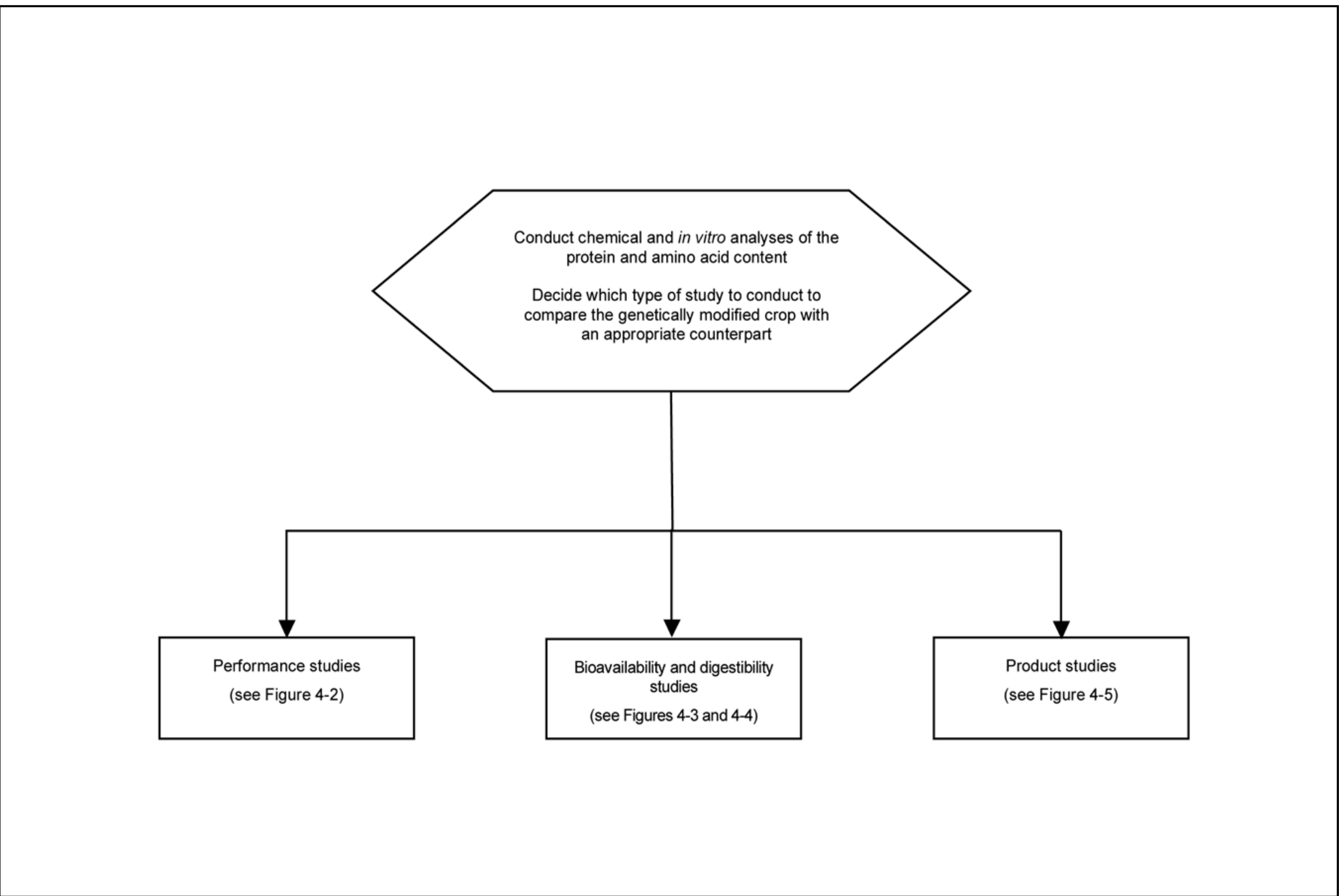


Figure 4-2. Performance studies

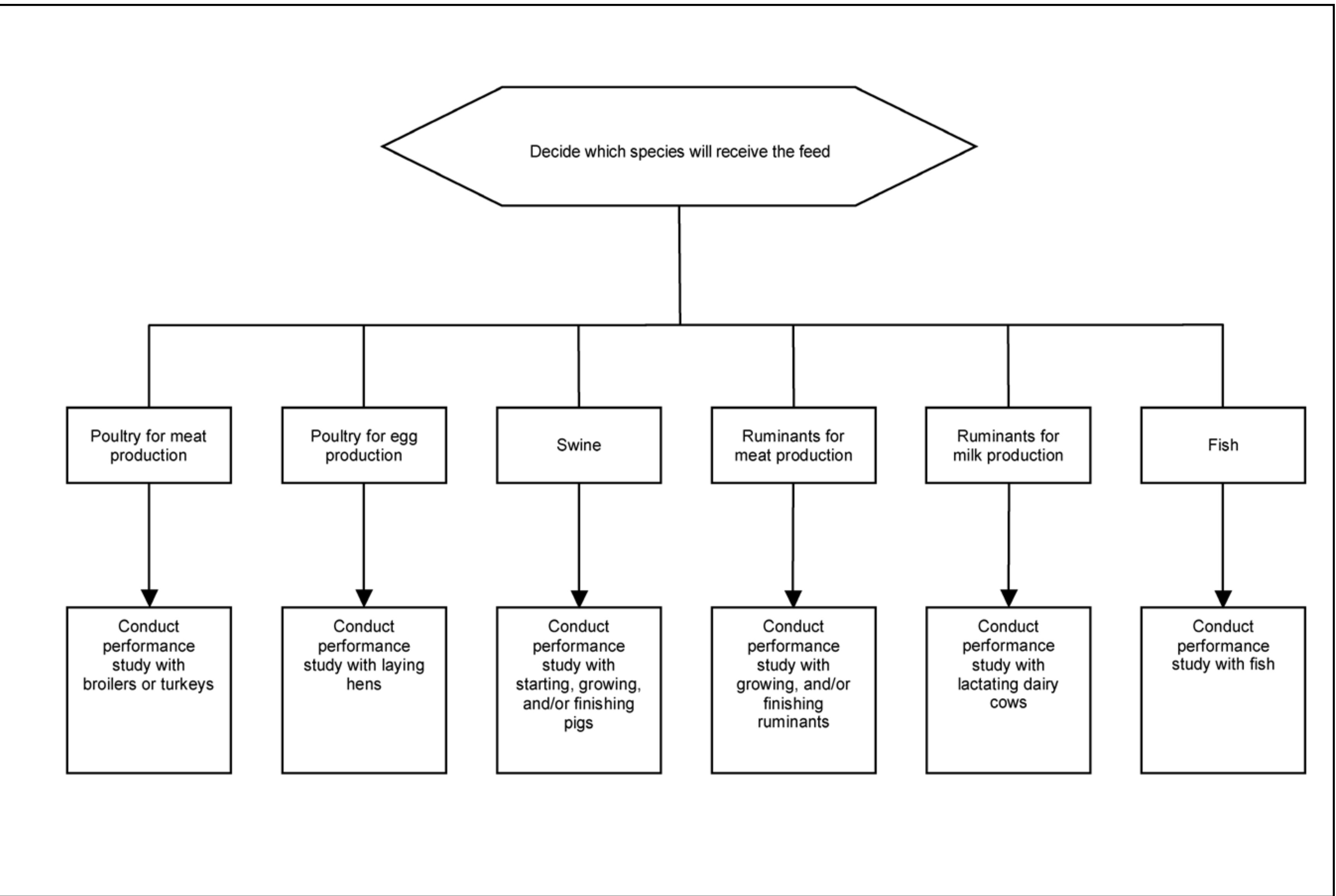


Figure 4-3. Bioavailability and digestibility studies

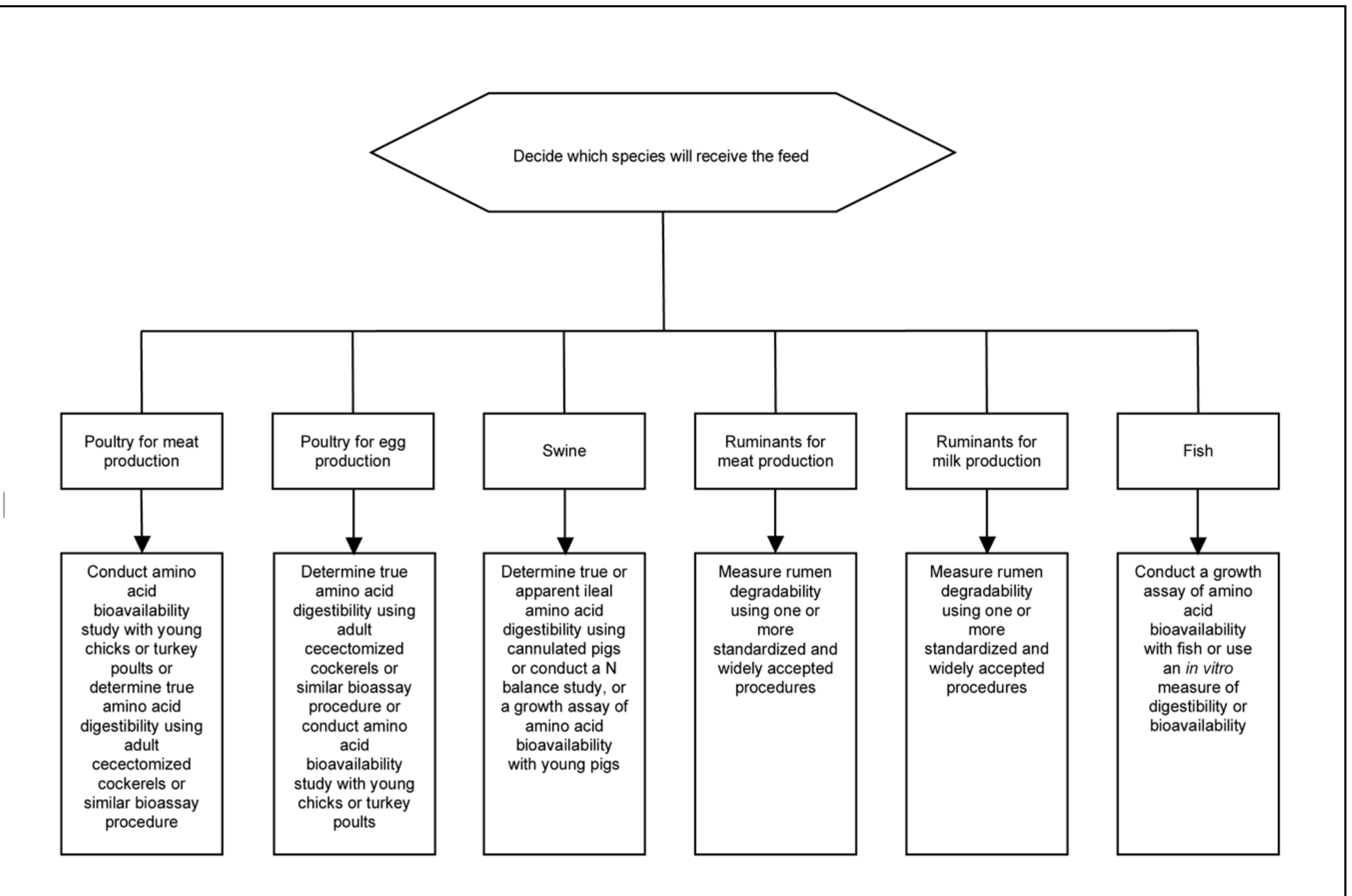


Figure 4-4. Digestibility and metabolism studies: apparent and true metabolizable energy

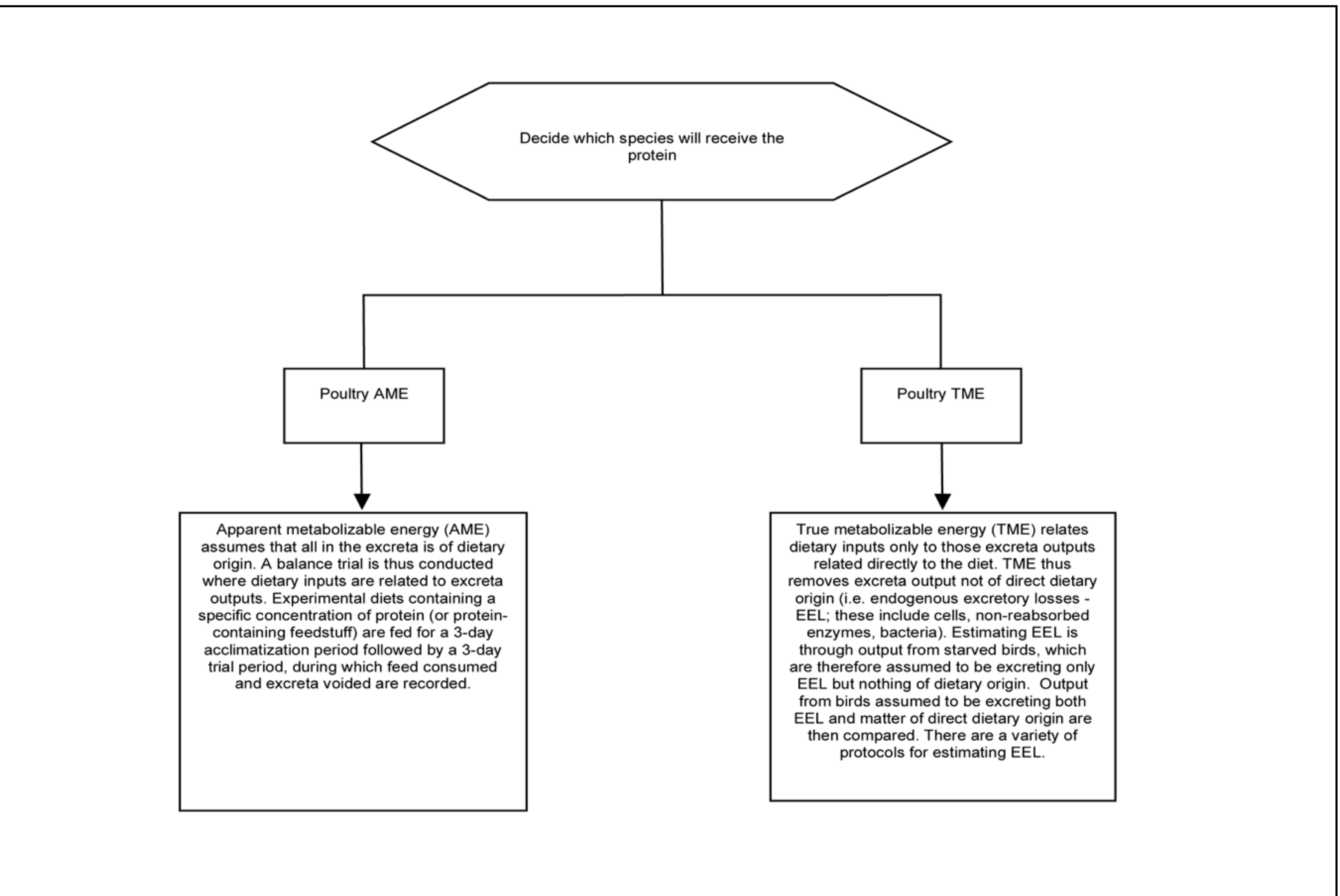
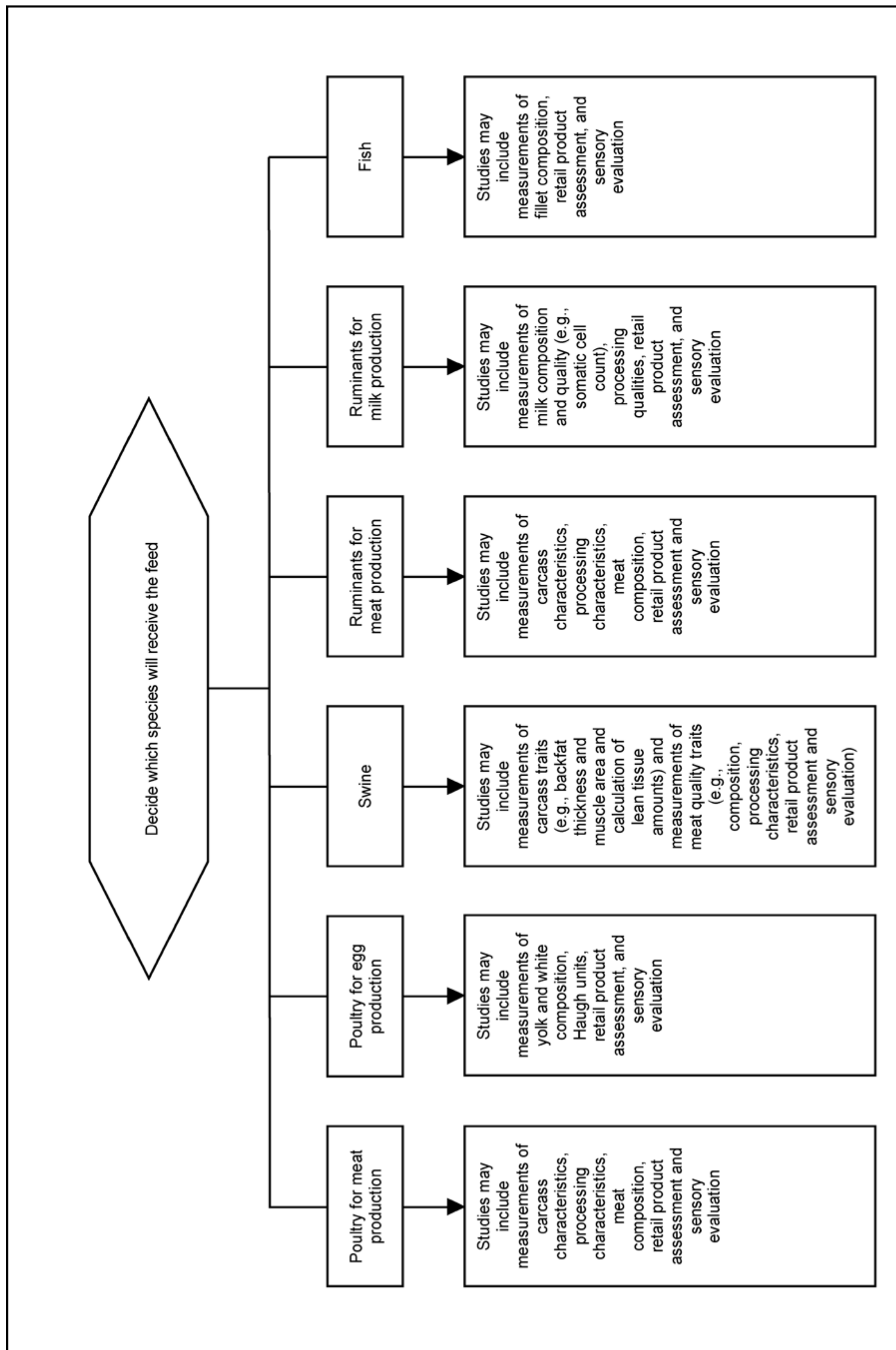


Figure 4-5. Product studies



Chapter 5

Carbohydrates

Carbohydrates in Animal Nutrition

Carbohydrates constitute the primary component found in livestock, poultry, and fish feed. Carbohydrates can be considered as a renewable resource because after they are consumed, they are converted into carbon dioxide, which plants use to form carbohydrates again through the process of photosynthesis. Carbohydrates comprise up to 70% of the dry matter in forages and as much as 80% of the dry matter in grains.

The primary role of carbohydrates in livestock, poultry, and fish is to serve as a source of energy. The more complex carbohydrates are digested and absorbed as simple sugars. These absorbed molecules enter various metabolic pathways that ultimately produce energy, much of which is trapped in the form of high-energy phosphate bonds such as adenosine triphosphate (ATP). In ruminant animals, most of the complex carbohydrates undergo anaerobic fermentation and produce volatile fatty acids (VFA), which in turn enter metabolic pathways that generate ATP.

From a chemical standpoint, carbohydrates are composed of carbon, hydrogen, and oxygen. The simplest form of a carbohydrate is classified as a monosaccharide (or simple sugar). The predominant monosaccharides in feeds contain either five (pentose) or six (hexose) carbons. Two linked monosaccharides are called disaccharides, three to nine monosaccharides linked together are called oligosaccharides, and longer chains are referred to as polysaccharides.

Glucose, fructose, and galactose are the most common monosaccharides, whereas sucrose, lactose, and maltose are the most common disaccharides. Glucose and fructose occur in both plant and animal tissues. Sucrose, a combination of glucose and fructose, is found in plants such as sugar cane and sugar beets. Lactose, which consists of glucose and galactose, is found only in milk. Maltose, consisting of two glucose units, is an intermediate breakdown product of starch.

Polysaccharides are generally referred to as starch and non-starch polysaccharides. These more complex carbohydrates are widely distributed in plants. Starch, in the form of amylose, consists of as many as 500 to 600 glucose units joined by chemical bonds between carbon no. 1 and carbon no. 4 with an α linkage (i.e., 1-4 α-linkage) with a limited amount of branching resulting from additional 1-6 α-linkages. Amylopectin, another type of starch, has similar linkages but with a greater amount of branching. Starch is concentrated in the cells of cereal grains, tubers, and other roots of plants. Typical maize starch is composed of 25% amylose and 75% amylopectin. Animals can easily digest both types of starch because the α-linkage between the glucose units is readily hydrolyzed by α-amylase enzyme, which is secreted by the pancreas gland into the digestive tract.

Non-starch polysaccharides are found in greatest concentrations in forages. Cellulose, hemicelluloses, and pectins are the most abundant non-starch polysaccharides in the cell walls of plants, representing up to 90% of the total (Selvendran and Robertson 1990). Fructans, glucomannans, and galactomannans are a smaller group of non-starch polysaccharides that serve as storage polysaccharides within the plant. Mucilages, β-glucans, and gums represent another group of non-starch polysaccharides in plants. Barley grain tends to have rather high concentrations of β-glucans.

The physiological impact of individual non-starch polysaccharides depends on the sugar units present and the type of linkages between the sugars. Cellulose, which occurs in tightly bound aggregates (microfibrils) in plants, consists of linear polymers of glucose linked between carbon atoms no. 1 and 4. However, the glucose units in cellulose are joined by a 1-4 β-linkage, which is resistant to enzymatic digestion in animals' gastrointestinal tract. β-glucans, like cellulose, are linear 1-4 β-linked glucose polymers. Mixed linkage β-glucans contain 1-3 β-linkages interspersed with 1-4 β-linkages. Only microorganisms have the enzymes to break the β-linkage. Thus, digestion of these non-starch polysaccharides occurs primarily in the rumen and lower gut of animals where microorganisms are abundant. The end products of microbial fermentation are the VFA (primarily acetic, propionic, and butyric acids). These VFA are utilized for energy in both ruminants and nonruminants.

Hemicelluloses are polymers of arabinose, xylose, glucose, fucose, mannose, and galactose. They contain a monosaccharide backbone of xylan, galactan, or mannan, and side chains of arabinose or galactose. Pectins are polymers of galacturonic acid containing side chains of other sugars such as glucose, galactose, and rhamnose (Butler and Bailey 1973, Aman and Graham 1990). Pectins also contain methoxy groups ($-OCH_3$) that are metabolized almost completely to methane gas by the gastrointestinal tract microflora.

The oligosaccharides generally contain only three

to nine monosaccharide units. They may have similar or different sugar units, various linkage structures, and may be in a linear or branched form. Like the non-starch polysaccharides, oligosaccharides are not hydrolyzed by mammalian enzymes, but are fermented by microorganisms in the gastrointestinal tract. Fructo-oligosaccharides are a mixture of fructose units linked by β-2-1-glucosidic bonds. These are preferentially used by bifidobacteria in the large intestine. The α-galacto-oligosaccharides consist of one, two, or three units of galactose linked by α-1-3-bonds and bound to a terminal sucrose. These α-galacto-oligosaccharides—raffinose (a trisaccharide), stachyose (a tetrasaccharide), and verbascose (a pentasaccharide)—are of particular concern in swine nutrition because they are poorly utilized and result in digestive disturbances and depressed growth in young pigs (Cromwell 2000). Soybeans and soybean meal contain high concentrations of these α-galacto-oligosaccharides. Poor utilization of the energy from soybean meal in poultry has been partially attributed to the presence of oligosaccharides (Leske et al. 1995).

There is quite a bit of interest in the mannan oligosaccharides, which are found in the cell walls of yeast. These oligosaccharides, when added as a feed supplement, are thought to be of benefit in that they saturate the binding sites on the pilli of potential pathogenic bacteria and prevent them from attaching to the intestinal wall and colonizing the lower gut (Newman 1994). The mannan oligosaccharides have been shown to improve pig performance in some cases (Pettigrew 2000); however, they have been ineffective in some other studies, especially when compared with dietary supplementation of antibiotics (Cromwell 1991).

Lignin is not a polysaccharide, but rather a high molecular-weight, heterogeneous polymer composed of phenylpropane residues formed by condensation of the aromatic alcohols, cinnamyl, guaiacyl, and syringyl alcohols (Southgate 1993). The lignin content of swine and poultry feed is typically low because cereal grain and oilseed meals usually have very little lignin (Dreher 1999), but the lignin content of mature forages can be quite high. Lignin not only is indigestible by animals, but its presence can interfere with the digestibility of other carbohydrates. These compounds can become intimately associated with cell wall carbohydrates rendering them recalcitrant to degradation (Hartley and Jones 1977).

Carbohydrates were originally quantified as crude fiber and nitrogen-free extract (i.e., sugars and starches) based on the Weende system of proximate analysis of feeds (AOAC 1984). In that system, crude fiber was a variable mixture of cellulose, hemicelluloses, some pectins and lignin, plus indigestible protein and lipids (Mertens 1992). Newer methods have been developed that more accurately delineate the form of the fiber component of feeds so that they describe the cellulose, hemicelluloses, and lignin fractions. The detergent methods of fiber analysis (Van Soest and McQueen 1973) partition fiber into neutral detergent fiber (NDF), which includes cellulose, hemicelluloses, and lignin, and acid detergent fiber (ADF), which includes cellulose and lignin. The difference between the NDF and ADF fractions is an estimate of the hemicelluloses in a feed. Although the detergent system is considerably better than the crude fiber estimation, both underestimate the amount of total fiber due to their inability to recover soluble fiber components such as the pectins, mucilages, gums, and β-glucans. Other methods have been developed to measure the total dietary fiber (TDF) in feeds, but these methods are more complex and time consuming (Theander and Aman 1982, Asp et al. 1983, Jeraci et al. 1989, Prosky et al. 1992, Lee et al. 1996). For additional information on fiber analysis, refer to Van Soest (1994) and Mertens (1997, 2002).

Grieshop et al. (2001) give an excellent review on non-starch polysaccharides and oligosaccharides.

Intended Compositional Changes

Either traditional plant breeding techniques (selection, crossbreeding, backcrossing, and propagation) or modern biotechnology methods can achieve changes in the amount or digestibility of various carbohydrates in feedstuffs. It generally makes little difference which method is used to achieve the improvement.

One of the compositional changes that can occur in plants is the type of carbohydrate. Reducing the amounts of carbohydrate fractions that have poor digestibility and replacing them with carbohydrates having higher digestibility should improve animal performance, improve efficiency of feed utilization, and reduce manure excretion. A potential reduction in manure dry matter excretion has major environmental impact.

An example of an altered carbohydrate that occurred using conventional breeding is waxy starch in maize and grain sorghum. This starch variant in maize was found in China in 1908 but it was not until 1936 that hybrids were developed in the US (Hartnell et al. 2005). Waxy endosperm maize contains 100% amylopectin starch rather than the normal ratio of 75% amylopectin and 25% amylose found in normal dent maize. Amylopectin is highly digestible in the rumen (Mohd and Wootton 1984), and waxy maize fed as silage and grain to lactating cows has 6% more apparently digestible starch (Akay and Jackson 2001). Some studies have shown that milk production in cows is improved with the feeding of waxy maize (Moreira et al. 2000, Akay and Jackson 2001), while other studies have not shown milk production

improvement (Schroeder et al. 1998). Responses in pigs to grains with waxy starch vs. conventional grains have been inconsistent (Camp et al. 2003).

The brown midrib trait was first reported in dent maize in 1924 (Hartnell et al. 2005). This trait is associated with a marked reduction in lignin in the maize leaf. Four brown midrib mutants have since been identified. The natural mutation that occurred in brown midrib maize caused a "knock-out" of one of the lignin biosynthesis enzymes, 0-methyltransferase, resulting in a 40% decrease in the lignin content of the maize plant (Barriere and Argillier 1993).

Other genetically enhanced forages with reduced lignin or with cellulose and hemicellulose that are more easily degraded are on the horizon (Hartnell 2000, 2004; Hartnell et al. 2005). Furthermore, lignase, cellulase, and hemicellulase enzymes have been bioengineered into plants to enhance utilizing these energy sources, thereby reducing manure output. Development of low-oligosaccharide soybeans (Anderson 1998) also offers potential for reducing energy digestibility in pigs and poultry, thereby potentially improving utilization of carbohydrates and reducing manure output.

Undesirable Effects

Any method inducing genetic changes, both traditional and transgenic, can potentially produce undesirable effects. Some undesirable changes are predictable, whereas others are unpredictable.

Plants and feedstuffs with improved traits often have undesirable agronomic characteristics. One of the most frequently seen undesirable effects is a yield reduction (or yield drag) when a modified trait is incorporated into a plant. There is also the possibility of a reduced germination rate in the planted seeds.

Evaluation Methods

The purpose of the evaluation methods is to establish whether the genetically modified feedstuff is of added benefit to a conventional feedstuff.

Deciding What Experiments Are Needed

A systematic, science-based process should be used to determine what experiments (if any) are needed. The need for and types of experiments will depend on the changes in the type, amount, or digestibility of the carbohydrates that have been made in the grain, oilseed, or forage. A systematic approach to deciding what experiments to conduct is provided in Figures 5-1 to 5-5 (see pages 62–66).

Animal experiments

Diet formulation: Appropriate diet formulation is crucial to assess an improvement in digestibility of carbohydrates. Simply substituting the nutritionally improved crop for a near-isogenic conventional crop in a standard diet formulation that is adequate in energy is not recommended because no benefit is likely to be observed. Instead, diets that are slightly deficient in energy should be formulated or animals should be limit fed so as to take advantage of the potential improvement in available energy to the animal.

To assess a potential improvement in an altered feedstuff, diets must be formulated such that they are adequate in all nutrients except for the carbohydrate source under investigation. The basic approach is to formulate diets that are marginal in energy for the animals so that the improvement in available energy from the genetically engineered forage or grain can be measured.

Animal performance (feed intake, weight gain, feed efficiency, milk production, egg production, etc): The experimental design will be similar to those for crops with improved agronomic traits (e.g., the importance of adequate replication, the identification of "pen" as the experimental unit [keeping in mind that a "pen" might be a pasture, an aquarium, or similar unit]). In some cases, differences among treatments, although important, are likely to be small. Therefore calculation of the number of replications needed and proper statistical design are crucial. It is frequently preferable to have fewer treatments and more replications than vice versa.

Digestibility: Digestibility experiments can often determine changes (either increases or decreases) in digestibility of carbohydrate fractions or of the total diet dry matter that are not detectable in performance experiments. In swine, ileal digestibility or total tract digestibility of the carbohydrate fraction or of diet dry matter would be usual measurements. In poultry, measurements of total tract digestibility would be the most common. In ruminants, rumen digestibility and total tract digestibility are a useful measure.

Product Quality

In nonruminants, improvements in carbohydrate or energy digestibility should not have a major effect on the quality of products derived from animals as long as the energy-to-protein (or energy-to-amino acid) ratios are kept in balance. In lactating ruminants, changes in carbohydrates may alter ruminal volatile fatty acids, microbial protein production, milk fat, protein, and yield.

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Figure 5-1. Initial steps

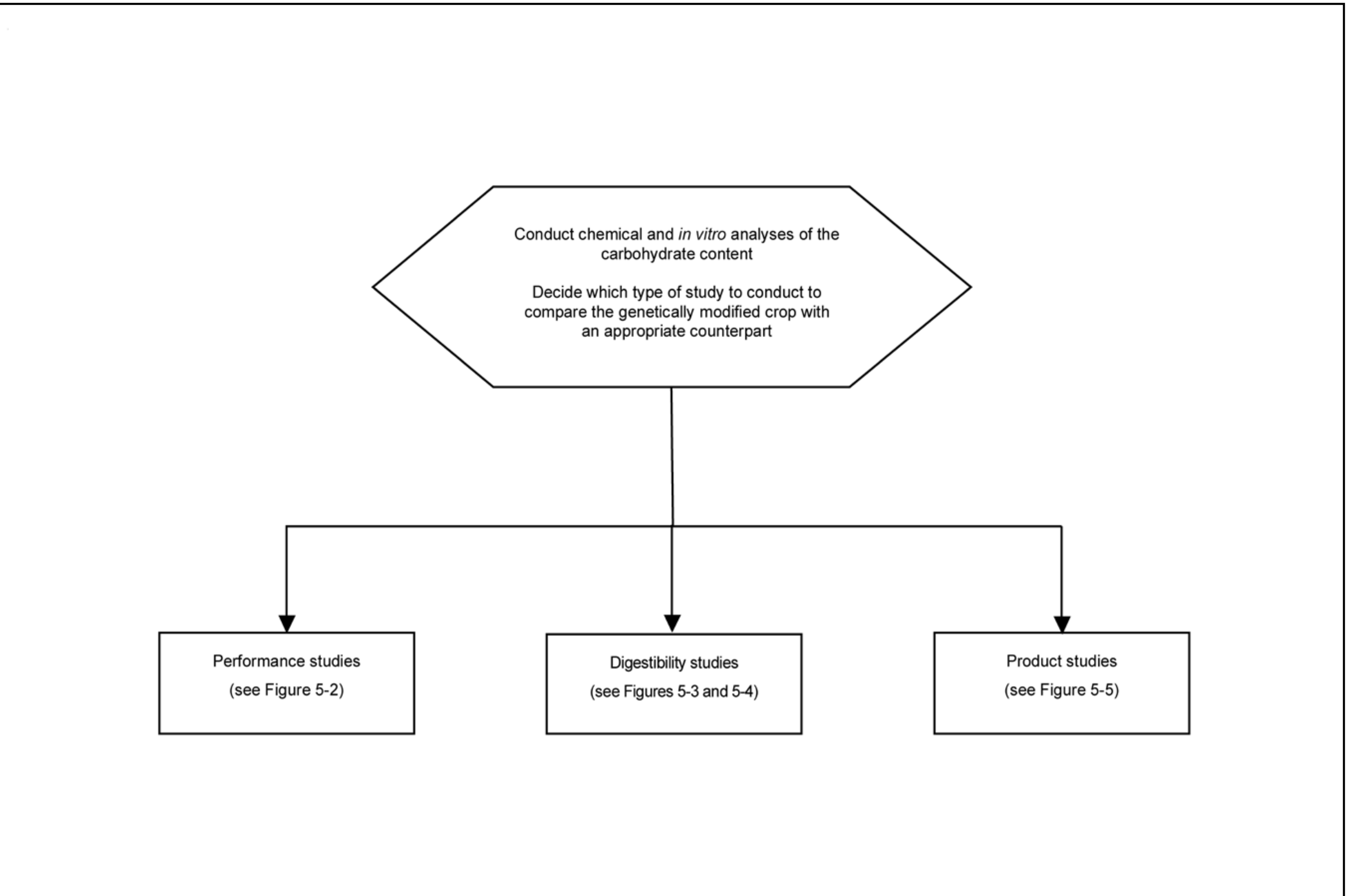


Figure 5-2. Performance studies

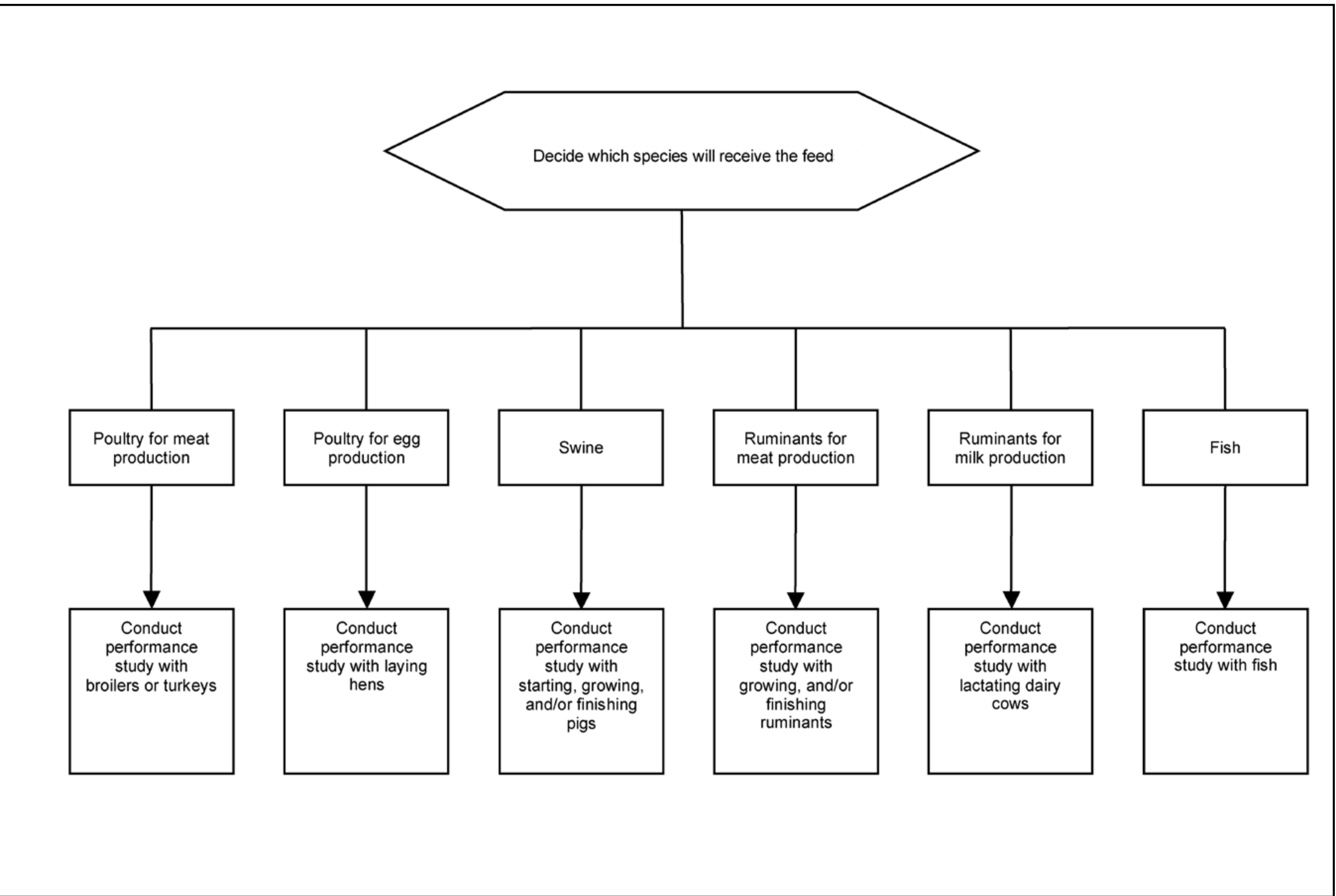


Figure 5-3. Digestibility studies

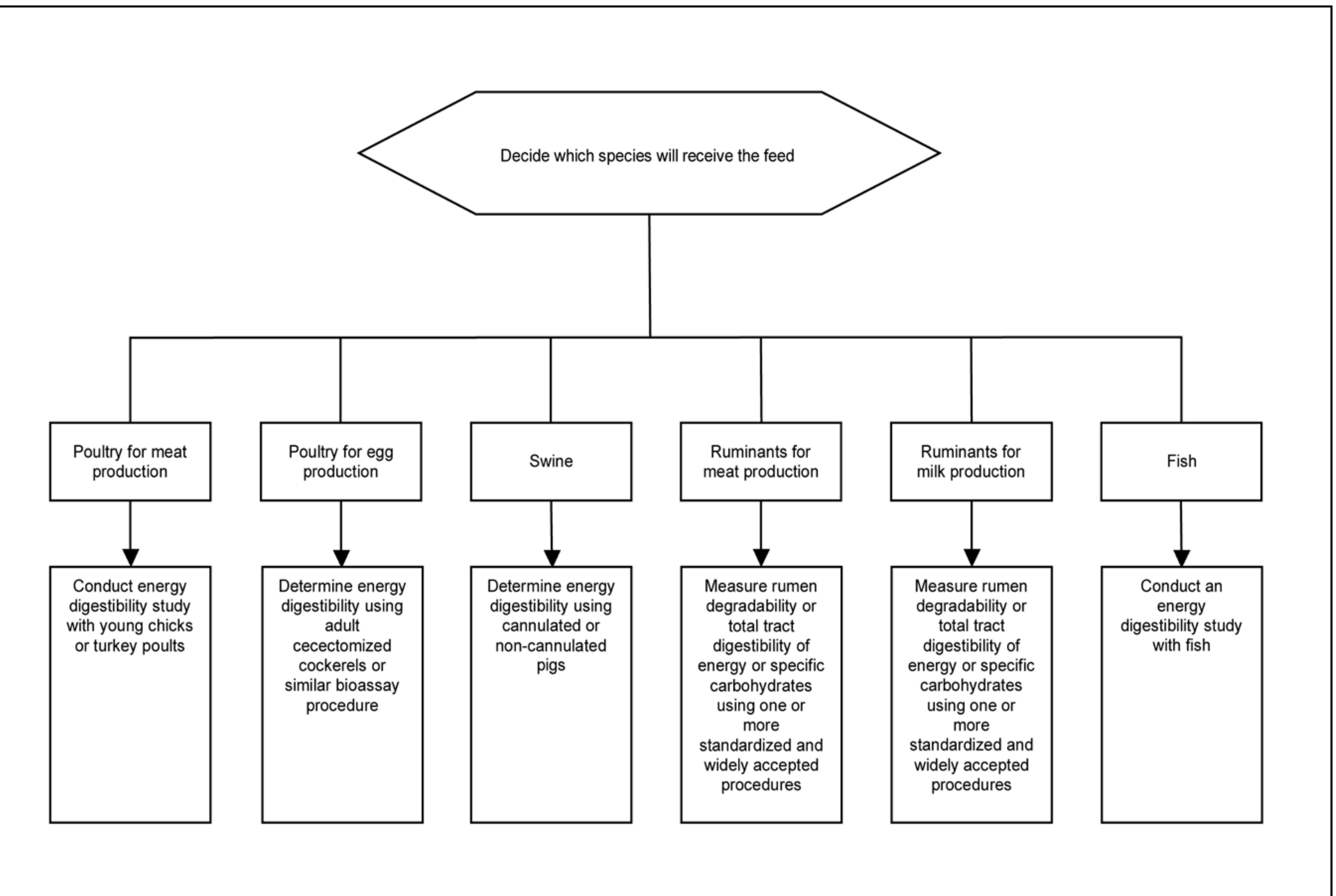


Figure 5-4. Digestibility and metabolism studies: apparent and true metabolizable energy

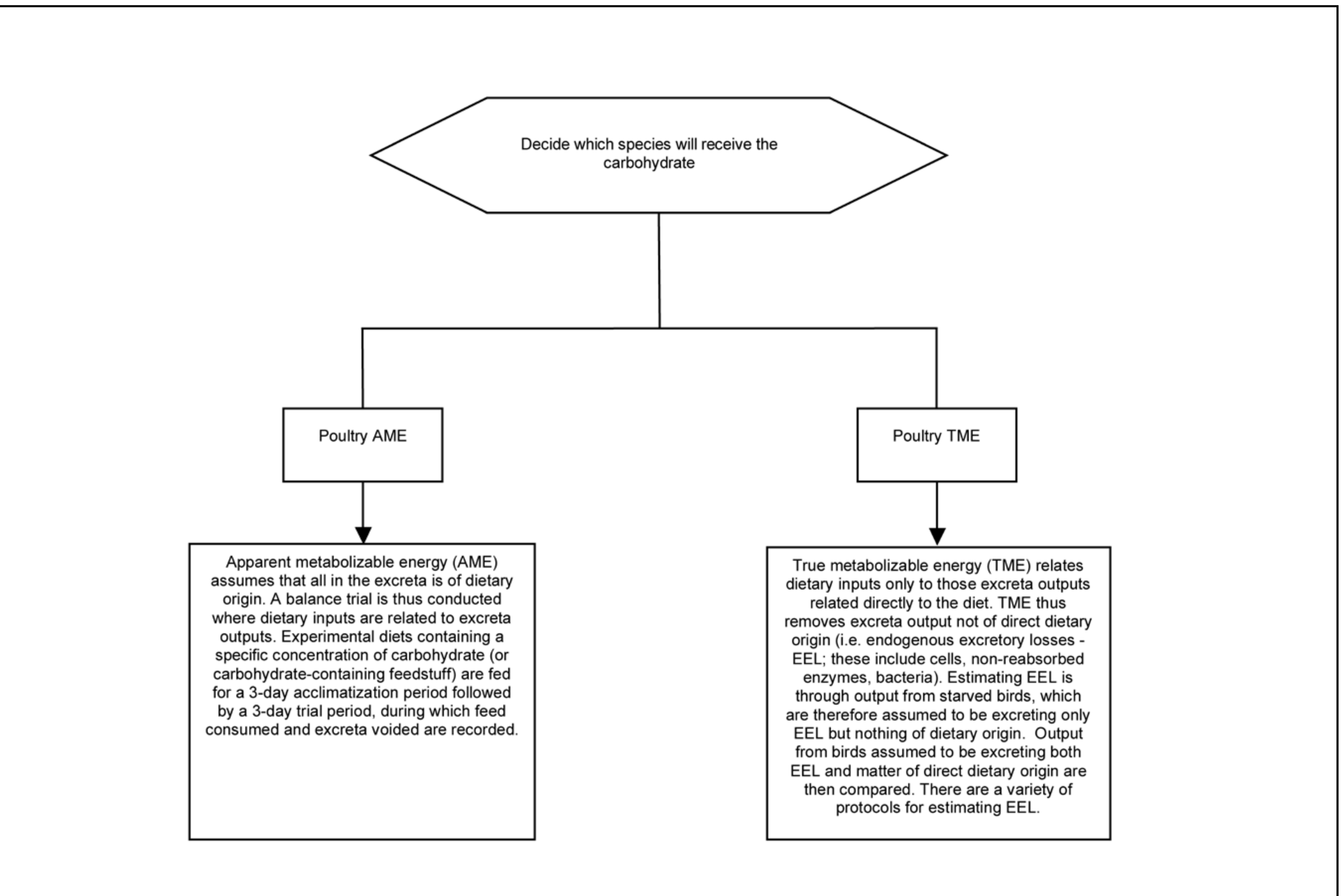
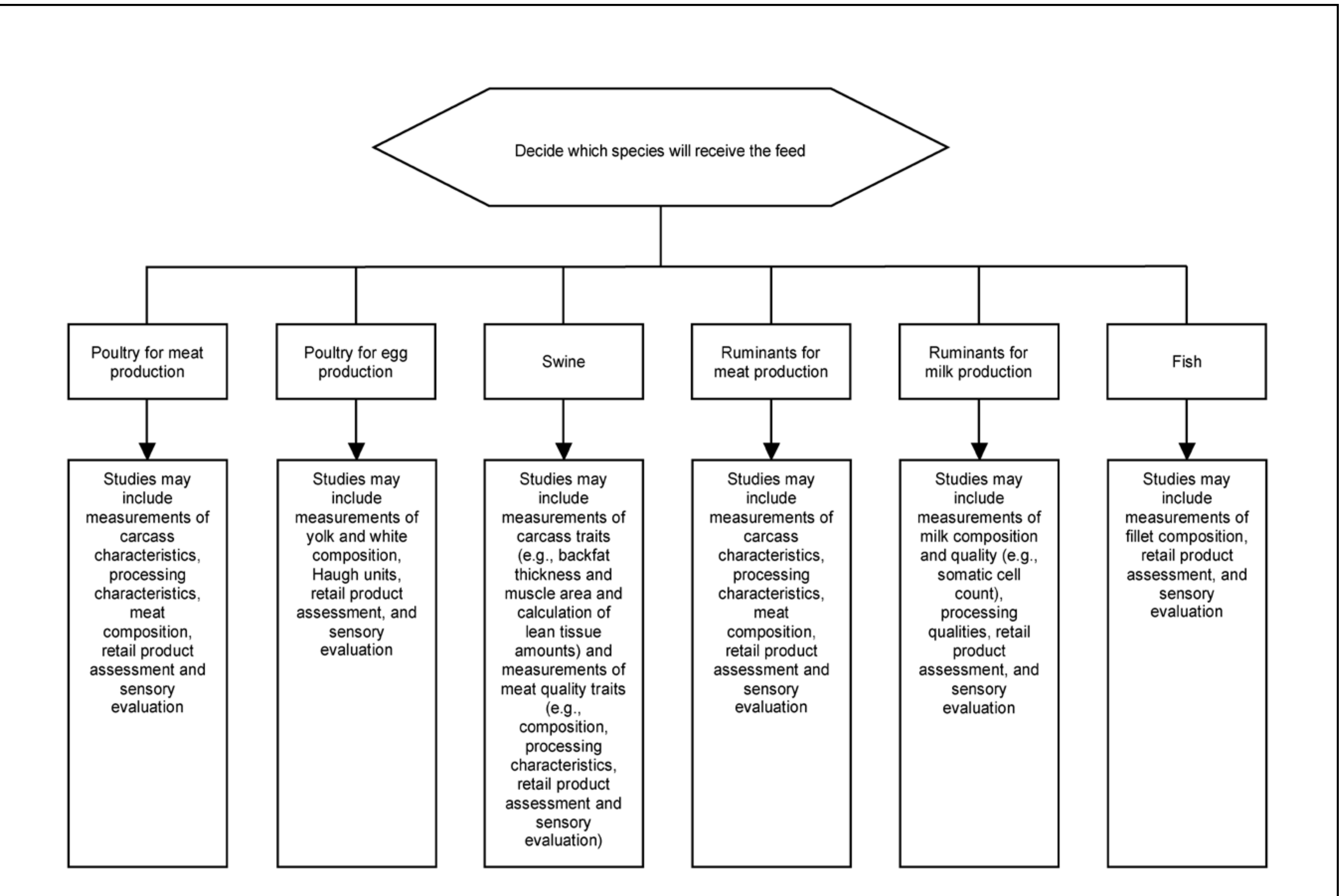


Figure 5-5. Product studies



Chapter 6

Lipids and Fatty Acids

Lipids in Animal Nutrition

Lipids destined for animal diets can come from several sources. First, there are small amounts of lipids in cereal grains. There is also residual oil within oil-extracted meals, typically between 1 and 2%. There is little differential extraction of individual fatty acids, so the fatty acid profile of oil within these meals will be similar to the oil within the original seed. In some countries, processed full-oil seeds (i.e., with no oil extraction) are often added to animal diets; an example is full-fat soybean meal with an oil content of approximately 20%. The extracted oil itself may also be made available for animal diets. Extracted oils undergo a number of processes during refining and the residues from these processes may also be used for animal diets.

The measurement of lipid content of oilseeds (either within the oil-containing seed or the residual oilseed meal after oil extraction) has been the subject of much debate (e.g., Sanderson 1986). Conventional analyses are based on petroleum ether extraction or the same extraction, preceded by hydrolysis in hydrochloric acid. The latter method extracts oil that is ‘bound’ and therefore would not be detected without acid hydrolysis.

Lipids, in the context of this chapter, are essentially glycerol esters of fatty acids that occur widely in nature (e.g., oilseed crops) to give triacylglycerols (neutral lipids). However, a small concentration of free fatty acids can sometimes be found, and these free fatty acids are often associated with an unstable oil that is undergoing oxidation.

Free fatty acids are straight-chain compounds with an even number of carbon atoms with a terminal carboxyl and methyl group; those of most relevance to animal nutrition are capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), and stearic (C18:0) and all are saturated. Unsaturated fatty acids include methylene-interrupted cis double bonds at specific positions within the carbon chain. Monoenoic or monounsaturated fatty acids (MUFA; with one double bond) include palmitoleic (C16:1; 16 atoms of carbon with one double bond) and oleic (C18:1); both are n-9 fatty acids with the double bond 9 carbon atoms from the terminal methyl group. The major dienoic fatty acid is α-linoleic (C18:2, n-6) and the most important trienoic fatty acid is α-linolenic acid (C18:3, n-3). Longer chain polyunsaturated fatty acids (PUFA) and those including “trans” double bonds are discussed under essential fatty acids and conjugated linoleic acids, respectively.

There are additional fatty acids that, although present in minor amounts, may be of nutritional significance. Two examples are cyclopropenoid fatty acids (sterculic [9,10-methylene octadec-9-enoic acid] and malvalic [8,9-methylene heptadec-8-enoic acid]; present at 0.3–0.5% and 0.7–1.5%, respectively, in cottonseed oil), which may result in egg whites becoming pink (Shenstone et al. 1965, Jeffcoat and Pollard 1977) and erucic acid (C22:1, n-13), which may give rise to cardiac lipidosis (Kramer et al. 1979) and activate certain enzymes (e.g., δ-9 desaturase).

Oilseeds, which are a major source of fatty acids (although high oil maize is also important), invariably contain a specific range of fatty acids that may characterize them. Common sources and major fatty acid profiles are presented in Table 6-1.

Lipids have two main functions in animal nutrition. Their high energy-yielding potential means that they are important components of diets fed to high-yielding livestock. They are also sources of essential fatty acids, which have a wide range of metabolic functions (e.g., precursors of mediators of cell function, such as prostaglandins, and components of phospholipids, contributing to the functionality and fluidity of cell membranes). Lipids may also be sources of fat-soluble vitamins on occasion, although it is uncommon to assume this in diet formulation.

A secondary function of lipids is to modify product quality. There is considerable interest in altering the fatty acid composition of meat, milk, and eggs through dietary routes.

Digestibility of Plant Lipids

How animals digest plant lipids is a crucial aspect of their nutritional value. This section will outline the major principles governing digestibility. Doreau and Chilliard (1997) published a review on the digestion and metabolism of dietary fat in farm animals.

Nonruminants

There have been numerous reviews on digestibility of fats and oils by nonruminants (e.g., Freeman 1984, Krogdahl 1985, Gu and Li 2003). The major site of fat digestion in pigs and poultry is the duodenum

Table 6-1. Fatty acid profile of major plant oils*

Oil	Fatty Acid %					
	16:0	18:0	18:1	α-18:2	α-18:3	Other
Coconut[a]	8	3	6	2	0	81
Maize	13		31	52		4
Cotton	24		19	53		4
Peanut	13		37	41		9
Olive	10		78	7		5
Palm	44		40	10		6
Palm kernel[b]	8	2	13	2	0	75
Rape	4		56	26	10	4
Rape: high erucic acid	3	1	16	14	10	56
Soybean	11		22	53	8	6
Sunflower	6	6	18	69		1
Sunflower: high oleic	6	2	74	16	2	
Sunflower: high linoleic	6	5	20	68	1	
Linseed	6		17	14	60	3

*Data from Gunstone et al. (1994), except [a]coconut oil, from Table 11-10 p. 141 of 1998 Swine NRC and [b]palm kernel oil from http://www.jarvm.com/articles/Vol1Iss1/BAVELJVM.htm.

and digestion consists of emulsifying dietary fat by conjugated bile salts, followed by hydrolysis of triglycerides by pancreatic lipase into mixtures consisting essentially of 2-monoacylglycerides and free fatty acids (FFA). The subsequent absorbability of these products depends on their solubility in bile salt micelles. Polar solutes are more readily incorporated into micelles; this explains the relatively higher absorbabilities of unsaturated fatty acids compared to saturated fatty acids and the well-established observation that unsaturated fatty acids have a higher digestibility than those that are saturated. Accordingly, unsaturated oils have a higher dietary energy value than more saturated ones (e.g., soybean oil has a higher dietary energy value than palm oil); this also explains why hydrogenation of oils (even partial) is associated with a reduction in dietary energy value.

The relative superiority of an intact triglyceride compared to hydrolyzed fat in terms of dietary energy value is also well known. Increasing the proportion of FFA (one of the residues remaining when oils are refined) will reduce the value.

Aquaculture

The use of lipids in fish feeds has been reviewed (NRC 1993, Webster and Lim 2002). Lipids are incorporated into feeds not only to meet energy and essential fatty acid requirements, but also to increase feed palatability and affect flavor of the fish (Robinson et al. 2001). As with nonruminants, saturated fats are less digestible than unsaturated fats (NRC 1993). For this reason, sources of unsaturated fats, such as vegetable and marine oils, are utilized in diets for aquaculture. Natural food organisms, such as zooplankton, may also be used as a source of essential fatty acids. Fish vary in their response to lipid concentration in the diet. Warmwater fish such as catfish are generally fed diets not exceeding 15% or more lipid, or weight gain and feed efficiency may be depressed; however, coldwater fish, such as salmon, require high levels of fat (Robinson et al. 2001, Storebakken 2002).

Fish cannot synthesize either 18:2 (n-6) or 18:3 (n-3) *de novo* (NRC 1993). Therefore, fish may require dietary sources of one or both of these fatty acids depending on their essential fatty acid requirement. Fish also vary in their ability to elongate 18 carbon unsaturated fatty acids to longer chain, more highly unsaturated fatty acids. Generally, stenohaline marine fish (those that can tolerate only a small variation in water salinity) require dietary eicosapentaenoic [EPA; 20:5(n-3)] and/or docosahexanenoic acid [DHA; 22:6(n-3)]; whereas freshwater fish require either dietary linoleic acid, 18:2 (n-6), or linolenic acid, 18:3 (n-3), or both. NRC (1993) has provided essential fatty acid requirements for several freshwater and marine fish species.

Ruminants

Bauman et al. (2003) provided an overview of lipid digestion and metabolism in the ruminant with special consideration of recent developments. Digestion of fats and oils by ruminants is complicated by the rumen's presence. Fats and oils can interfere with microbial digestion in the rumen through detergent effects on microbial cell walls and by coating feed particles, rendering them inaccessible to microbial enzymes (Garnsworthy 1997). The presence of polyunsaturated fatty acids (PUFA), particularly in free oils, is inhibitory to rumen microorganisms. For this reason, rumen microorganisms attempt to saturate PUFA using isomerase and reductase enzymes in a process called biohydrogenation. Biohydrogenation has a major impact on the fatty acid profile of digesta leaving the rumen and the subsequent profile of ruminant meat or milk (as discussed in the section on product quality). Under normal circumstances, biohydrogenation increases the proportion of saturated fatty acids unless feed ingredients are processed to render them inert in the rumen (see section on processing).

Another feature of lipid digestion in ruminants is that triglycerides are almost completely hydrolyzed by bacterial enzymes in the rumen. Fatty acids presented for absorption in the small intestine are, therefore, in the form of FFA. Apart from this, digestion of fatty acids in the duodenum is similar to that in nonruminants. Bile salts and pancreatic secretions emulsify fatty acids to form micelles for absorption. Triglycerides are reformed in the gut wall postabsorption, which can put a considerable strain on glucose requirements (Garnsworthy 1997). Although their form and degree of saturation change in the rumen, the quantity of fatty acids leaving the rumen is normally similar to the quantity ingested. With low-fat diets, however, the quantity may increase because of microbial synthesis of lipids. Absorption of fatty acids is generally more efficient in ruminants than in nonruminants, and there is little effect of degree of unsaturation on fatty acid digestibility (Doreau and Ferlay 1994).

Intended Compositional Changes

There has been considerable interest in modifying the fatty acid composition of oilseed crops (Topfer et al. 1995, Thelen and Ohlrogge 2002, Burton et al. 2004). Interest has been shown in raising the concentration of oleic acid, although there have been investigations into increasing the levels of longer-chain polyunsaturated fatty acids of the n-6 and n-3 families, which are of considerable relevance to human nutrition (Abbadi et al. 2004, Sayanova and Napier 2004, Domergue et al. 2005) both directly and also indirectly through elevating levels in products for human consumption. Accordingly, it is important to consider protocols for evaluating the nutritional benefits of plant commodities containing these altered fatty acid profiles (and crops with increased total oil content). A systematic approach to deciding what experiments to conduct is provided in Figures 6-1 to 6-5 (see pages 75–79).

Data are also presented in Table 6-1 for oilseeds that have been modified to generate different fatty acid profiles from conventional ones (e.g., high-oleic and high-linoleic sunflower).

Undesirable Effects

A number of undesirable effects may occur with the improper feeding of lipids. For example, excessive dietary levels may result in reduced growth and poorer feed efficiency, poorer fiber digestion, reduced milk fat concentration, sub-optimal mineral levels, and poorer pellet quality; such excessive levels may require adjustment to prevent deficiencies. Certain undesirable effects may occur when using fat sources with a higher degree of unsaturation. For instance, softer pork bellies create processing problems in bacon production; off-flavors in meat, milk, and eggs; reduced shelf-life unless the product is properly stabilized; and increased chance of rancidity, resulting in a less palatable feed.

Evaluation Methods

Determination of the Dietary Energy Value of Plant Oils

Diet formulation requires accurate information on the dietary energy value of all constituent raw materials. This section will consider methodologies and protocols that are used to obtain such values.

Nonruminants: Dietary energy values are conventionally expressed as digestible or metabolizable energy (DE or ME; pigs) or apparent or true metabolizable energy (AME or TME; poultry). Although biological evaluation of oils using animals or birds is the conventional means of deriving values (and will be described later in this chapter), it is a time-consuming activity with significant resource implications. Accordingly, there has been much interest in predicting values from chemical analyses (Wiseman et al. 1998b, Wiseman 2001a, Wiseman 2004).

Prediction of the dietary energy value of oils: The section on digestibility of plant lipids described the two major structural determinants of an oil's digestibility, being the degree of saturation of constituent fatty acids and the FFA content (expressed as g/kg oil). The former

can be expressed in terms of the ratio of unsaturated to saturated fatty acids (U/S). Following a comprehensive series of metabolism trials, prediction equations were generated (Wiseman et al. 1998b).

There have been modifications to the equations in terms of the chain length of fatty acids. Thus "saturated" fatty acids consist only of C14:0, C16:0, and C18:0; those < C14:0 are considered as "unsaturated" fatty acids in the context of the prediction equations.

There are a number of components of oils that may reduce the dietary energy value and it is conventional to determine these. Routinely, assessments of moisture, impurities, and unsaponifiable (conventionally referred to as MIU) content are undertaken; because their collective dietary energy value is assumed to be zero, they act merely as diluents but must be accounted for in correcting the equations. Although it is unlikely that oils and oilseeds would have been heated excessively, this is not impossible, particularly if processing and/or refining coproducts are being considered. A further chemical test that is suggested is non-elutable material determined during GC (gas chromatography) analysis for fatty acids; the values derived from the equations should also be corrected for this component (Edmunds 1990).

If the novel crop contains a higher concentration of long-chain PUFA, then it is potentially unstable. Accordingly, an assessment of oxidative potential is required. The quality of products is measured by the amount of free fatty acid (acid value), primary oxidation products (peroxide value), secondary oxidation products (anisidine value), and its *Totox value*, which is an overall indicator of oxidation (Fritsch 1981).

The comments within this section are summarized in Figure 6-1, which presents a flow diagram for initial assessment of oils for nonruminants.

Determination of the dietary energy value of oils and digestibility of constituent fatty acids using poultry and pigs: It is unlikely that biological assessments of the DE or ME and AME or TME of oils will be necessary on a routine basis. Any changes to fatty acid profiles through plant breeding technologies can be assessed through GC and the prediction equations discussed above (section on prediction of the dietary energy value of oils) employed in assigning dietary energy values. However, should *in vivo* assays be considered necessary, then there are established protocols that have been designed to determine values. There has been much debate over the relative merits of true vs. apparent ME in birds (e.g., McNab 1990), although the closer to *ad libitum* intakes, the smaller the difference. If adult birds are used in TME assays for oils, then data generated may not be applicable to younger birds as a result of the well-established effect of age on oil digestibility. Protocols to measure TME for younger birds have been developed. Wiseman (1990) presented descriptions of the principles surrounding determination of DE, ME, AME, or TME.

It is possible that one may require measurements of the digestibility of individual fatty acids, particularly if one suspects that the fatty acids may be bound to other components. The hydrogenating environment of the large intestine of pigs and ceca of poultry means that reliance on fecal or excreta output is not valid. Collection of digesta from the terminal ileum is recommended. This can be achieved by inserting "T" piece or re-entrant cannulae in pigs. Such surgical intervention is more difficult in poultry and cecectomized birds are preferred or birds are humanely euthanized and ileal digesta are collected directly. Both interventions require the use of inert dietary markers.

Aquaculture: In fish, intake energy minus the energy lost in fecal excretions is termed digestible energy (DE). Metabolizable energy (ME) is the remaining energy from DE after substracting energy from gill and urine excretions. NRC (1993) provides an entire chapter on the methods for determining digestibility in fish and factors affecting digestibility. Furthermore, DE and ME values of selected feedstuffs are also provided. Since fecal energy accounts for most of the excretory losses, there is little advantage to using ME instead of DE. Also, DE values are generally easier to determine and the fish feed voluntarily. Special care needs to be exercised in feces collection to minimize any leaching of nutrients.

Ruminants: Dietary energy values for ruminants are expressed in terms of metabolizable energy (ME) or net energy (NE). Single ME values can be assigned to individual dietary ingredients, but NE values vary according to whether animals are in maintenance, growing, or lactating. Energy evaluation systems based on ME use efficiency factors to convert total ME intake to total NE supply for each productive process.

Both ME and NE values can be determined by *in vivo* energy balance studies. True ME or NE determinations require whole-animal calorimeters, which are very expensive to install and operate, so it is usual to perform digestibility studies. Energy intake and losses in feces and urine are measured; losses of energy as methane and heat are assumed to be constant proportions of energy intake. Because few dietary ingredients can be used as a sole feed for ruminants, it is necessary to determine energy values of test raw materials as components of a standard diet. Values for the test raw materials can then be calculated by difference.

In vitro techniques are available to predict the energy content of fermentable feed materials (Deaville

et al. 1998), but these are not appropriate for fats and oils, which are not fermented in the rumen. Energy values for compound feeds, including those containing oilseeds and their derivatives, are calculated from chemical analysis using the equation given in the Feeding Stuffs Regulations 1995:

ME (MJ/kg dry matter) = 0.14 × %NCGD + 0.25 × %AHEE

where NCGD is neutral detergent cellulase plus gamanase digestibility, determined by the method detailed in MAFF (1993); AHEE is acid hydrolyzed ether extract (oil content) determined by Procedure B of Method 3 of the methods of analysis specified in Schedule 2 of the Feeding Stuffs (Sampling and Analysis) Regulations 1982 (MAFF 1993).

Animal Performance

As discussed in the section on determining the dietary energy value of oils and digestibility of constituent fatty acids using poultry and pigs, it is unlikely that performance studies, following alterations to total oil content or concentration of individual fatty acids through plant biotechnology developments, would be necessary. As long as the dietary energy content of new products was known accurately (either through prediction or *in vivo* assessments), these data can be used to produce diets of required energy value.

If performance assessments were required to confirm that modifications to oil content and fatty acid profile had no detrimental effects on nutritional value of oilseeds/high-oil cereals, then practical end-points should be assessed. These would include weight gain, feed intake, and feed conversion ratio for meat animals, and dry matter intake, milk yield, and milk composition for dairy animals (see Figure 6-2).

Product Quality

Nonruminants and Aquaculture

The fatty acid profile of carcass storage, as opposed to structural, lipid in fish, pigs and poultry is influenced substantially by diet. It is comparatively easy to modify storage lipid by altering the diet's fatty acid composition (Wiseman et al. 2000). Studies with pigs (Wiseman and Agunbiade 1998) and broilers (Wiseman 2001b) determined that most of the carcass changes take place within 2.5 weeks of diet change.

There are, however, two major problems associated with an increase in the degree of unsaturation of carcass storage lipid. Such lipid is by definition "softer" and, accordingly, there may be problems during meat processing. It is comparatively easy to measure carcass fat firmness through penetrometer tests. A much more important problem is related to the inherent instability of fat that is more unsaturated. Oxidative degradation is a real possibility (more so if suboptimal dietary levels of vitamin E have been used), as it generates a number of volatiles that may give rise to off-odors and flavors. Such instability may be assessed through the thiobarbituric acid (TBA) test. A comprehensive data set describing protocols for obtaining meat samples for analysis, measuring headspace through GC-mass spectrometry in both raw and cooked meat, determining TBA number, and conducting sensory analyses was provided by Darling et al. (1998) as described in Figure 6-5.

Ruminants

Altering fatty acid profiles of products by diet manipulation is far more challenging in ruminants than in nonruminants because of biohydrogenation of unsaturated fatty acids in the rumen. Complete biohydrogenation results in the conversion of linoleic and linolenic acids to stearic acid. Biohydrogenation is normally only 80–90% complete (i.e. the degree of rumen hydrogenation of unsaturated to saturated fatty acids is around 80–90%); however, conversion of dietary PUFA to PUFA in milk and meat is between 3 and 10% (i.e., because of extensive biohydrogenation in the rumen, only a very small amount of dietary PUFA is transferred to milk and meat; the range is 3–10%). Some desaturation of stearic acid occurs in mammary and adipose tissues, where the delta-9 desaturase enzyme system adds a double bond to create oleic acid.

Biohydrogenation can be reduced by processing raw materials. Incomplete biohydrogenation can be encouraged by diets with a high starch or oil content or coating the oil with a substance that is resistant to microbial attack (Garnsworthy 1997). In addition to increasing the flow of linoleic and linolenic acids to the small intestine, incomplete biohydrogenation has other benefits. The final step of biohydrogenation is conversion of vaccenic acid (C18:1 trans-11) to stearic acid. This step is performed by only one group of bacteria, which is easily inhibited (Bauman et al. 2003).

Accumulation of vaccenic acid raises exciting possibilities for product manipulation because vaccenic acid is converted to conjugated linoleic acid (CLA; 18:2 cis-9, trans-11) by the δ-9 desaturase enzymes in mammary and adipose tissue (Bauman et al. 2003). Conjugated linoleic acid has been shown to have many beneficial effects on human health, including reductions in cancer, atherosclerosis, diabetes, osteoporosis, and immune deficiency (Nagao and Yanagita 2005). Dairy products and meat from ruminants are the predominant sources of CLA in the human diet.

Current challenges for manipulating product quality in ruminants include reducing the effects of fatty acids on the rumen and reducing the effects of the rumen on fatty acids. Increasing PUFA concentrations in crops would have little benefit unless they are inert in the rumen and lead to a real increase in PUFA absorption from the duodenum.

Maintaining Stability of Raw Materials and Effects of Processing

Oils and oilseeds are particularly prone to deterioration during storage and require specific protection to avoid this. Those involved in storing and/or using these commodities have a wealth of experience in minimizing deterioration, ranging from simple environmental control (temperature, moisture, and light) to antioxidant usage. Such controls are much more important for ground oilseeds, which have less "natural" protection against deterioration (e.g., oil present in the free state as opposed to being located in intact spherosomes for example). It is unlikely that unique and new storage protocols need to be developed for novel oilseeds with altered total oil content and that existing successful practices should be entirely acceptable.

Nonruminants and Aquaculture

There are a considerable number of processes both raw materials and mixed diets are subjected to. For raw materials, these can range from physical to include simple grinding (with particle size being a major variable) and heat treatment (with a range of equipment; with the temperature, time, and moisture being variables). Mixed diets are frequently pelleted, but again under a wide range of conditions (e.g., pressure, pelleting shear, extrusion, and expansion).

There have been reports of the effects of processing on the digestibility of oil (which is the key nutritional issue when considering oilseeds/oilseed meals). For example, Agunbiade et al. (1991) examined full-fat rapeseed and oil-extracted rapeseed meal and found that oil digestibility varied depending on the form in which it was found. In a later study, Agunbiade et al. (1992) found the same situation with extruded full-fat soybeans and oil extracted soybean meal.

It is unnecessary to routinely evaluate the effects of processing on all the possible variables with novel oil seeds/high oil cereals. The effects of processing on oil digestibility are significant but it is suggested that, if any biological evaluations are required, then those processing conditions that will be used in practice should be employed. Screening products *in vitro* may be used as an initial test; this was an approach adopted by Carew et al. (1962) where the amount of oil extracted in one hour of petroleum ether extraction was related to the digestibility of oil in full-fat soybean meal.

Ruminants

As discussed in the section on the effect of oil/fatty acid intake on product quality, the major obstacle to improving the fatty acid profile of ruminant products is biohydrogenation of fatty acids in the rumen. Treating raw materials with chemicals or heat renders lipids relatively inert in the rumen so that they do not interfere with microbial fermentation and are less susceptible to biohydrogenation.

Heat treatment of oilseeds is an effective way to deliver fatty acids to the small intestine; heat treatment reduces rumen degradation of protein in oilseeds, and oil is trapped within the undegraded protein matrix. Grinding or crushing oilseeds is usually required to break the seed coat and allow access by rumen microorganisms to fermentable carbohydrates and protein. These processes have little effect on fatty acid digestibility, but they do affect the extent of biohydrogenation and the degree to which free oils interfere with rumen fermentation. Some oilseeds (e.g., cottonseed) can be fed whole, which offers a degree of protection to fatty acids.

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Figure 6-1. Initial steps

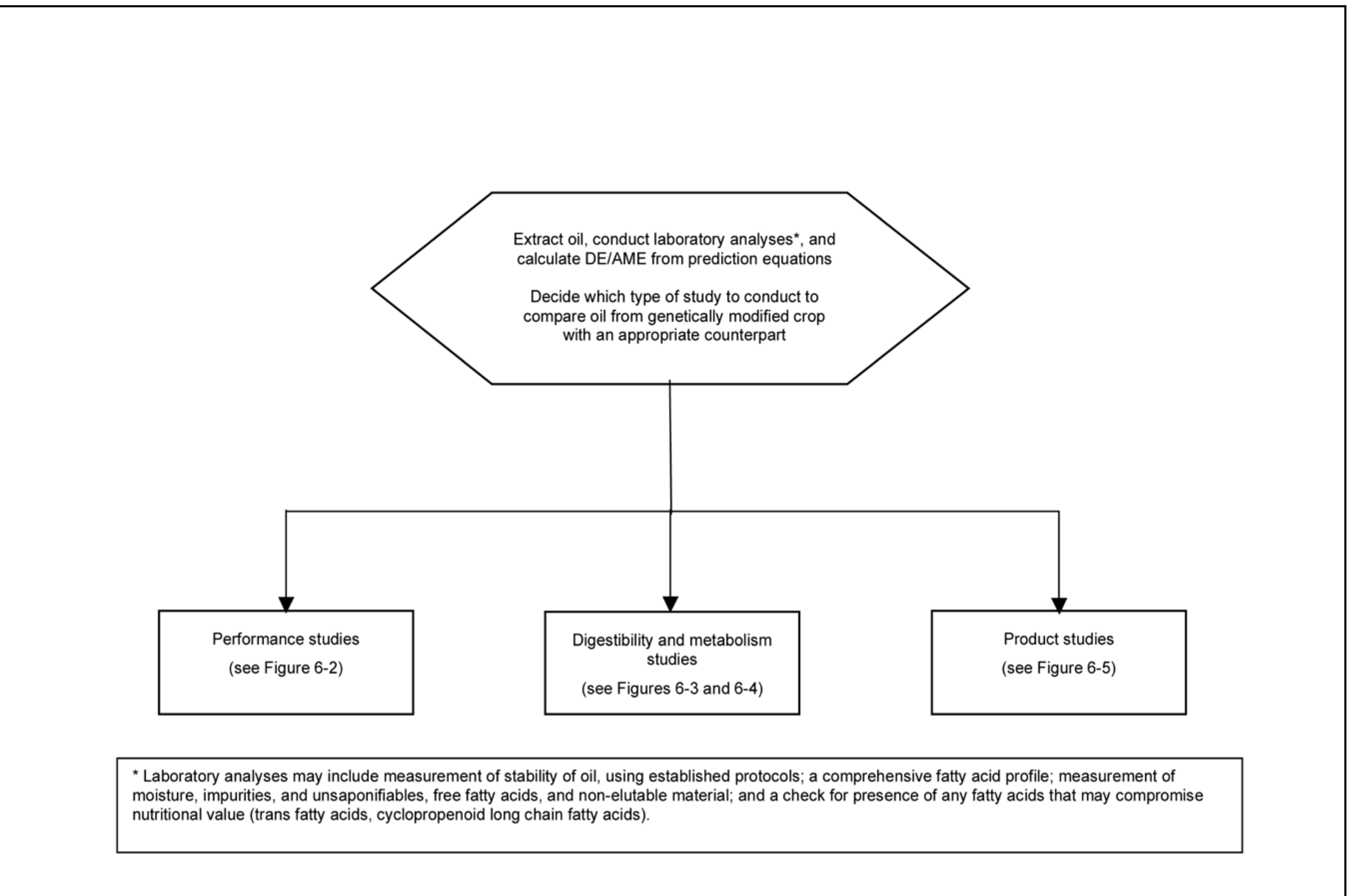


Figure 6-2. Performance studies

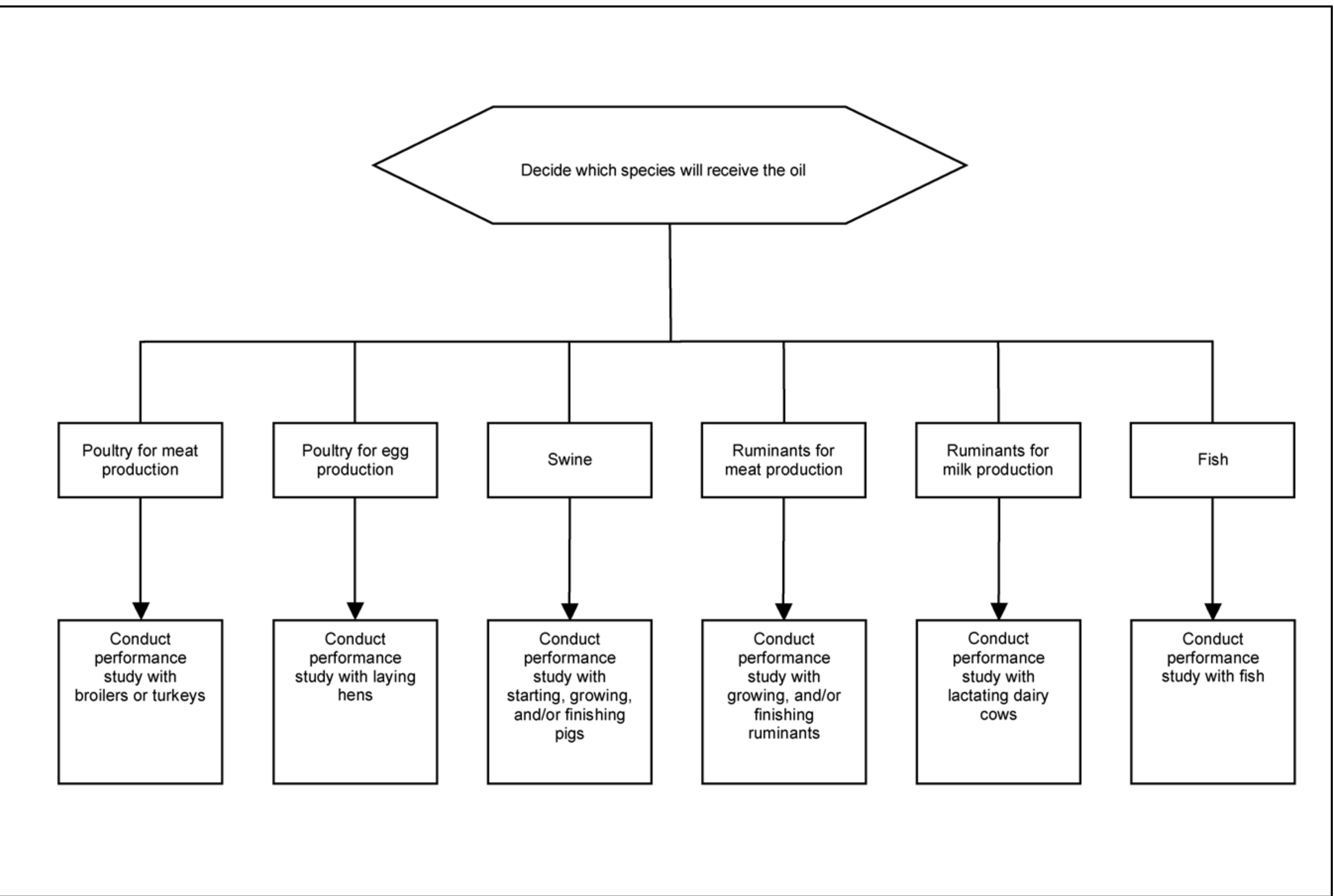


Figure 6-3. Digestibility and metabolism studies

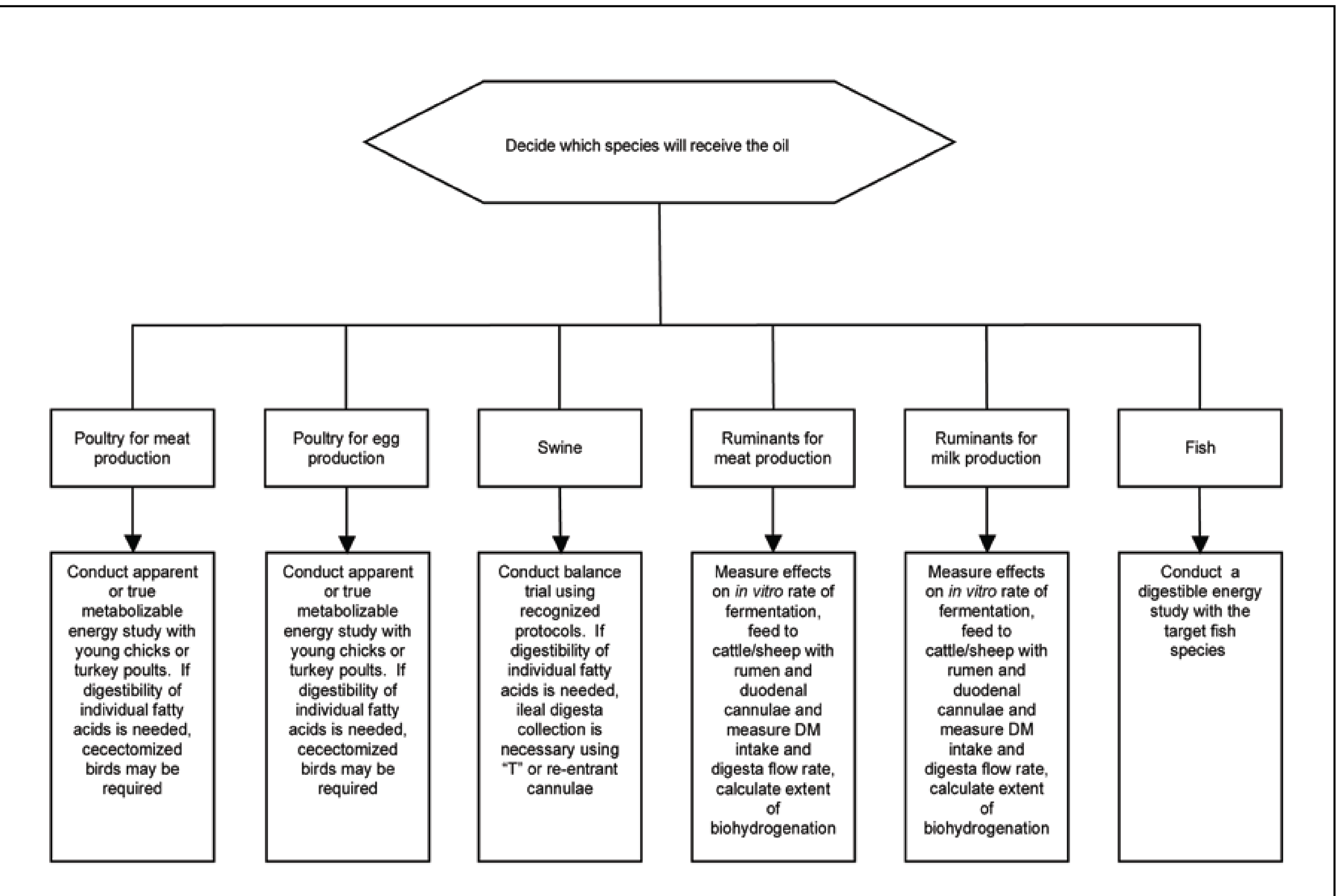
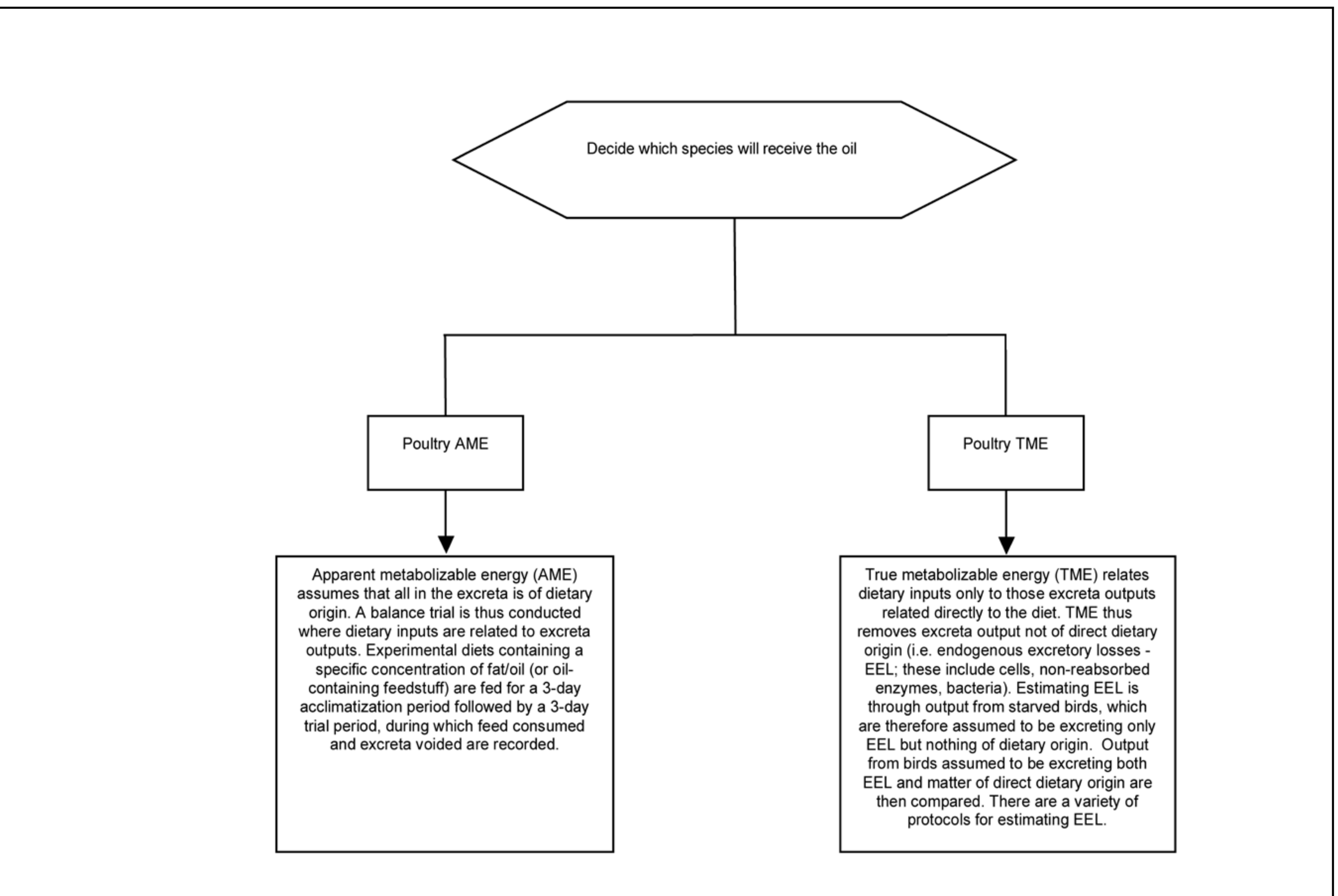


Figure 6-4. Digestibility and metabolism studies: apparent and true metabolizable energy



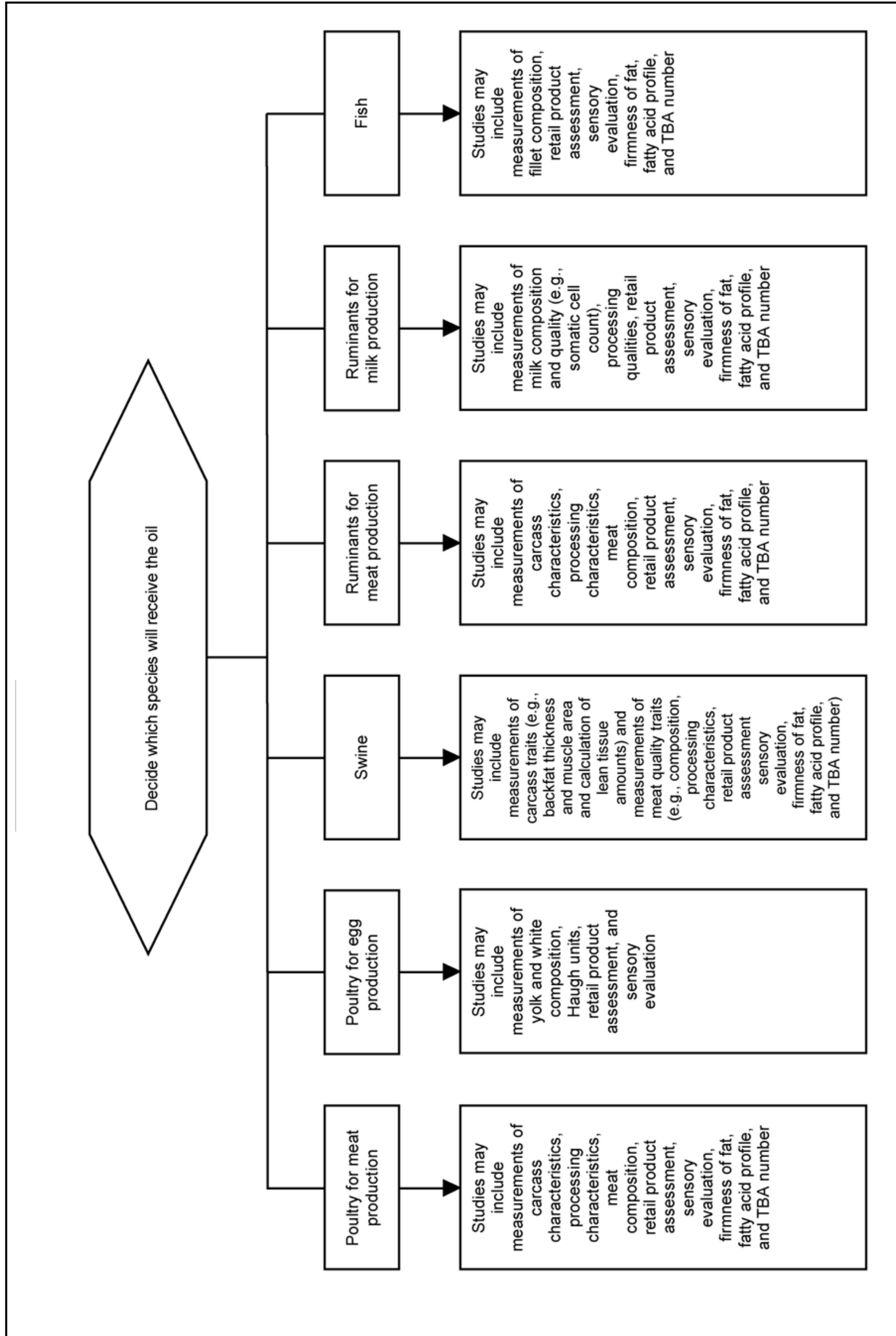


Figure 6-5. Product studies

Chapter 7

Vitamins and Antioxidants

The Value of Increased levels of Vitamins and Antioxidants

A major emphasis of biotechnology, as applied to micronutrients (trace elements, vitamins, antioxidants), is to improve micronutrient levels important to human health and well-being (Tanksley and McCouch 1997, Galili et al. 2002). Hence, nutrients, such as vitamin A and iron where worldwide deficiencies exist, have been studied in great detail. Interest also exists, however, in seeking means to improve the levels and (or) bioavailability of calcium, selenium, and iodine among mineral elements and vitamin E, folate, biotin, thiamin, and ascorbic acid among vitamins. The rationale for emphasizing these nutrients has been their role in human health.

For nonruminant diets that are based on a cereal grains (mainly maize) and oilseed meal (mainly soybean meal), only the four fat-soluble vitamins (vitamins A, D, E, and K), together with choline, riboflavin, pantothenic acid, niacin, and vitamin B_{12}, are routinely supplemented. Wheat-based diets, however, often also require biotin supplementation. All vitamins for fish must be supplied in the diet since vitamins are not synthesized in sufficient quantities *in vivo* for fish development, maintenance, and growth (Webster and Lim 2002). Diets for ruminant animals are generally supplemented with vitamins A, D, and E (Hutjens 2005). Rumen bacteria synthesize the other vitamins required for functional metabolism in adequate quantities.

In addition to essential nutrients, plants synthesize an estimated 80,000 secondary metabolites (Harborne 1993). This review will illustrate how biotechnology is applied to vitamins and antioxidants using the following examples: vitamin A, a fat-soluble vitamin; folacin a water-soluble vitamin; and vitamin E, an antioxidant.

Quantity in Food Crops

Vitamin bioactivity in food crops for animals varies considerably. There is very little preformed vitamin A, D, or K in the cereal grains and oilseed meals fed to animals, and vitamin E levels are considered low in plant-based diets fed to animals. Among the water-soluble vitamins, riboflavin and vitamin B_{12} stand out as being severely deficient in feedstuffs fed to animals. Thiamin and folacin are generally more-than-adequate in animal feedstuffs, but thermal processing (e.g., pelleting, extrusion, drying) can markedly reduce the thiamin and folacin bioactivity in plant-based animal feeds. Thiaminases, present in certain feedstuffs, can also reduce the amount of thiamin activity. Vitamin C is relatively low in most plant-based animal feeds, but this is unimportant for food animals (and most other animals as well) because ascorbic acid can be synthesized in these animals' bodies (guinea pigs, apes, and most fish species are exceptions). Biotin is low in wheat products and much of the niacin bioactivity is unusable in cereal grains. Cereal grains are also low in choline (a nutrient that most nutritionists place in the vitamin category), but most oilseeds are rich in this essential nutrient. Pantothenic acid and vitamin B_6 are generally adequate in plant-based feedstuffs fed to animals. A comparison of the vitamin content of maize, wheat, and soybean meal with the vitamin requirements of swine, poultry, cattle, and fish is presented in Table 7-1.

Chemical Forms in Food Crops

With few exceptions, most of the vitamin bioactivity present in feedstuffs designed for animal consumption does not exist in free form, but instead is present as either coenzymes that are bound to either nucleotides or phosphorus, or is bound in some manner to proteins, carbohydrates, or lipids. Thus, digestive processes in the gut are necessary to free the vitamin moiety from its bound state.

Fat-soluble Vitamins

Vitamin A bioactivity in plant-based feedstuffs exists as provitamin A carotenoids. Of the 600 naturally occurring carotenoids in plants, about 50 are thought to have vitamin A (i.e., retinol) bioactivity. Among these, however, only ß-carotene, α-carotene, γ-carotene, cryptoxanthin, and ß-zeacarotene are quantitatively important (Ullrey 1972, Lee et al. 1999, Solomons 2001). Beta-carotene has the most vitamin A activity of all carotenoids, with a theoretical molar vitamin A yield of 200%. Both α- and γ-carotene have less than half the vitamin A activity of ß-carotene. Ullrey (1972) estimated that maize carotenoids consist of 50% cryptoxanthin, 25% ß-zeacarotene, and 25% ß-carotene. There are other carotenoid compounds in plants that may have significant health benefits, although they have no vitamin A bioactivity. Among these are lutein and zeaxanthin

Table 7-1. Vitamin Levels in Maize, Wheat, and Soybean Meal Compared with Vitamin Requirements of Pigs, Poultry, Beef Cattle, Lactating Dairy Cattle, and Fish[a]

	Vitamin Levels			Vitamin Requirements				
Vitamin	Maize	Wheat	Soybean meal, dehulled	Pig 35 kg	Chick 3-6 wk	Beef Steer[bc] 300 kg	Dairy cow[cd] lactating	Trout rainbow
Vitamin A, IU/kg	213	0	0.5	1,300	1,500	2,000	2,780	2,500
Vitamin D, IU/kg	0	0	0	150	200	250	760	2,400
Vitamin E, IU/kg	8.3	11.6	2.3	11	10	13–25	20	50
Vitamin K, mg/kg[e]	0	0	0	0.5	0.5	-	-	-
Biotin, mg/kg	0.06	0.11	0.26	0.05	0.15	-	-	0.15
Choline, g/kg	0.62	1.09	2.73	0.30	1.00	-	-	1.00
Folacin, mg/kg	0.15	0.35	1.37	0.3	0.55	-	-	1.00
Niacin, available, mg/kg	0	0	22.0	10.0	30.0	-	-	10.0
Pantothenic acid, mg/kg	6.0	9.9	15.0	8.0	10.0	-	-	20.0
Riboflavin, mg/kg	1.2	1.4	3.1	2.5	3.6	-	-	4.0
Thiamin, mg/kg	3.5	4.5	3.2	1.0	1.8	-	-	1.0
Vitamin B_6, mg/kg	5.0	2.2	6.4	1.0	3.5	-	-	3.0
Vitamin B_{12}, μg/kg	0	0	0	10.0	10.0	-	-	10.0
Ascorbic acid, mg/kg[f]	0	0	0	-	-	-	-	50
Myoinositol, mg/kg[g]	-	-	-	-	-	-	-	300

[a]NRC (1982, 1993, 1998, 2000, 2001)

[b]Assumes growth rate of 1.5 kg/day and feed intake of 5.5 kg/day.

[c]Assumes that sufficient levels of the B-complex vitamins are synthesized by the rumen microorganisms.

[d]Assumes Holstein cow producing 45 kg/day of milk.

[e]Assumes that sufficient vitamin K is synthesized by intestinal microorganisms to meet the requirement of pigs, poultry, and fish, and that sufficient vitamin K is synthesized by rumen microorganisms to meet the requirement of ruminants.

[f]Assumes that sufficient amounts of ascorbic acid (Vitamin C) are synthesized metabolically to meet the requirement.

[g]Required only by fish.

that are thought to benefit vision by preventing macular degeneration and cataract formation, and lycopene that may effectively prevent various types of cancer (Frazer and Bramley 2004). Another carotenoid that is commonly used in the feed of farm raised salmon and trout is astaxanthin (3,3'-dihydroxy-ß-ß-carotene-4,4'-dione) (Baker 2002). This compound is used as a meat colorant as it is absorbed and deposited in the meat of the fish and imparts the pink color that is characteristic of wild salmon. Astaxanthin also has significant antioxidant activity estimated to be 1000 times that of vitamin E (Tso and Lam 1996). Yet another carotenoid pigment, canthaxanthin, is extracted from marigold flowers and paprika fruit and is often used in the feed of laying hens to generate egg yolks with a deep orange color (Galobart et al. 2004).

Vitamin D is very deficient in plant-based feedstuffs, although some previtamin D_2 (ergosterol) is present in some products. Much of the focus of recent vitamin D research has been on animal-based 7-dehydrocholesterol in the skin and its conversion to cholecalciferol (vitamin D_3), and subsequently to the hormone 1α, $25(OH)_2D_3$ (calcitriol). This compound is required not only for calcium and phosphorus homeostasis, but also for cell differentiation, insulin secretion, and hematopoiesis.

Vitamin E is associated with the lipid fraction in plants and exists in eight different forms: α, ß, γ, and δ tocopherol and α, ß, γ, and δ tocotrienols (Bieri and McKenna 1981, Baker 1995). The tocopherols predominate in plants. The tocopherols (and α-tocotrienol to some extent) are amphipathic compounds that act as recyclable chain-reaction terminators of polyunsaturated fatty acid (PUFA) free radicals formed by lipid oxidation (Kamal-Eldin and Appelqvist 1996, Grusak and DellaPenna 1999).

Thus, vitamin E functions as a lipid-soluble antioxidant to protect biological membranes. Most of the tocopherol content of seed oils (including maize, soybean, and canola oil) is γ-tocopherol. This isomer, which has only about 10% vitamin E activity (relative to α-tocopherol), represents about 70% of the total tocopherol content of most plant oils. There are complex interrelationships between vitamin E and other dietary antioxidant factors (selenium, cysteine, glutathione) as well as other fat-soluble vitamins. Cleavage of ß-carotene into retinol requires vitamin E. High doses of vitamin E, however, may inhibit ß-carotene absorption or conversion to retinol in the gut, and may also be antagonistic to vitamin K by reducing prothrombin formation. The dietary need for vitamin E is closely related to the level of PUFA in the diet.

Vitamin K is present in plant-based feedstuffs as phylloquinone, also referred to as vitamin K_1. This compound is rich in alfalfa leaves, but is marginal in most feedstuffs for nonruminant animals. The metabolic role of vitamin K in posttranslational carboxylation of specific glutamic acid residues (to γ-carboxyglutamate) affects not only blood clotting, but also osteocalcin synthesis and bone mineralization. In poultry, blood clotting follows a different pattern from that in mammals, and the synthetic forms of vitamin K (menadione in its various forms, also known as vitamin K_3) may be more active than the plant (vitamin K_1) and microbial (vitamin K_2) forms.

Water-soluble Vitamins

Thiamin, riboflavin, niacin, and pantothenic acid are largely present in plant-based feedstuffs in their coenzyme forms: mono-, di-, and triphosphate for thiamin; flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) for riboflavin; nicotinamide adenine dinucleotide (NAD) and dinucleotide phosphate (NADP) for niacin; and coenzyme A, acyl carrier protein, and acyl CoA synthetase for pantothenic acid.

Much of the niacin is in a bound form, either niacytin (carbohydrate binding) or niacinogens (peptide binding) in both cereals and oilseed meals. Also, tryptophan in feedstuffs is a precursor of NAD.

Vitamin B_6 can exist in foods and feeds as free pyridoxine, pyridoxal, or pyridoxamine, and also as each of these compounds in their phosphorylated coenzyme forms. In plants, free pyridoxine predominates but pyridoxal glycosides and pyridoxal lysine may also be present.

Some of the biotin activity present in plants may exist as ε-N-biotinyl-lysine (biocytin),which is bound to protein. Folacin (also called folic acid) exists in feeds as pteroylpolyglutamates, of which over 150 different forms have been identified (Sauberlich 1985). Generally, two to nine glutamate residues are present in these compounds, thus being distinct from folacin itself, which has only one glutamate residue. The main folate precursor compounds in foods and feeds are N^5-methyltetrahydrofolate and N^{10}-formyltetrahydrofolate. A class of gut enzymes known as conjugases (folyl polyglutamate hydrolases) is required to remove all γ-linked glutamate residues except the last one, thus forming folic acid, per se.

Vitamin B_{12} is not present in plants, and exists only in animal tissues and fermentation products as either methylcobalamin or adenosylcobalamin. Cobalt is located at the center of the vitamin B_{12} organic matrix. Ruminant animals have access to vitamin B_{12} that is synthesized by microbes in the rumen, as long as dietary cobalt is adequate. Synthetic vitamin B_{12} is available as either cyanocobalamin or hydroxocobalamin, compounds in which either a (-CN) or (-OH) group is attached to the cobalt moiety of vitamin B_{12}. Choline (synthetic) is a water-soluble (and very hygroscopic) compound, but in feedstuffs choline activity is present in the lipid fraction as phosphatidyl choline (lecithin) or sphingomyelin. Betaine, the oxidation product of choline, is also present in many feedstuffs but is particularly rich in sugar beets.

Bioavailability

Bioavailability is generally defined as the portion of a food-containing nutrient (or precursor compound) that can be digested, absorbed, and utilized by the species in question. In animal nutrition, relative bioavailability is generally measured, and this refers to the percentage utilization of a feed or precursor compound relative to the pure crystalline form of the nutrient.

It is important to note that even with pure forms of most nutrients, digestion/absorption and utilization are generally less than 100% efficient. Complex radiotracer studies are required to establish true utilization efficiency, and with vitamins, little information exists in areas such as true absorption and turnover. Also, the precise location of vitamin compounds in feedstuffs (e.g., germ, endosperm, bran portions) is largely unknown. A paucity of information is available on vitamin bioavailability for both animals and humans.

Fat-soluble Vitamins

Provitamin A carotenoids have variable vitamin A bioactivities and there are vast differences across species. Theoretically, ß-carotene (the most active precursor) can yield two molecules of retinol per one molecule of ß-carotene; α-carotene and ß-cryptoxanthin (less active) can yield one molecule of retinol per molecule

consumed of these carotenoids. In practice, however, these theoretical yields are not realized. Moreover, recent evidence suggests that cis vs. trans configuration of ß-carotene can influence vitamin A bioactivity (Deming et al. 2002), and this further complicates bioavailability assessment. Because food source, animal species, and the particular carotenoid mix can influence vitamin A yield from carotenoids, new *in vivo* stable isotope procedures are being applied to vitamin A potency of food carotenoids (Anonymous 1999). The US National Academy of Sciences proposed a new activity unit called the retinal activity equivalent (RAE). Using this bioactivity scale (designed for humans), retinol, ß-carotene, and other provitamin A carotenoids have values (in μg) of 1:12:24, respectively. Thus, 1 RAE = 12 μg of all-trans ß-carotene from food (Solomons 2001). Chickens and rats derive three times more vitamin A bioactivity from maize carotenoids than pigs (Baker 1995). In a recent experiment with vitamin A-depleted gerbils, Howe and Tanumihardjo (2006) reported that the RAE for provitamin A carotenoids in maize was 2.8. This value was much lower than for carrots and the authors hypothesized that the oil content of maize may enhance carotenoid absorption. The pro-vitamin A activity of carotenoids for ruminants is unknown, but is thought to be lower than that for nonruminant animals (Ullrey 1972). Feline species and mink cannot convert ß-carotene or any other carotenoid to retinol. Therefore, they have a dietary requirement for preformed vitamin A. Knowledge concerning bioavailability (in this case gut absorption) of other high-interest carotenoids (e.g., lutein, zeaxanthin, lycopene) is lacking.

Vitamin E activity in animal feedstuffs exists largely in the oil fraction where γ-tocopherol is the principal component. On an antioxidant rating scale based on molecules of tocopherol required to protect molecules of PUFA, one molecule of α, ß, γ, and δ tocopherol is proposed to protect 220, 120, 100, and 30 molecules of PUFA, respectively (Fukuzawa et al. 1982). More recently, Kamal-Eldin and Appelqvist (1996) proposed relative vitamin E activity values (%) of 100, 50, 10, and 3 for α, ß, γ, and δ-tocopherol in foods, respectively. α-tocotrienol is thought to have a relative vitamin E bioactivity of about 25% (Bieri and McKenna 1981).

Although relative bioavailability estimates have been made for synthetic vitamin K (menadione) products (Oduho et al. 1993; Baker 1995, 2001), no information is available on bioavailability of phylloquinones (K_1) in foods or feeds for animals or humans.

Water-soluble Vitamins

Relative to the pure crystalline form, bioavailability of most B-vitamins is considerably less than 100%. Processing (e.g., heat) and storage can further lower the bioavailability. No information is available for thiamin or folacin bioavailability in animal feeds, but due to their heat lability (Maillard reaction), low bioavailability is likely. Riboflavin is thought to be about 60% bioavailable in a maize-soybean meal diet for chicks (Chung and Baker 1990; Baker 1995), and pantothenic acid was estimated to be 100% bioavailable in both maize and soybean meal for chicks, but only 60% bioavailable in wheat, barley, and sorghum (Southern and Baker 1981, Baker 1995). Vitamin B_6 bioavailability has been estimated at 40% for maize and 60% for soybean meal (Yen et al. 1976).

Choline is rich in oilseed meals, particularly canola meal. Bioavailability of choline has been estimated at 83% for soybean meal, 76% for peanut meal, and 24% for canola meal (Emmert et al. 1996, Emmert and Baker 1997). Choline is largely degraded in the rumen, so a protected choline product has been developed for ruminant animals (Hutjens 2005). Any consideration of choline for mammalian species must also include methionine, because methionine at levels above its requirement for protein accretion and growth can be used to synthesize choline. Thus, excess dietary methionine can eliminate the requirement of mammalian animals for preformed choline. This is not true, however, in avian species. Like methionine, betaine (the metabolic oxidation product of choline) is also present in feedstuffs, and this compound likewise has choline-sparing activity—in both mammals and avians. Recent evidence shows that soybeans and soybean meal have a substantial quantity of S-methylmethionine, a compound having choline-sparing activity (Augspurger et al. 2004), and suggests that previous estimates of choline bioavailability in oilseed meals may be overestimated.

The niacin activity in plant-source feed ingredients is poorly bioavailable, with 85% to 90% present in a bound unavailable form in cereal grains and 40% bound and unavailable in oilseeds (Baker 1995). Although only 2% efficient (wt:wt), excess dietary tryptophan can be metabolized by animals to niacin. Felids, however, cannot carry out this conversion. Biotin bioavailability (relative to pure D-biotin) has been estimated at 100% for both maize and soybean meal, but is only about 50% for wheat, barley, triticale, and sorghum (Baker 1995).

Antioxidants

In considering plant-derived antioxidants, numerous compounds must be considered: tocopherols (already discussed), selenium-containing compounds, ascorbic acid (Frei et al. 1989), uric acid, thioredoxin, ß-carotene, lycopene, other carotenoids, certain enzymes (superoxide dismutase, glutathione peroxidase, catalase), and several

reducing agents with a free SH group (e.g., cysteine, reduced glutathione, α-lipoic acid) (Atmaca 2004). Bioavailability of most of these compounds is not known. Antioxidant capacity of carotenoids depends on absorption of these compounds intact. Retinol, per se, thus has no antioxidant activity. There are great species differences in absorption capacity of carotenoids, per se, including ß-carotene (Lee et al. 1999).

Selenium (Se) is an important water-soluble antioxidant compound, and its bioavailability in foods and feeds is a subject in itself. Dietary levels above the minimum requirement have been shown to have anti-cancer properties (Ip 1998). Thus, because low soil levels of Se (in many parts of the world) result in low crop Se levels, there may be incentive to engineer food crops to have more Se. Wedekind et al. (1998) used multiple indices of Se bioavailability in chicks and reported (relative to Na_2SeO_3) that animal-derived feed ingredients had average Se bioavailabilities of 28% whereas plant-derived feed ingredients had Se bioavailabilities of 47%.

Compositional Changes

Traditional plant breeding (Graham and Welch 1996), together with molecular genetic approaches to increasing nutrient concentrations in food crops, represents a relatively new and emerging field in plant biochemistry. With vitamins, antioxidants, and trace minerals, the major emphasis has been on food crops destined for human consumption. Thus, not surprisingly, focus has been on nutrients such as iron and vitamin A where worldwide deficiencies occur. However, progress has also been made in understanding the biosynthesis of tocopherols, folacin, biotin, thiamin, vitamin B_6, and ascorbic acid (DellaPenna 1999). Modern biotechnological approaches have used gene databases, protein and DNA homologies, and computer searches to identify enzymes and metabolic pathways that can be applied to the target food crop (Saier 1998). Identified genes can be functionally studied, and heterologous expression or gene mutational approaches can, and have been, used to enhance nutrient levels in maize, rice, and tomatoes.

Vitamin A

The molecular approaches to increasing vitamin A bioactivity of food crops have focused on increasing the concentration of the most active pro-vitamin A precursor, ß-carotene. Unlike most vitamins where the upper safe intake level is far above the requirement, vitamin A (i.e., retinol) per se can be toxic at five to 10 times the minimally required intake level. ß-carotene, however, has an upper safe intake level at least 20 times that of retinol. The entire biosynthetic pathway of ß-carotene in plants is now known (Hirschberg 2001, Park et al. 2002). From phytoene, a series of enzymatic reactions result in lycopene, which is a precursor for γ-carotene, which in turn is a precursor for both ß-carotene (two ß rings) and α-carotene (one ß and one ε ring). ß-cryptoxanthin and zeaxanthin can then be made from ß-carotene and lutein can be made from α-carotene. These three carotenoid compounds produced from α- and ß-carotene require a series of complex hydroxylation reactions. Identification of the carotenoid biosynthetic genes in model systems has led to engineering of three carotenoid biosynthetic enzymes (two from plants, one from bacteria) for expression in rice endosperm. The first generation transgenic rice had a yellow endosperm (i.e., golden rice) and a substantially increased level of ß-carotene (Ye et al. 2000).

Vitamin E

Green leafy (i.e., photosynthetic) tissues are rich in α-tocopherol, the compound with the highest vitamin E- and PUFA-protecting antioxidant activity. In contrast, the seed portion of plants contains mostly γ-tocopherol, a compound having about 10% vitamin E bioactivity (i.e., relative to α-tocopherol). Soybean oil, for example, is rich in total tocopherol content (1 g contains 1.2 mg of tocopherols), but only 7% is present as α-tocopherol. Plant biochemists have now worked out the entire biosynthetic pathway for α-tocopherol synthesis from homogentisic acid (Grusak and DellaPenna 1999, DellaPenna, 2001). The last enzymatic reaction in this pathway is the methylation of γ-tocopherol to α-tocopherol via the enzyme γ-tocopherol methyltransferase (γ-TMT). This enzyme is limiting in most agricultural food crops. Scientists have used Arabidopsis seeds to overexpress γ-TMT, and the resulting seeds contained a 10-fold increase in α-tocopherol with no change in total tocopherol content (Shintani and DellaPenna 1998).

Folacin

Folate-related birth defects and homocysteinemia have caused considerable interest in increasing the folacin level in food crops. Bacteria, fungi, and plants synthesize folacin *de novo*, but animals do not. Folic acid is comprised of three parts: pteridine, p-aminobenzoate (pABA), and glutamic acid. These three components are made in different parts of the plant: pteridine in the cytosol, pABA in the chloroplast, and the final glutamylation step in the mitochondrion (Rébeillé and

Douce 1999, Quinlivan et al. 2003). Biochemists have focused much of their folacin biofortification approaches toward increasing either pABA or pteridine synthesis (Basset et al. 2004a, 2004b; de la Garza et al. 2004).

De la Garza et al. (2004) engineered tomatoes to have a 10-fold increase in folacin. To do this, they used molecular approaches to markedly increase pteridine biosynthesis. This procedure increased fruit folate only two-fold. When combined with administration of pABA via the fruit stalk, however, a 10-fold increase in fruit folate was accomplished. They noted that detachment of tomatoes from the plant for ripening resulted in considerable folacin breakdown, and this likely explains why store-bought tomatoes are so low in folacin. Thus, future attempts to modify food crops to contain more folacin will need to address both biosynthetic and catabolic enzymatic reactions.

Undesirable Effects

Whether produced by conventional selection and breeding or by transgenic approaches, plants with higher levels of any given nutrient may have different agronomic characteristics (e.g., yield, disease resistance, etc.) as well as higher (or lower) levels of other chemical entities (Anonymous 2004). From the standpoint of vitamins and antioxidants, one could question the implications of having more ß- and less α-carotene in a food crop, or more α- and less γ-tocopherol in plant oils. Likewise, in plants manipulated to produce more folacin, the implications of elevated levels of pteridines and pteridine glycosides that are present in the engineered fruit should be considered.

When dealing with increased levels of vitamins or antioxidants in a feedstuff, one must consider questions concerning safe upper limits of the vitamins, per se. With the exceptions of vitamins A (i.e., retinol) and D, a wide margin of safety exists between required levels and levels well above the requirement for most vitamins (NRC 1987, Groff et al. 1995).

Evaluation Methods

Evaluating a food/feed crop that has been genetically improved through genetic engineering or other techniques requires one to determine both the quantity and bioavailability of the targeted nutrient. The literature is extensive on best methodology for chemical analysis of vitamins and antioxidants in feedstuffs, and each vitamin or antioxidant has unique features concerning its analysis.

A systematic approach to deciding what experiments to conduct is provided in Figures 7-1 to 7-4 (see pages 92–95). One should keep in mind that animal performance experiments may not show an improvement in a feedstuff with a superior vitamin trait because growth rate and feed efficiency are insensitive indicators to vitamin adequacy. More specific experiments are ultimately required and are discussed below.

Experiments needed

Generally speaking, necessary components of an experiment to determine efficacy include supplementing a purified or semi-purified diet with the new feed ingredient and then assessing animal responses. To illustrate the experiments needed for evaluation, a high riboflavin variety of maize is used here. Two types of experiments are desirable and are outlined below:

Experiment 1 (three treatment diets)

- Riboflavin-deficient diet (riboflavin set at 80% of requirement) and new maize furnishing all the riboflavin
- Near isogenic control maize at the same dietary level as the new maize in treatment 1 (note this diet is expected to be markedly deficient in riboflavin)
- Same as diet 2 but supplemented with crystalline riboflavin up to the riboflavin level furnished in diet 1

Experiment 2 (eight treatment diets)

- Riboflavin-free diet (standard curve, riboflavin deficient)
- As diet 1 + 0.8 mg riboflavin/kg (standard curve, riboflavin deficient)
- As diet 1 + 1.6 mg riboflavin/kg (standard curve, riboflavin deficient)
- As diet 1 + 5.0 mg riboflavin/kg (riboflavin at 2-x requirement)
- As diet 1 + 30% new maize replacing dextrose (furnishing 1.4 mg/kg riboflavin)
- As diet 1 + 30% isogenic control maize replacing dextrose (furnishing 0.7 mg/kg riboflavin)
- As diet 4 + 30% new maize replacing dextrose
- As diet 4 + 30% isogenic control maize replacing dextrose

For both experiments, it is desirable to use a low-riboflavin pretest diet (for 5–7 d) to deplete riboflavin stores. The subsequent experiments should then be of sufficient duration to allow maximum expression of the riboflavin response. In this example (Experiment 2), both growth rate and erythrocyte glutathione reductase (EGR) are expected to respond linearly to bioavailable riboflavin intake. Each of these (Y) criteria are regressed on supplemental riboflavin intake (X) to establish a standard curve (diets 1, 2, and 3). Then, the Y responses occurring for diets 5 and 6 are substituted into the standard curve regression equation to arrive at calculated bioavailable riboflavin intake (X) values. These values are then divided by maize intake to arrive at estimated bioavailable riboflavin levels in both maize varieties. To obtain a percentage bioavailability estimate, one can then divide the bioavailable riboflavin level in each maize variety by total riboflavin level in that variety.

Sometimes adding substantial quantities of a feedstuff to a purified diet can either positively or negatively affect the dependent Y variable being measured. Thus, treatments 7 and 8 in Experiment 2 are used to measure this influence, such that possible adjustments can be made for the nonriboflavin aspects of the maize responses occurring in treatments 5 and 6.

Questions also sometimes arise concerning the accuracy of the chemical assay used to quantify a given vitamin in a given feedstuff. If this is the case, at least three levels of the new food/feed (e.g., maize at say 0, 20, and 40%) can be added to the riboflavin-free diet to establish a linear regression equation for Y (dependent variable) regressed on maize intake. Then dividing this regression coefficient (i.e., g gain/g maize intake) by the crystalline riboflavin regression coefficient (g gain/mg riboflavin intake, i.e., for diets 1 to 3) yields mg bioavailable riboflavin/g maize. This procedure therefore provides a value for bioavailable content of riboflavin in maize, but it does not give a prediction of the percentage of (total) riboflavin in maize that is bioavailable.

The standard-curve experiment illustrated in Experiment 2 is considered most efficient for evaluating several different varieties of a nutritionally improved feed ingredient. If, however, a single variety or sample of the feedstuff in question is to be evaluated, the slope-ratio procedure is considered superior. In slope-ratio experiments, at least three graded levels (zero being one of the levels) of the new feedstuff need to be fed. Common-intercept multiple linear regression is then performed so that the slope of maize riboflavin (or maize itself) can be compared to the crystalline riboflavin slope.

Diet Formulation

Preparation of the right kind of experimental diets is critical to the success of vitamin experiments. The protein-amino acid source should be essentially free of the vitamin being studied. Thus, vitamin-free casein, soy protein isolates (essentially devoid of riboflavin, vitamin B_6, thiamin, and choline), or a crystalline amino acid mixture are often used in vitamin experiments (Baker et al. 1976, Anderson et al. 1978, Southern and Baker 1981, Erdman et al. 1986, Chung and Baker 1990, Oduho and Baker 1993, Oduho et al. 1993, Patel and Baker 1996, Emmert and Baker 1997, Blair et al. 1999, Baker et al. 1999, Scherer and Baker 2000). Crystalline amino acid purified diets are particularly useful in vitamin experiments that employ rats or mice as animal models (Hirakawa et al. 1984, Raines et al. 1997, Blair et al. 1999). Because milligram quantities of vitamins are supplemented in these experiments, precise and careful formulation, mixing, and ultimate feeding are required.

A purified vitamin mix devoid of the vitamin being studied must be prepared or purchased. Adding the vitamin under study to individual diets must be done accurately and quantitatively. Making a (diluted) dry premix of the vitamin under study should be discouraged. Instead, after establishing solubility and stability in an array of possible liquid solvents, it is best to dissolve fat-soluble vitamins in an organic solvent (e.g., ethanol, propylene glycol) and water-soluble vitamins in either deionized water or a water-based buffer to achieve a known concentration (e.g., 1 mg/ml) of the vitamin. Then, pipeting (or weighing) can be done, and this may be followed by premixing and screening prior to mixing each final diet. Final diets should be kept in a cool dry place prior to being used in the experiment.

The dietary carbohydrate source may be important in experiments involving heat-labile vitamins like thiamin and folacin. These B-vitamins have free amino groups that can react with free carbonyl groups of reducing sugars (e.g., glucose, lactose), rendering them unavailable as a bioavailable source of thiamin and folacin. Hence, Maillard-type reactions can occur because the feeding environment of the experiment generally involves warm and sometimes humid conditions. Sucrose or a sucrose-starch mixture is preferable as a carbohydrate source in diets designed to study thiamin and folacin bioactivity.

Animal Responses

Suitable response criteria are a function of each particular vitamin. Growth assays work well for several

vitamins including thiamin, riboflavin, niacin, biotin, vitamin B_6, pantothenic acid, and choline. Although growth assays are considered the best measures for these B-vitamins, other measures are often used as well (Groff et al. 1995). Enzymatic-specific activity can be helpful in some cases: erythrocyte transketolase activity in whole hemolyzed blood for thiamin, EGR for riboflavin, and pyruvate carboxylase for biotin. Plasma biotin concentration and urinary biotin excretion are also used to assess biotin status.

Growth assays for niacin (Oduho et al. 1994) and choline bioactivity (Emmert et al. 1996, Emmert and Baker 1997) in a feedstuff measure more than just niacin and choline per se (Baker 1995). Tryptophan at levels above its need for protein synthesis can be converted (except in felids) to biological niacin activity (i.e., NAD), and methionine at levels above its protein synthesis need can furnish methyl groups (as S-adenosylmethionine) for choline biosynthesis—in mammals, but not avians. Also, betaine and S-methylmethionine at varying levels are present in some feedstuffs and these compounds also have choline-sparing activity.

Liver vitamin A accumulation is generally used to assess the retinol activity of carotenoids, and bone ash is the preferred dependent variable for vitamin D activity. Prothrombin time has been used effectively to quantify vitamin K activity (Baker 1995).

Vitamin E bioactivity in foods and feeds is more complex, and it is generally agreed that there is no truly accurate measure of vitamin E responses in animals. Oxidative damage to lipids can be measured by assessing the disappearance of PUFAs, formation of lipid hydroperoxides, or formation of conjugated dienes. These, however, are *in vitro* measurements. In animals, measure of thiobarbituric acid-reactive compounds (TBARS assay) in erythrocytes is often used to measure antioxidant bioactivity. In this assay, malondialdehyde (MDA) or the TBAMDA adduct is quantified (McCall and Frei 1999). Newer measures of free radical generation (prostaglondin F-2 isoprostanes) as well as markers of oxidative damage to proteins and DNA have been proposed; these procedures were reviewed by McCall and Frei (1999).

Folacin and vitamin B_{12} cannot be measured accurately by animal growth assays. Red blood cell level of folacin is a common measure, as is the serum concentration of B_{12}. Deficient vitamin B_{12} or folacin intake can result in low red blood cell levels of folacin. Another measure of folate status is the deoxyuridine suppression test wherein the enzyme thymidylate synthetase is measured in cultured lymphocytes or bone marrow cells. An oral histidine loading test, in which N-formiminoglutamate (FIGLU) is quantified in urine, may have potential to assess folacin because folacin is required to remove formimino groups from FIGLU. Excretion of FIGLU may be elevated up to 10-fold when folacin intake is inadequate, but vitamin B_{12} deficiency also causes elevated FIGLU excretion.

With some vitamins, specific vitamin antagonists are helpful in achieving a distinctly deficient state, thus facilitating a more definitive growth experiment. For biotin, addition of excess avidin renders dietary biotin ineffective, and this can be useful in detecting nonbiotin effects of feedstuff addition to a purified diet (Anderson et al. 1979). With choline, dietary addition of 2-amino-2-methyl-l-propanol effectively blocks phosphatidyl choline (and subsequently choline per se) biosynthesis from phosphatidyl aminoethanol and S-adenosylmethionine. This, therefore, is useful in separating choline responses from methionine responses when a feedstuff containing both nutrients is added to a choline-deficient purified diet (Emmert and Baker 1997).

Species Selection

Vitamin experiments generally employ a laboratory animal species such as chicks, rats, or mice. However, as discussed previously, evaluating plant carotenoids for vitamin A activity or antioxidant capacity is very species specific (Lee et al. 1999). Other examples of species specificity are avians' lack of ability to spare choline with excess dietary methionine, and felines' inability to convert excess dietary tryptophan to niacin and β-carotene to vitamin A.

Young chicks make up an ideal animal model for most vitamin experiments, and results with chicks are thought to be good predictors of bioactivity values for other nonruminant species, but not feline species. Young chicks grow fast and generally respond markedly to graded dosing of a vitamin in short-term (14 d) experiments. Moreover, unlike rodents, chicks do not practice coprophagy, which is a major confounding factor in vitamin experiments. Also unlike rodents, feed wastage during *ad libitum* feeding is minimal in chick experiments, assuming knowledgeable people are supervising the feeding experiment. If a urinary metabolite needs to be measured for a specific vitamin, however, chicks are not ideal since they excrete urine and feces together.

It is generally too costly and time consuming to attempt vitamin experiments with large-animal species. Besides, vitamin studies with nonruminant animals have little relevance to ruminant animals because ruminants are able to synthesize most vitamins.

Bioavailability

Genetically altered food crops with increased vitamin and (or) antioxidant levels are unlikely to have 100% bioavailability compared to the crystalline forms of the vitamins. By design, the vitamin experiments described previously reveal estimates of the bioavailable level of the vitamin, vitamin precursor, or vitamin complex in a feedstuff. Bioavailability (i.e., percentage utilization relative to the standard) can then be calculated if an accurate analytical value for the feedstuff vitamin is available.

Rumen Degradability

With the exception of fat-soluble vitamins, rumen microbes synthesize most of the vitamins in adequate supply to satisfy the host. Nonetheless, responses have sometimes been seen to certain supplemental B-vitamins (e.g., choline, biotin) in certain situations (Hutjens 2005). Choline added to the diet of dairy cows is thought to be almost totally degraded, but some biotin is thought to escape rumen degradation. Rumen-protected choline is currently being researched. It seems possible that a genetically altered feedstuff (e.g., maize) with a higher vitamin and (or) antioxidant concentration might be more beneficial for ruminant animals than the crystalline vitamin itself. Thus, if a meaningful quantity of the feedstuff escapes rumen degradation and passes to the abomasum and upper small intestine instead, it seems likely that the vitamin component of the feedstuff would also bypass the rumen. In situations like this, bioavailability of the vitamin in question may actually exceed 100%, perhaps by a wide margin.

Product (Food) Quality

Enhancing vitamin levels in a food/feed crop would generally not be expected to affect the quality of the meat, milk, and (or) eggs produced by the food-animals involved. When comparing deficiency to adequacy to excess, however, bioavailable vitamin intakes can directly affect vitamin levels in meat, eggs, and milk. With antioxidant compounds like vitamin E, there may be some benefit to organoleptic quality features (e.g., color, shelf life, etc.) of meat products containing higher levels of vitamin E and other antioxidant compounds.

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Figure 7-1. Initial steps

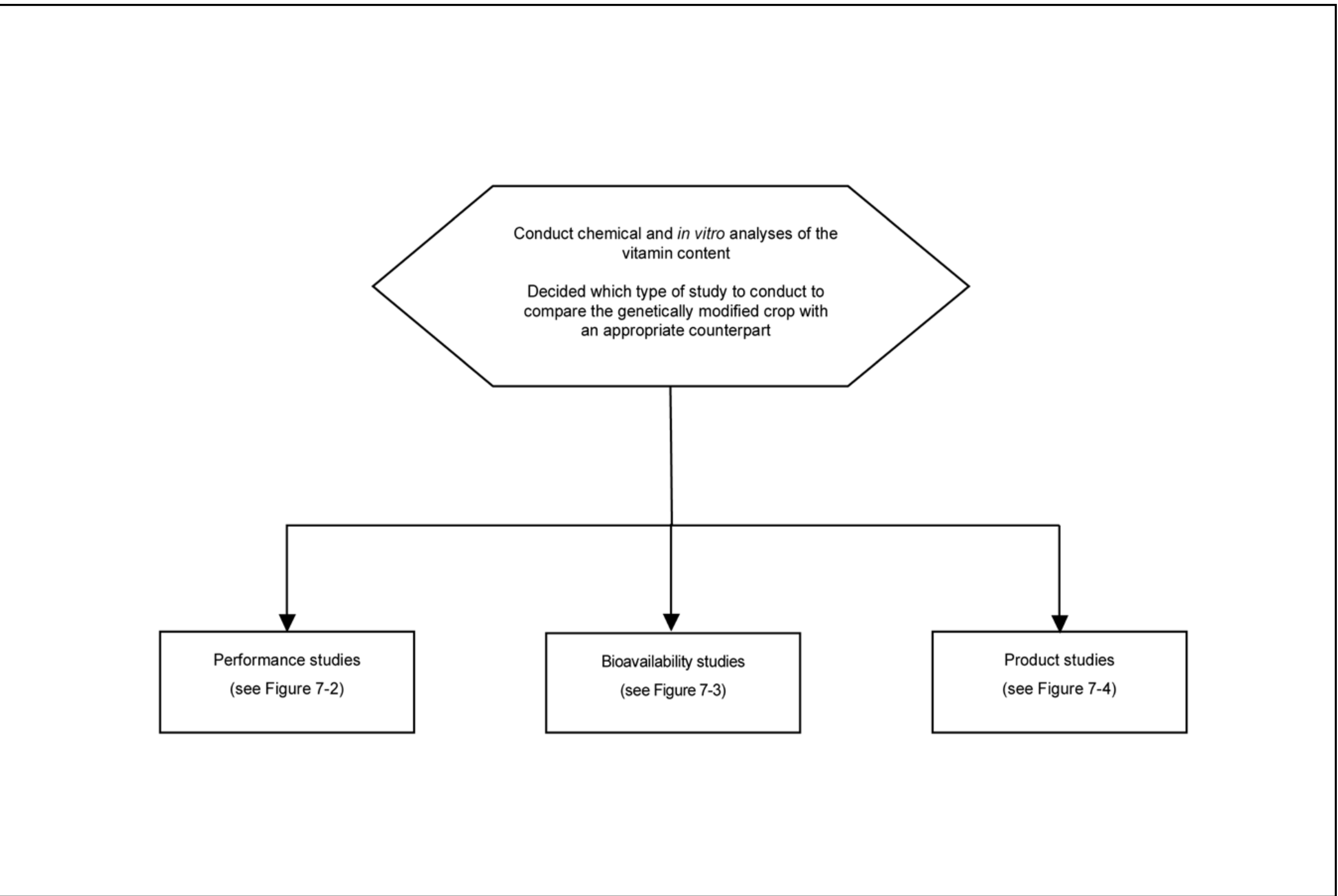


Figure 7-2. Performance studies

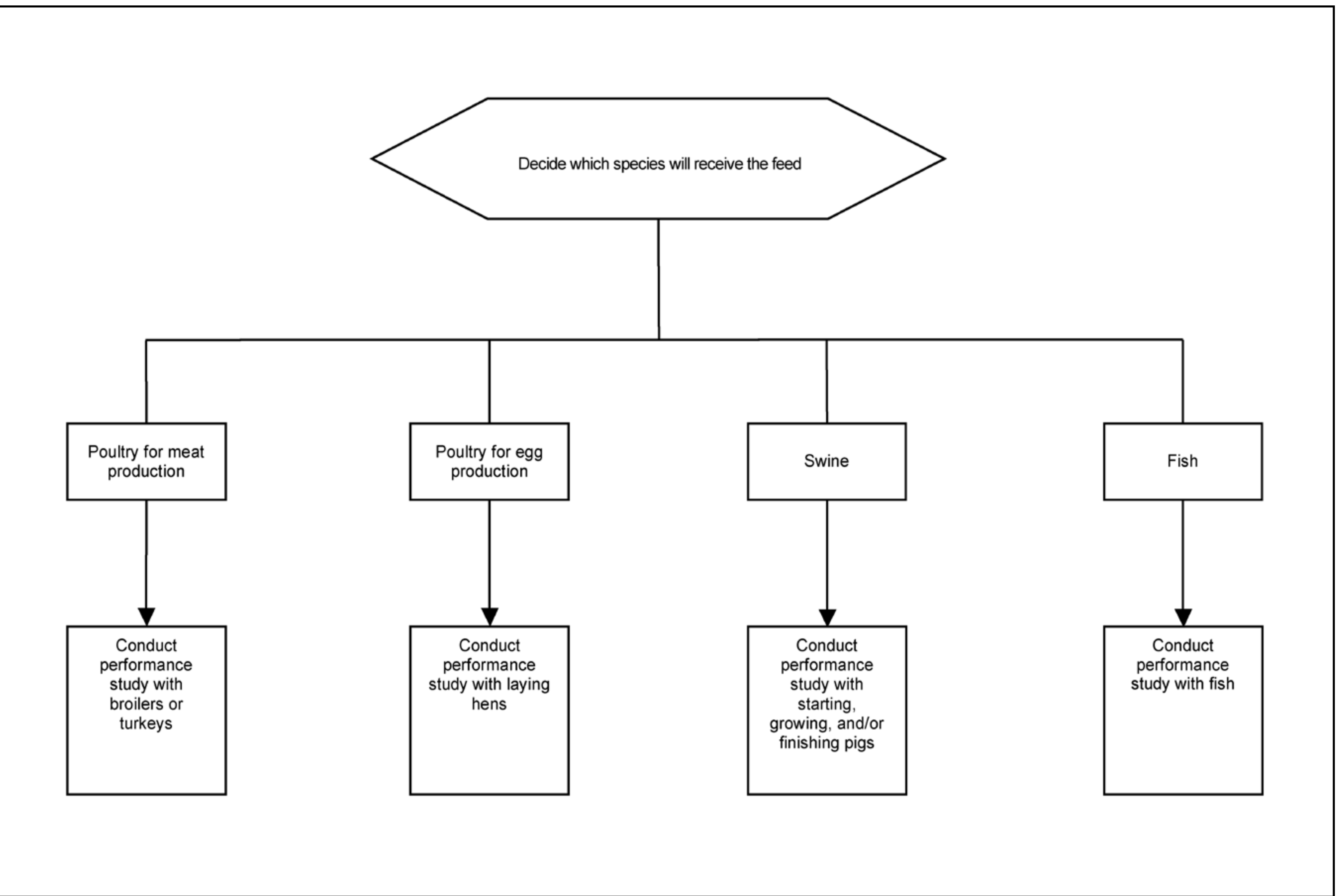


Figure 7-3. Bioavailability studies

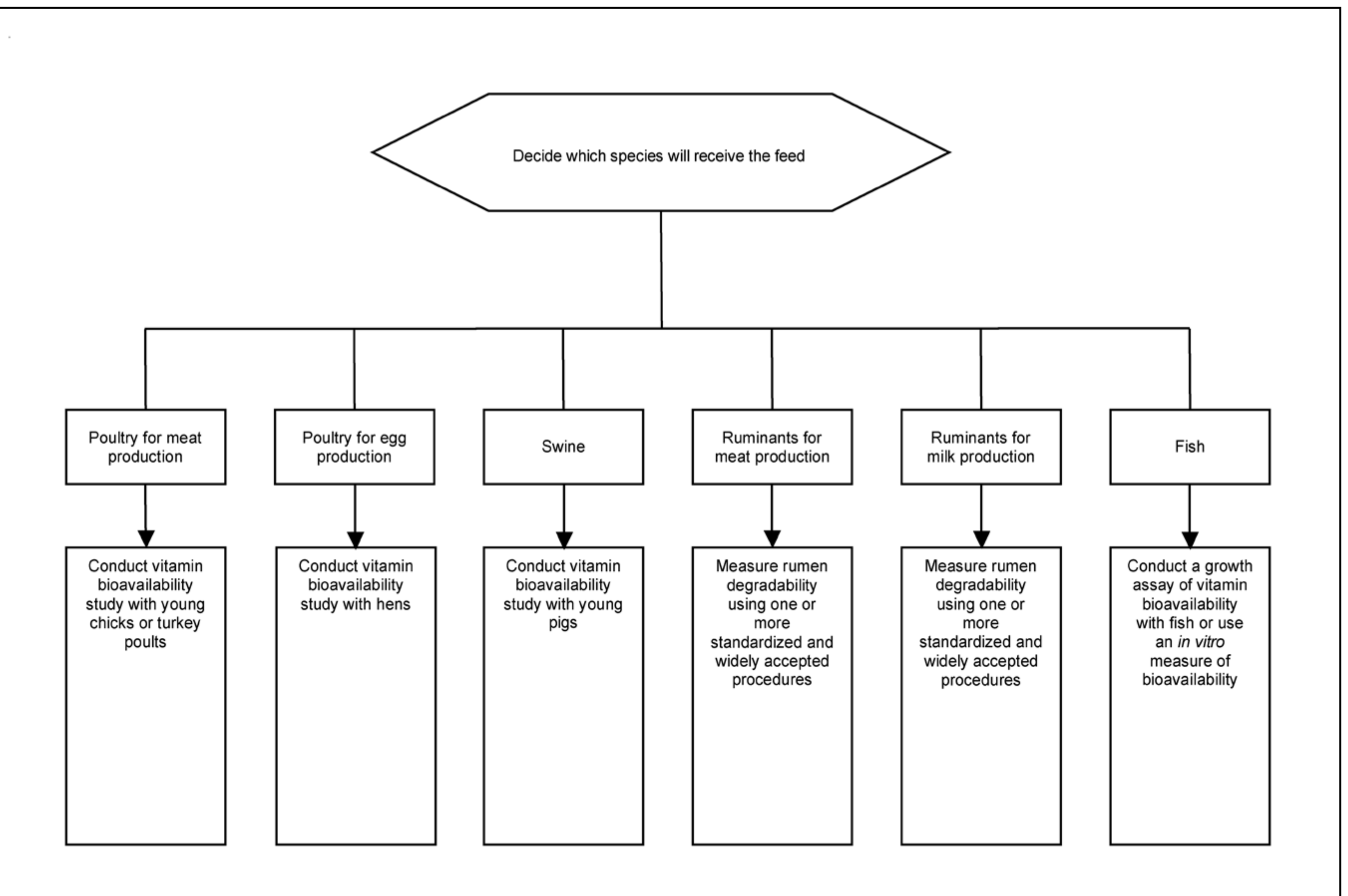
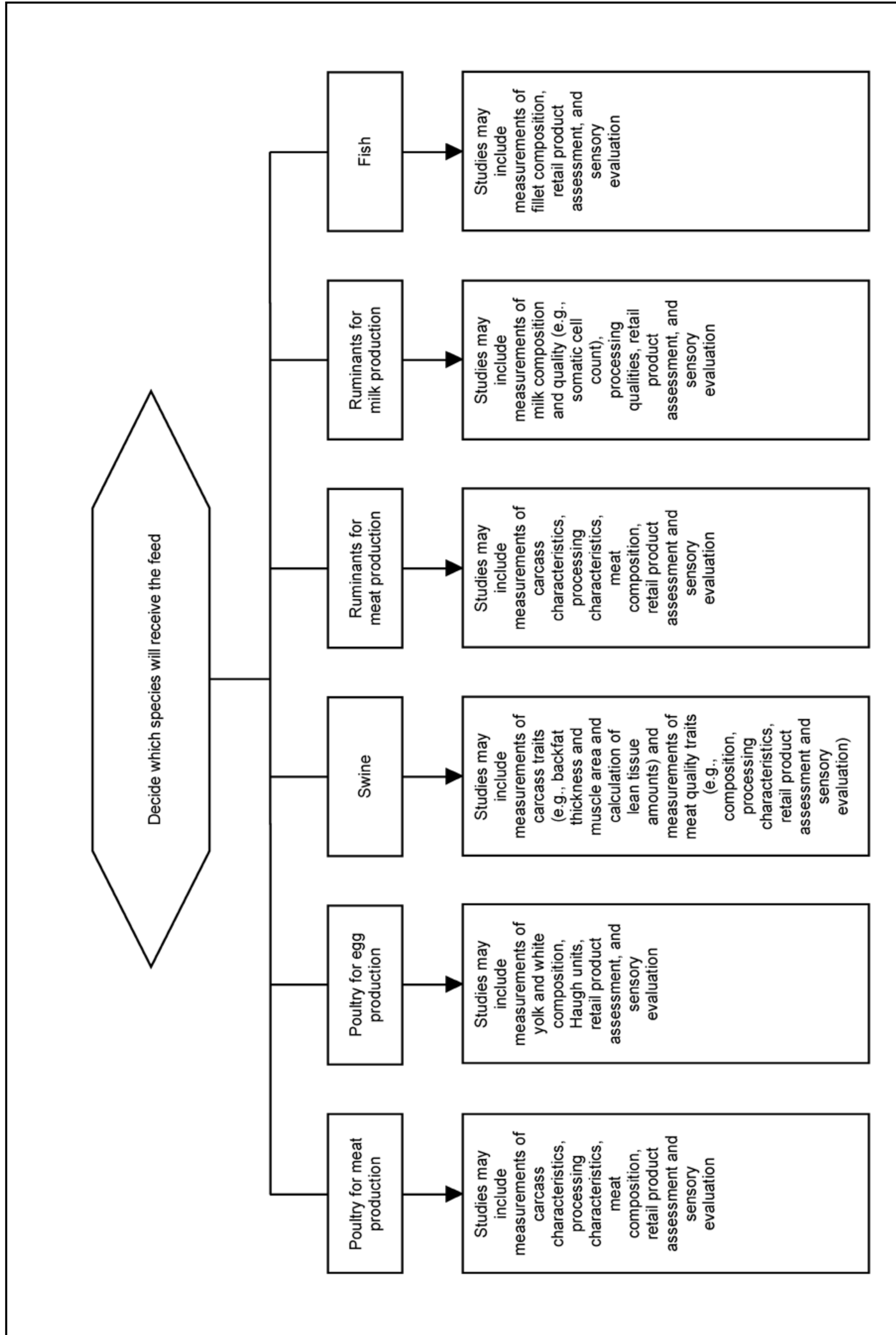


Figure 7-4. Product studies



Chapter 8

Minerals

Minerals in Animal Nutrition

Minerals are inorganic elements that have many important functions in the body. Their functions are quite varied. Some are important for developing and maintaining the skeleton (e.g., calcium, phosphorus), while others have important regulatory functions. Some minerals function as constituents of amino acids (e.g., sulfur for methionine, cystine), vitamins (e.g., cobalt and vitamin B_{12}), hormones (e.g., iodine and thyroxin; zinc and insulin), and other important compounds (e.g., iron and hemoglobin), while other minerals are integral constituents of metalloenzymes (e.g., copper and superoxide dismutase) or are essential for the activity of physiologically important enzymes.

Fifteen minerals are known to be required by most domestic animals (Table 8-1). Compared with energy and protein, the minerals are required in very small amounts. The essential minerals include seven macro-minerals: (these are classified as "macro" because they are required in considerably greater amounts than the micro-minerals) calcium, phosphorus, sodium, chlorine, potassium, magnesium, and sulfur; and eight micro- or trace-minerals: copper, iron, zinc, manganese, iodine, selenium, chromium, and cobalt. Cobalt is classified as essential for ruminant animals because rumen microorganisms can utilize cobalt to synthesize vitamin B_{12}. However, nonruminant animals cannot perform this function so cobalt is not considered essential for pigs and poultry. Some additional trace elements have been shown to have a physiological role in laboratory species. Large animals may also require some trace elements, but the requirement is so low that essentiality has not been conclusively proven. These include arsenic, boron, bromine, fluorine, molybdenum, nickel, silicon, tin, and vanadium.

Calcium and phosphorus are the two minerals required in the greatest amounts in the animal diet. Approximately 99% of the calcium and 77% of the phosphorus in the body is found in the skeleton with the remaining amounts in the soft tissue. Phosphorus, in particular, has more roles in the body than any of the other minerals. It is a component of high-energy and low-energy phosphate bonds; is involved in protein synthesis; is a component of phospholipids, nucleic acids, and numerous metabolic enzymes; is an activator of many enzyme systems; is an important intracellular buffer; and is involved in osmotic and acid-base balance. Almost every series of metabolic reactions in the body involves this important mineral. Besides its role in bone formation, calcium is a regulator of key enzymes involved in blood coagulation, is required for cell membrane integrity, and is involved in nerve function and muscle contractibility. Calcium and phosphorus are the major minerals in milk, where they are predominately complexed with casein in the casein micelle. These two minerals contribute to the buffering capacity and the maintenance of pH, ionic strength, and osmotic pressure of milk.

Adequate levels of both minerals must be included in the diet for strong skeletal structure. Calcium is also important for proper egg shell formation in poultry. If either calcium or phosphorus is deficient, poor bone mineralization will occur and can lead to leg deformation (rickets), lameness, or bone fractures. Sound feet and legs are especially important in animals reared on concrete or slatted floors in confinement facilities. Also, a phosphorus deficiency will result in slow and inefficient growth. Calcium deficiency seldom depresses growth unless the deficiency is severe. Calcium and phosphorus need to be kept in proper balance in the diet. Excess calcium can cause problems, especially if the phosphorus level is marginal.

Cereal grains are extremely deficient in calcium for both ruminants and nonruminants. In addition, grains are deficient in phosphorus for nonruminants (Table 8-1). Most of the phosphorus in grains and oilseed meals (60 to 80%) is in the form of phytate, an organic complex containing six phosphorus radicals that is not utilized by nonruminant animals due to the lack of phytase enzyme in their digestive tract. Adding phytase to the diet is an effective means of degrading the phytate and increasing the bioavailability of phosphorus in maize, soybean meal, and other feedstuffs of plant origin (Jongbloed et al. 1992, Cromwell et al. 1993). Using phytase in the diet allows for feeding of lower phosphorus diets, which reduces the amount of phosphorus excreted by nonruminants (Carter et al. 2003; also see chapter 9). A reduction in phosphorus in livestock and poultry manure has important implications because excess phosphorus from animal wastes is considered a potential environmental pollutant (CAST 2002).

Sodium and chlorine (chloride) are the principal extracellular cation and anion, respectively, in the body. Chloride, as hydrochloric acid, is the chief anion

Table 8-1. Mineral Levels in Maize, Wheat, and Soybean Meal Compared with Mineral Requirements of Pigs, Poultry, Beef Cattle, Lactating Dairy Cattle, and Fish[a]

	Mineral Levels			Mineral Requirements				
Mineral	**Maize**	**Wheat**	**Soybean meal, dehulled**	**Pig 35 kg**	**Chick 3-6 wk**	**Beef Steer[b] 300 kg**	**Dairy cow[c] lactating**	**Trout rainbow**
Calcium, %	0.03	0.04	0.34	0.60	0.90	0.75	0.67	1.00
Phosphorus, %	0.28	0.39	0.69	0.50	-	0.35	0.35	0.60
Bioavailable, %	0.03	0.19	0.16	0.23	-	-	-	-
Nonphytate, %	0.08	0.13	0.22	-	0.35	-	-	-
Sodium, %	0.02	0.01	0.02	0.10	0.15	0.06	0.22	0.60
Chlorine, %	0.05	0.08	0.05	0.08	0.15	-	0.28	0.90
Magnesium, %	0.12	0.11	0.30	0.04	0.06	0.10	0.20	0.04
Potassium, %	0.33	0.46	2.14	0.23	0.30	0.60	1.06	0.70
Sulfur, %[d]	0.11	0.16	0.43	-	-	0.15	0.20	-
Copper, ppm	3	8	20	4	8	10	11	3
Iron, ppm	29	32	176	60	80	50	17	60
Manganese, ppm	7	38	36	2	60	20	13	13
Zinc, ppm	18	47	55	60	40	30	48	30
Iodine, ppm	0.03		0.16	0.14	0.35	0.50	0.50	1.1
Selenium, ppm	0.07	0.28	0.27	0.15	0.15	0.10	0.30	0.30
Cobalt, ppm[e]	0.05		0.09	-	-	0.10	0.11	-
Chromium[f]	-	-	-	-	-	-	-	-

[a]NRC (1982, 1993, 1994, 1998, 2000, 2001)

[b]Assumes growth rate of 1.5 kg/day and feed intake of 5.5 kg/day.

[c]Assumes Holstein cow producing 45 kg/day of milk.

[d]Assumes that sufficient sulfur is provided by the sulfur-containing amino acids to meet the requirement of pigs, chicks, and fish.

[e]Pigs, poultry, and fish have no cobalt requirement for vitamin B_{12} synthesis; instead, vitamin B_{12} must be provided to these species.

[f]Required, but amount unknown.

in gastric juice. These two minerals are required for normal growth and body functions. Cereal grains are low in sodium and chloride; the requirements can be met by supplementing the diet with common salt.

Potassium is the third most abundant mineral in the body and the most abundant mineral in milk. It is involved in electrolyte balance and neuromuscular function. Magnesium is a cofactor in many enzyme systems and is a constituent of bone. Sulfur is a constituent of a number of sulfur-containing compounds (e.g., methionine, cystine, taurine, chondroitin sulfate, etc.). These three minerals do not need to be supplemented to nonruminant diets because the natural feedstuffs contain ample amounts of them. Ruminant animals sometimes require additional sulfur for optimal rumen function, especially when sources of nonprotein nitrogen are added to the diet. Also, supplemental magnesium is often needed by grazing ruminants during periods of lush pasture growth (with high potassium and nitrogen content) to prevent a metabolic condition called "grass tetany."

Zinc is a component of over 200 metalloenzymes in the body and numerous digestive enzymes, and is associated with the hormone insulin. Hence, it plays an important role in protein, carbohydrate, and lipid metabolism. Zinc is also thought to bolster the immune system. This element is required for normal growth and healthy skin in all animals. Zinc deficiency most commonly occurs in swine and causes slow growth and a hyperkeratinization (scabby appearance) of the skin, a condition called "parakeratosis." A high level of dietary calcium interferes with zinc absorption. Also, high levels of phytate tend to bind zinc and render it unavailable. Thus, the zinc requirement is much higher in a grain-soybean meal diet (which is high in phytate) than in a diet with a significant portion of animal protein.

Pharmacological levels of zinc (1,500 to 3,000 ppm, as zinc oxide) have been shown to stimulate growth and feed intake in early-weaned pigs (Hill et al. 2000).

Iron exists in complex organic forms in the body (e.g., hemoglobin, myoglobin, transferrin, uteroferrin, and lactoferrin) and plays an important role in the body as a constituent of several metabolic enzymes. Dietary iron is especially needed for hemoglobin synthesis in young animals, and an iron deficiency results in depressed hemoglobin levels in the blood (anemia). Suckling pigs are most susceptible to iron deficiency and will become anemic within a week after birth if not given supplemental iron. The reasons are that: 1) newborn pigs have low iron reserves at birth; 2) they grow exceptionally fast, quadrupling their weight in only two to three weeks; and 3) their only food is milk, which is extremely deficient in iron, and they have little opportunity to obtain any iron from their environment when raised in confinement. Anemia can easily be prevented by administering an intramuscular injection of iron dextrin before three days of age. Copper is also needed for the synthesis of hemoglobin and for synthesis and activation of several oxidative enzymes necessary for normal metabolism. This mineral, like zinc, stimulates growth and feed intake when pharmacological levels (100-250 ppm) are fed to pigs (Braude 1967).

Manganese functions as a component of several enzymes involved in carbohydrate, lipid, and protein metabolism. It is also required for the synthesis of chondroitin sulfate, a component of mucopolysaccharides in the organic matrix of bone. The manganese requirement is quite low in most animals, but very high in birds; thus supplementation is crucial in poultry diets to prevent perosis, a leg malady called "tibia dyscondylphasia" or "slipped tendon."

Iodine is present in the thyroid as a component of thyroxin and its precursors, which are important regulators of metabolic rate. Iodine is deficient in feedstuffs grown in certain areas of the world. Also, some feedstuffs (e.g., soybeans, rapeseeds, linseed, etc.) contain goitrogens, compounds that interfere with iodine absorption or the incorporation of iodine into thyroxin. Failure to supplement diets with iodine can cause goiter in pigs, calves, and lambs. One can conveniently provide iodine by adding the recommended level of iodized salt to the diet.

Selenium is a component of the glutathione peroxidase enzyme, which detoxifies lipid peroxides and protects membranes against peroxide damage. Selenium deficiency in pigs, calves, and lambs is frequently a problem in many parts of the world. Maize and soybean meal will vary by a factor of 15 or more depending on where the grain or beans are grown (Cromwell et al. 1999). Selenium supplementation is required in areas where low-selenium feedstuffs are fed.

Chromium functions as a cofactor with insulin and is an important regulator of glucose metabolism. Animals require this mineral at very low dietary levels, but the quantitative needs have not been determined. Cobalt serves as the nucleus of the vitamin B_{12} molecule and can be used by ruminants to synthesize this essential vitamin.

Additional information on mineral nutrition can be found in other publications (Underwood and Suttle 2001, NRC 2005)

Quantity and Bioavailability of Minerals

Quantity of Minerals

For most nonruminant animals raised in modern production systems, cereal grains (e.g., maize, sorghum, barley, wheat, etc.) form the basis of the diet because they provide the most economical way of meeting the animal's energy needs. To meet the animal's amino acid needs, cereal grains are supplemented with oilseed meals such as soybean meal (the predominant one used), canola meal, cottonseed meal, and peanut meal, sometimes in combination with animal by-product meals such as meat and bone meal, meat meal, fish meal, and blood meal. Diets fed to ruminant animals vary more than those fed to most nonruminants. In some ruminant feeding systems, diets are based on forages and other roughages. In finishing ruminants and dairy cattle production, diets are based on a blend of forages and cereal grains.

Unfortunately, the cereal grains, oilseed meals, and in some cases, the forages are deficient in many minerals as shown in Table 8-1. Therefore, diets must include mineral supplements (e.g., salt, limestone, inorganic phosphates, trace-mineral mixes, etc.) to correct the deficiencies in the natural ingredients and meet the animal's needs. Having higher levels of minerals or greater bioavailability of minerals in the natural feedstuffs would reduce the amount of mineral supplements that would need to be added. In some instances, it could also reduce the amount of minerals that would be excreted into the environment.

Bioavailability of Minerals

Most of the minerals in cereal grains, oilseed meals, and forages occur in organic compounds or complexes. Some of these minerals may be highly available to animals, but others may be poorly available. Unfortunately, this is an area that still needs a lot of

research. However, one area that is quite well understood is the form and role of phosphorus in seeds. Most of the phosphorus in seeds is organically bound in a complex that is chemically defined as myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, or more commonly called "phytic acid" or its salt, "phytate." Phytic acid constitutes 60 to 80% of the total phosphorus in most cereal grains and oilseed meals (Nelson et al. 1968). Most of the phosphorus in this form is not available to pigs, poultry, and other nonruminants because they do not have sufficient levels of phytase in their digestive tract to degrade the phytate and release the phosphorus from the organic complex. Ruminant animals have no problem utilizing phytate phosphorus because of the abundance of rumen microbes that secrete phytase. The inability of pigs, chickens, and fish to utilize phytate phosphorus results in relatively large amounts of undigested phytate phosphorus in the feces, which can lead to environmental pollution (CAST 2002). In addition, phytate has been shown to bind other divalent minerals and reduce their absorption (Adeola et al. 1995).

The use of microbial phytase in feeds has allowed swine and poultry producers to feed diets with lower levels of supplemental phosphorus, and this practice has reduced phosphorus excretion by these species. Also, the discovery of low-phytate maize (Raboy et al. 1990) and low-phytate soybeans (Anderson 1998) has expanded the potential for further reducing phosphorus excretion by pigs and poultry. Furthermore, the phytase gene has been inserted into canola (McHughen 2000) and alfalfa (Ullah et al. 2002). These transgenic plants could have a major positive impact on phosphorus pollution in the environment in the future.

Overall Benefit

In general, genetic improvements in the mineral components of feedstuffs are unlikely to have major effects on increasing growth rate, feed efficiency, carcass leanness, milk production, etc. The greatest benefits of improved traits in feedstuffs (especially increased bioavailability of minerals) are more likely to result from reducing excretion of nutrients, such as phosphorus, that have the potential to cause environmental pollution.

Intended Compositional Changes

Changes in the amount, digestibility, or biological availability of minerals in a feedstuff can be achieved through either traditional plant breeding techniques (selection, crossbreeding, backcrossing, and propagation) or modern biotechnology methods. It generally makes little difference which method is used to achieve the improvement.

One of the major compositional changes with respect to minerals has been the change in the type of phosphorus in feedstuffs. Raboy and coworkers (1990) discovered that the synthesis of phytic acid in maize containing the lpa mutant gene was markedly depressed, whereas the total phosphorus in seeds was not impacted by the mutant gene. In other words, the maize containing this gene had about half as much phytic acid phosphorus and twice as much inorganic phosphorus. In the initial studies conducted with pigs (Cromwell et al. 1998a, Spencer et al. 2000) and chicks (Cromwell et al. 1998b), the bioavailability of phosphorus in the mutant maize was two to three times greater than the phosphorus in a near-isogenic conventional maize. Similar trends were reported for soybean meal produced from low-phytate soybeans and near-isogenic conventional soybeans (Cromwell et al. 2000). In addition, the reduced amounts of phytic acid result in less chelation of zinc, manganese, copper, and other divalent cations, rendering them more available to the animal (Adeola et al. 1996).

Undesirable Effects

Any method of achieving genetic change, using either traditional or transgenic approaches, can potentially produce undesirable effects. Some undesirable changes are predictable, whereas others are unpredictable.

Plants and feedstuffs with improved traits often have undesirable agronomic characteristics. One of the most frequently seen undesirable effects is a yield reduction (or yield drag) when a modified trait is incorporated into a plant. There is also the possibility of a reduced germination rate in the planted seeds. Reduced germination in seeds with reduced amounts of phytate phosphorus has been one problem that has delayed the commercial introduction of the low-phytate grains. Other possible undesirable conditions might include the creation of mineral imbalances should a trait be introduced that markedly increases the amount of a certain mineral. A reduction in the palatability of a feedstuff is another possible undesirable effect that could occur.

Evaluation Methods

The purpose of the evaluation methods is to establish whether the genetically modified feedstuff is of added benefit when compared to a conventional feedstuff.

Deciding What Experiments Are Needed

A systematic, science-based process should be used to determine what experiments (if any) are

needed. The need for and types of experiments will depend on the changes in the amount or bioavailability of minerals that have been made in the grain, oilseed, or forage. A systematic approach to deciding what experiments to conduct is provided in Figures 8-1 to 8-4 (see pages 104–107). One should keep in mind that animal performance experiments may not show an improvement in a feedstuff with a superior mineral trait because growth rate and feed efficiency are insensitive indicators to mineral adequacy. Exceptions are phosphorus, sodium, and zinc, in which case animal performance is markedly reduced when diets are deficient in any one of these three minerals.

Animal Experiments

Diet formulation: Appropriate diet formulation is crucial for assessing improvement in a mineral level or mineral bioavailability. Unlike the situation with improved agronomic traits, simple substitution of the nutritionally improved crop for a near-isogenic conventional crop in a standard diet formulation that is adequate in all nutrients is not recommended because no benefit is likely to be observed. Instead, diets should be formulated that are deficient in the mineral under investigation to take advantage of the potential nutritional improvement.

In order to assess a potential improvement in an altered feedstuff, diets must be formulated such that they are adequate in all nutrients except for the mineral under investigation. One approach is to have a minimum of three dietary treatments: 1) a diet low in the mineral being tested and containing the genetically altered feedstuff; 2) a similar diet but with a conventional (preferably near-isogenic) feedstuff substituted for the genetically altered feedstuff on an equal-weight basis; and 3) a positive control diet consisting of the conventional feedstuff supplemented with an inorganic form of the mineral in question added to meet the animal's requirement for that mineral.

Bioavailability studies are a bit more complex. This example assumes that the bioavailability of phosphorus in a genetically altered crop will be determined. A semi-purified, negative control diet consisting of a pure carbohydrate source (maize starch, dextrose, etc.), a protein source, and supplemented with vitamins and minerals (except for the mineral under investigation) should serve as the basal diet. Graded levels of the genetically altered feedstuff (e.g., a low-phytate maize) or the near-isogenic conventional feedstuff are added to the basal diet in place of, and in order to provide, the carbohydrate source. A single level of each feedstuff can be used, but two or three levels are recommended. Another series of two, three, or more diets consisting of the basal diet with the same levels of phosphorus added in the form of a highly bioavailable source of phosphorus (e.g., monosodium phosphate or monocalcium phosphate) is included as the standard. The highest level of the supplemental phosphorus source must be below the animal's requirement so that the response is linear. Bone traits are measured and slopes are calculated for the three phosphorus sources when regressed on the level of added phosphorus consumed. Slope ratio procedures are used to determine the bioavailability of the phosphorus in the two maize sources relative to the standard.

Animal performance (feed intake, weight gain, feed efficiency, milk production, egg production, etc.): The overall experimental design will be similar to those for crops with improved agronomic traits (e.g., the importance of adequate replication, the identification of "pen" as the experimental unit, etc.). As indicated previously, except for experiments that evaluate phosphorus, sodium, or zinc, differences among treatments are likely to be small or nonexistent in growing nonruminants, so performance experiments are not recommended for other minerals. With small possible differences, the calculation of the number of replications needed and proper statistical design are crucial.

Digestibility/bioavailability: Changes (either increases or decreases) in digestibility/bioavailability of the macrominerals that are not detectable in performance experiments can often be determined in digestibility or bioavailability experiments. In swine, ileal digestibility or total tract digestibility of minerals would be usual measurements. In poultry, total tract digestibility measurements would be the most common. Slope ratio experiments are most commonly used for bioavailability experiments.

Assessment of traits sensitive to the mineral under investigation: It is extremely critical to assess traits that are sensitive to the mineral under investigation. For calcium or phosphorus, bone traits are the most sensitive traits to measure. Bone breaking strength and bone ash weight are very sensitive to calcium and/or phosphorus levels in the diet. These two traits respond in a linear manner to graded levels of bioavailable calcium and/or phosphorus in the diet provided that the diet is in the deficient range. Thus, they are the best criteria to use in slope ratio studies.

Other traits are sensitive to levels of certain minerals in the diet. Blood hemoglobin levels are an excellent measure of iron adequacy in studies involving iron. Serum concentration of glutathione peroxidase is often used as an indicator of selenium status in selenium studies. The level of radioactive iodine uptake by the thyroid or weight of the thyroid gland may be used in studies involving iodine adequacy. Bone zinc

levels are often used in zinc studies. Such responses to these minerals are generally more interpretable if the animals are fed depletion diets prior to the test period. Unfortunately, many of the minerals do not have specific, easily measurable traits that can be used for bioavailability studies.

Product quality: In general, improvements in mineral content or bioavailability are not expected to have major effects on the quality of products derived from animals. However, in some instances, a higher level or greater bioavailability of a mineral in the feed may increase the level of that mineral in meat, milk, or eggs. Therefore, the emphasis of experiments designed to assess product quality will be to measure whether there are any unexpected/unintended effects that either increase or decrease product quality.

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Figure 8-1. Initial steps

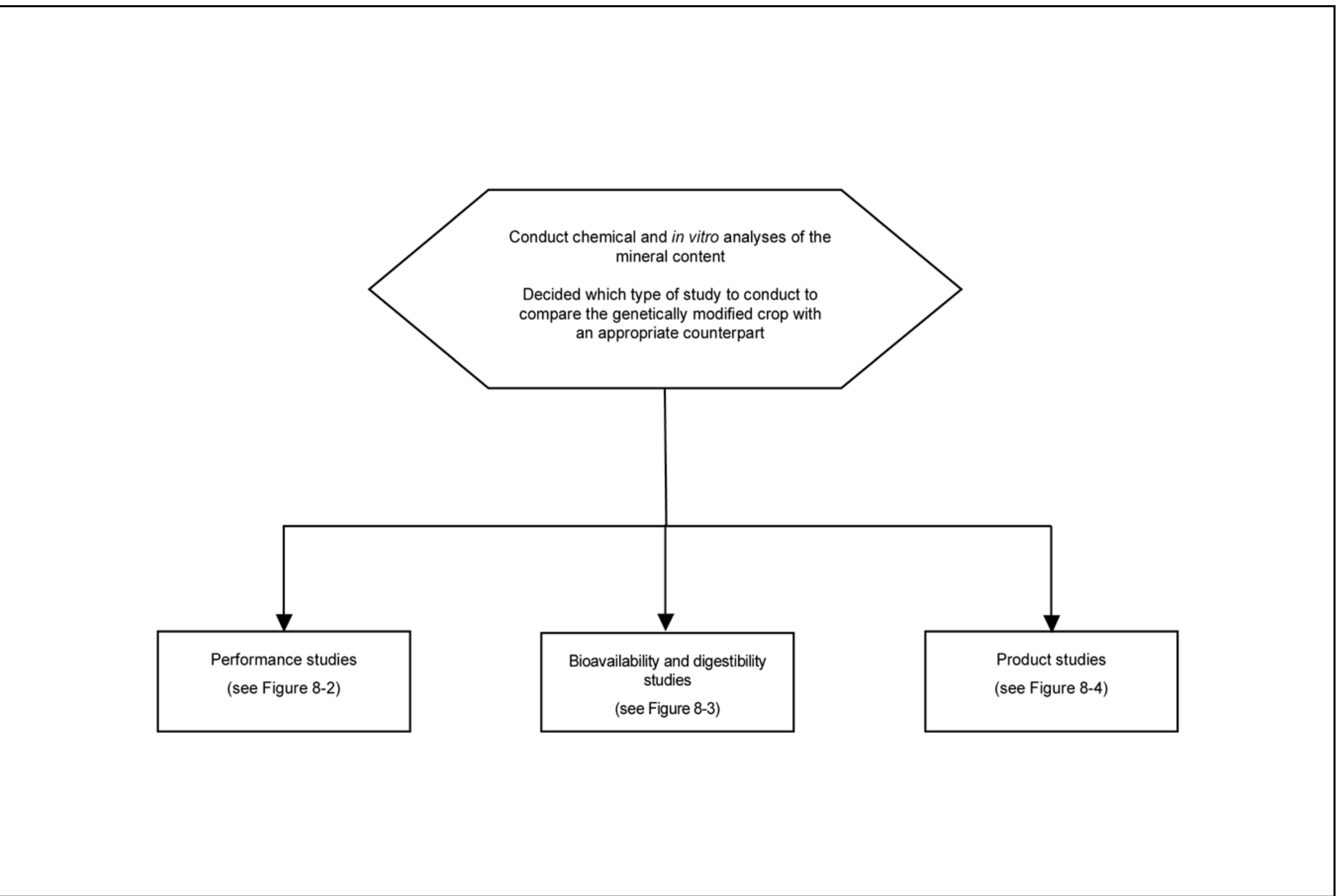


Figure 8-2. Performance studies

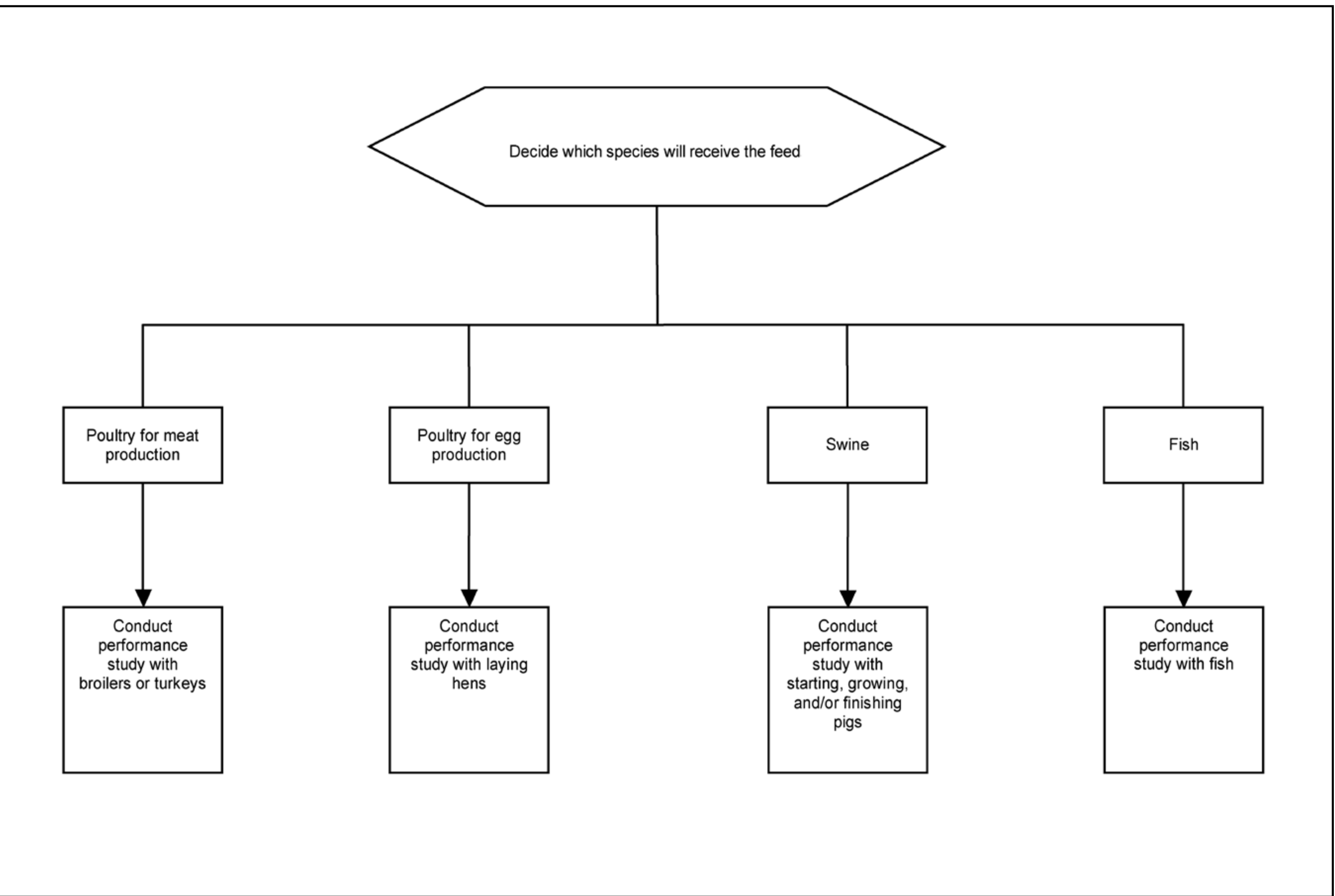


Figure 8-3. Digestibility and bioavailability studies

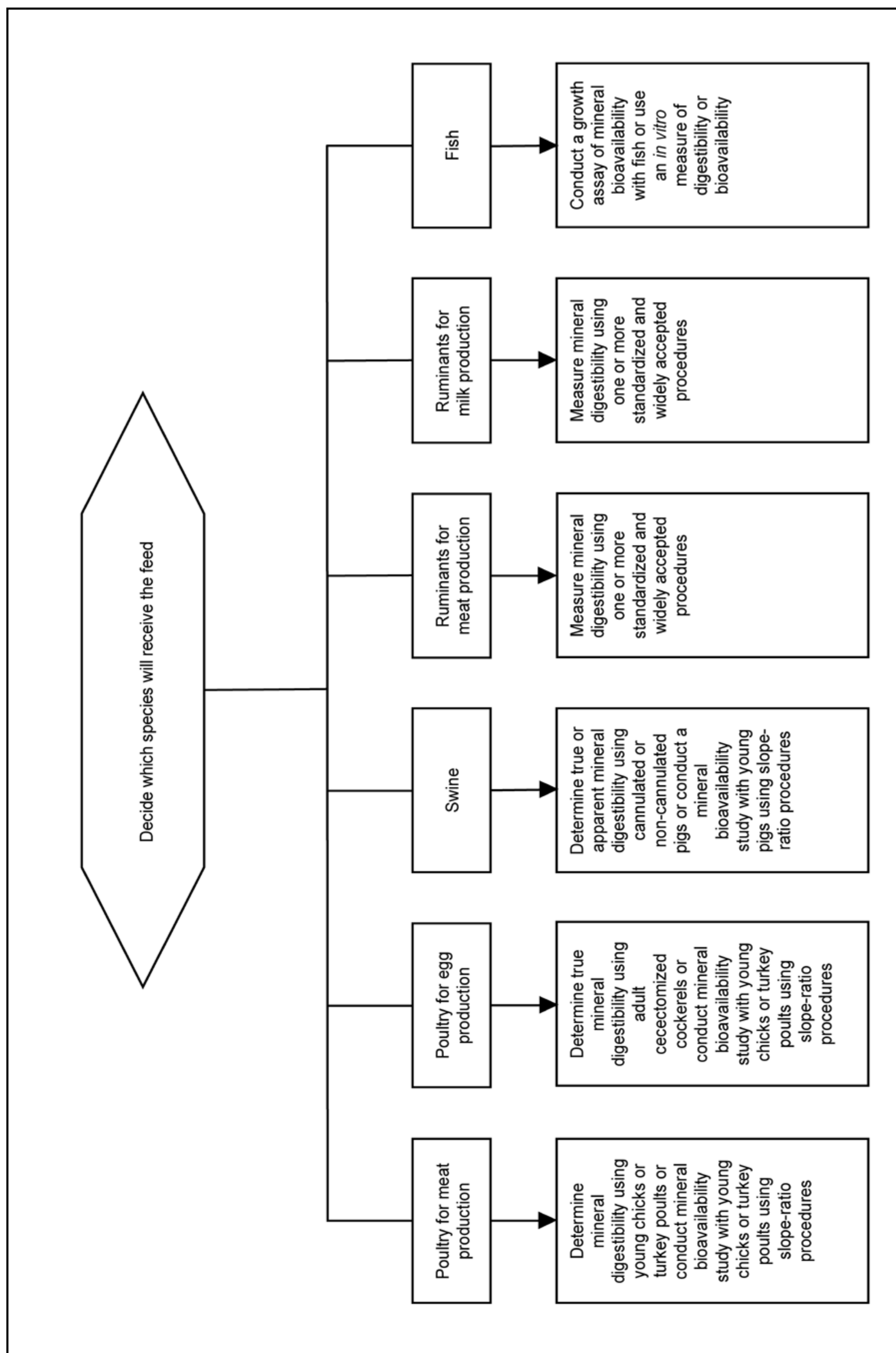
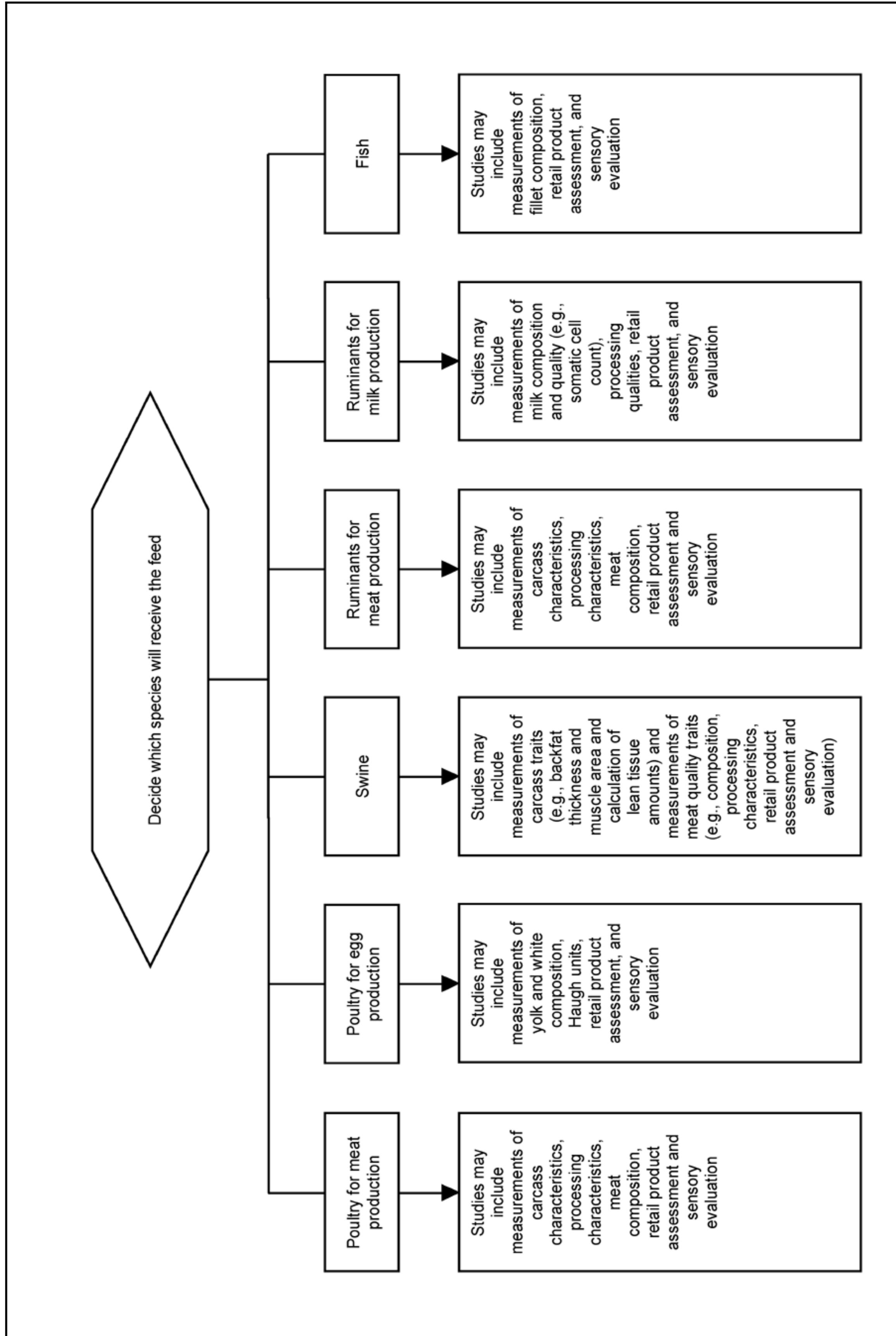


Figure 8.4 Product studies



Chapter 9

Enzymes

The Concept of Enzymes in Animal Nutrition

From an evolutionary viewpoint, it may not be obvious why exogenous enzymes would benefit animals offered feeds to which they are accustomed. Animals that eat grains, for example, would be expected to have evolved with a digestive system tailored to efficient degradation of grains. It is evident, however, that live performance (among other metrics) of many classes of livestock is improved when they are offered appropriate exogenous enzyme(s) with their diet. Part of the reason for such benefits is that grains and forages contain indigestible or poorly digestible fractions that may also act as antinutrients if present in sufficient quantities. When animals are not given a choice to select their own diets, the concentrations of antinutrients may be greater than what the animal is accustomed to.

Delivery of exogenous enzymes (fermentation derived) that address specific antinutrients has been shown for many years to benefit poultry, swine, and, to a lesser extent, ruminants and fish (Beauchemin et al. 2003, Bedford and Schulze 1998, Rodehutscord and Pfeffer 1995). Research during the last 10 years has shown that digestibility in poultry is limited during early life stages, and birds may benefit from the addition of enzymes during this period. The advent of techniques enabling expression of such enzymes in plants during the past 15 years has raised interest in such plant-derived enzymes and about whether such enzymes would differ from those derived from fermentation. This chapter focuses on the status of the current market in terms of enzymes used and their purported mode of action. It also recommends methods to determine the relative worth of plant-derived versus fermentation-derived animal feed enzymes.

Enzyme Classes

Generally, exogenous feed enzymes are used to digest fractions of the feed that the animal ordinarily does not digest because of an inadequate amount or total lack of a relevant enzyme. Examples are phytases, which degrade phytate (hexa-inositol phosphate); enzymes that target plant cell walls (e.g., endo- and exocellulases); xylanases; pectinases; galacto-mannanases; phenolic acid esterases; ligninases; and others such as α-galactosidases. Enzymes used currently also include classes that are present in the host animals, but target the fractions of the diet that the host may not completely digest. Examples are amylases, proteases, and, to a far lesser extent, nucleases and lipases/esterases.

Although all exogenous enzymes currently used in animal nutrition are fermentation derived, it is clear there is interest in delivering such products from plants. The most commonly cited potential advantage of plant delivery is reduced cost. Other potential benefits are also foreseen and are discussed later in this chapter. It is envisaged that feed enzymes produced in plants may be delivered as low inclusion rate additives (much as the current fermentation derived products), where a small dosage of plant-derived enzyme is sufficient to treat the whole diet, or as much larger inclusion rate feed ingredients (e.g., as genetically modified corn that contains the enzyme of interest but which is fed at much higher levels of inclusion, as a feed component rather than a feed additive).

Current Enzyme Usage and Potential for Plant-derived Enzymes

Commercial usage of enzymes is most common in poultry and swine diets, where the most consistent benefits have been shown and are generally cost effective (Bedford and Schulze 1998). Plant delivery and efficacy of phytase has been demonstrated (Pen et al. 1993, Beudeker and Pen 1995), but the technique has yet to reach market. Potential reasons for the lack of commercialization are discussed below.

Non-starch polysaccharide digesting enzymes, for ruminants in particular, have been shown to enhance rate and, less frequently, the extent of digestion of forage and grains, with the response often being quite extensive but variable (Beauchemin et al. 2003, Beauchemin et al. 2004). Enzyme dosage rates generally need to be very high to elicit such responses, however, and this has limited commercial usage. In fish nutrition, responses can be significant but varied (Rodehutscord 1995, Rodehutscord and Pfeffer 1995), but commercial use is constrained by the extreme pelleting temperatures used in the industry, which limits methods of inclusion to post-pellet liquid application. This is a problem because it is more costly and is difficult to achieve even distribution.

As previously stated, use of the appropriate enzyme

enables greater use of dietary ingredients that contain poorly digestible structures. As a result, ingredients that previously were used with caution can be used at greater inclusion rates and, in general, there is improved nutrient extraction from such diets. The combination of these two effects enables a reduction in the inclusion rate of expensive concentrates (fats, protein supplements, and inorganic phosphates) needed to meet nutrient requirements. Nevertheless, few enzyme classes are currently used. Several explanations exist, the first four being generic and the last two being more specific to expression in plants:

- Enzyme production costs are excessive relative to the value created and thus the product is unsustainable.
- The availability of suitable sources of a particular enzyme may be limiting (e.g., ferulic acid esterases), and those available for test may be impure thus clouding interpretation as to whether the putative enzyme class is indeed of value.
- Target substrate concentrations may vary markedly in complete rations due to variable inclusion rates of the ingredient of interest, resulting in inconsistent economics (e.g., α-galactosidase targeting soybeans).
- The benefit of the enzyme may not be well proven or may even be negative despite evidence that digestion of the target substrate is poor (e.g., lipases).
- The enzyme of interest may be detrimental to the growth of the plant-production system. For example, cellulases would be expected to degrade production plant cell walls if produced in such a way that they were active in the plant. See section on undesirable effects.
- Enzyme release may be inconsistent. Fermentation enzymes are either sprayed onto a carrier or coated with a known thickness of a protective agent. Plant-derived enzymes are produced within plant cells and thus need to either be extracted, in which case they are largely equivalent to fermentation produced enzymes, or applied as milled plant material, in which case delivery from within cells may be varied.

Nevertheless, production of feed enzymes in plants may provide several benefits compared with traditionally fermented products. These potential benefits include:

- Production cost could be much lower than that of the fermentation-derived counterpart. This cost advantage not only provides the enzyme producer with a better competitive position, but may also allow use of far greater enzyme concentrations than would be considered economical with conventionally produced enzymes. This is of considerable interest for enzymes such as phytase, where the current rate of commercial usage is clearly well below the optimum for animal performance because of cost constraints (Rosen 2002). As a result, plant-derived production of enzymes may enable extended use in nonruminants and particularly in ruminants, where use is currently not economical.
- There is a potential for physical protection from processing and/or digestion. If an enzyme were to be expressed in a seed that is ground before feeding, then the physical location of enzyme within intact cells will likely provide significant protection from the steam applied during pelleting (see comments under *Initial Enzyme Characterization*). Traditional fermentation-derived enzymes are usually sprayed onto a carrier and thus are externalized compared with plant-derived enzymes. In some cases, this drives the manufacturer to coat the enzyme granule with a hydrophobic carrier, which protects the enzyme but may reduce efficacy because the enzyme must escape the coating *in vivo* to be active (Klein Holkenborg and Braun 2001, Wilson and Ward 2002). There is no such apparent delay in release of plant-derived phytase because the efficacy compared with an uncoated fermentation product was similar, if not marginally superior (Pen et al. 1993).

Intended Outcomes

Cell Wall Degrading Enzymes

Generally, the goal of enzyme use is to improve digestibility of the entire diet by disrupting both insoluble and soluble cell wall material and, hence, accelerating digestion. Xylanases (also referred to as pentosanases, hemicellulases, and "glycanases") and cellulases (also referred to as β-glucanases) constitute the vast majority of this class of enzyme in commercial use. Xylanases are used predominantly in diets rich in wheat, triticale, and rye (because these cereals contain significant quantities of antinutritive arabino-xylan), whereas cellulases are used in diets rich in barley or oats (because these cereals are rich in antinutritive β-glucans). It is thought that their beneficial activity is brought about by:

- Destruction of cereal cell walls, which increases exposure of the contents of the cells to the intestinal lumen, hence increasing the rate of digestion (Campbell and Bedford 1992)

- Depolymerization of soluble arabinoxylans or β-glucans may reduce intestinal viscosity, thereby increasing solute diffusion rates and hence accelerating digestion (Bedford and Classen 1992).
- Depolymerization of soluble and insoluble arabinoxylans, which subsequently provides soluble oligomeric and monomeric sugars that can be used by certain beneficial species of luminal bacteria. Thus, there is a benefit to the health and integrity of the intestine and hence improved animal performance (Bedford and Apajalahti 2001).
- Disruption of cell walls by exogenous enzymes will increase binding sites for rumen bacteria and increase the rate, but perhaps not the extent, of diet digestibility as a result (Nsereko et al. 2000a, Wang et al. 2001)

Other Cell Wall Degrading Enzymes

Pectinases: The goal is disruption of cell walls, principally of dicotyldenous protein sources such as soybeans and pea, to gain access to contents (Cowan et al. 1999a, 1999b). Some pectins may be viscous, but this does not seem to be their principal antinutritive activity.

Mannanases: The purpose of employing mannanases is cell wall dissolution, particularly in soybean meal (Jackson et al. 1999). The mechanism seems to be split between cell wall disruption, reduced viscosity, and provision of oligosaccharides, which either act as substrate for beneficial bacteria or act as an alternate binding site to the intestinal wall for growth-depressing bacteria, in which case they act to flush out potential pathogens (Lee et al. 2003, Jackson et al. 2004)

Enzymes that target lignin and phenolic acid esters: It is recognized that the digestion rate of many raw materials, particularly for ruminants, may be limited by the content of lignin and ester linkages (Black 2001, Yu et al. 2003) because these are responsible for much of the structural integrity of plant cell walls. As a result, there has been some interest in using enzymes targeting these substrates, although it has been suggested that current offerings are not cost-effective, possibly because of the lack of such products that are small enough to penetrate the cell wall and bring about the desired effect (Chesson 1994).

Phytases

Increasing the digestibility of plant phytate (hexa-inositol phosphate) through application of an exogenous phytase will release phosphorus for use by the animal (Selle et al. 2000, 2006; Hatten et al. 2001), which is of most benefit in phosphorus deficient diets. The benefit in terms of reduced phosphorus pollution is evident. Phytase action will also provide inositol, which is an important nutrient for some aquatic species.

Not only is phytate a source of nutrients, it is also an antinutrient that binds to metal ions with various degrees of tenacity and to proteins and starch, thereby reducing the rate of nutrient digestion/absorption. Some recent data also suggest that phytate promotes increased rates of endogenous losses and thus its removal will not only enhance dietary nutrient digestion, but also limit expenditure of endogenous resources in the digestion process (Selle et al. 2000, Hatten et al. 2001, Cowieson et al. 2004).

Other Enzymes

Other enzymes currently employed include enzymes that augment those already produced by the host, but are not produced in sufficient quantities due to disease or neonatal metabolism. As a result, amylases and proteases are regularly found in commercial products today. The scale and frequency of responses observed from use of proteases or amylases alone is limited and often associated with other activities such as cell wall hydrolyzing enzymes.

α-Galactosidases target the raffinose series of oligosaccharides, which are indigestible but fermentable carbohydrates present in soybeans and other legumes. They are thought to disturb digestive processes, particularly in young pigs, through their effects on intestinal proliferation of undesirable bacteria.

Undesirable Effects

The potential undesirable effects of plant-derived enzymes can be segregated into two main topics, effects on agronomic traits of the production host and effects on the animal subsequent to feeding.

Effects on Agronomic Traits

With reference to potential undesirable effects on agronomic traits, a clear challenge exists for a plant-derived enzyme designed to digest plant components. If the enzyme is activated during the synthetic/storage process and comes into contact with its substrate, then it will start to digest the substrate in the host plant manufacturing it. Depending on the enzyme of interest, the effects can be varied but generally fall into three categories:

- Reduced seed fertility and/or plant yield due to destruction of a critical component for growth or reproduction. Phytate, for example, is a phosphorus source for the seed, so significant reduction of seed phytate may reduce fertility. Cell

wall degrading enzymes will clearly compromise growth (yield) if activated at the wrong time by degrading the very cell walls encapsulating the enzyme. Therefore, a clear requirement for plant-derived enzymes is that they must not interfere with the target substrates in the host plant.

- Reduced quality of the plant-derived product. For example, delivery of proteases and amylases in seeds risks significantly degrading the quality and integrity of endosperm starches and proteins. This may not only influence yield, but could also potentially cause production of an appetite suppressant (e.g., presentation of bitter peptides) or accelerate storage losses (e.g., through production of free sugars).
- Neutral to positive effects. Marginal degradation of high-amylose starch by amylases and removal of oligosaccharides before feeding, such as the α-galactosides in soybean meal, may improve the intrinsic feeding value of the seed employed. This is only of significant relevance, however, if the GM seed is a dominant constituent of the diet, since the enzyme will be less effective on the non-GM version of the enzyme delivery plant material in the diet.

Effects on Animal Performance

The response to enzymes generally increases with dose to a maximum, with further increments in dose either resulting in few, if any, further problems in terms of performance (e.g., phytase) or a reduction in performance as a result of enzyme product overproduction. Thus, undesirable effects of an exogenous enzyme on animal performance fall into one of two categories:

- *Underdosage.* A lack of adequate enzyme dosage may be caused by either accidental underdosing or the presence of more substrate/inhibitors than anticipated. Undesirable effects due to underdosage will be common to all enzymes
- *Overdosage.* This can lead to production of excessive amounts of "product" that interact negatively with the host. Undesirable effects due to overdosage tend to be enzyme-related.

Effects from specific enzyme groups

Phytases: Using phytases in diets already sufficient in phosphorus will result in degradation of insoluble phytate phosphorus into soluble phosphorus, which will be excreted in the urine. This will likely result in increased content of soluble runoff phosphorus in the manure, which is the most damaging form of this element. However, it is clear that if diets are properly formulated, phytase addition can contribute significantly to reducing phosphorus pollution (Ibrahim et al. 2000, Waldroup 2002). Some data suggest that if reformulating the diet to account for the phosphorus released from phytase does not also consider the benefits on calcium digestibility, then a wet litter problem may ensue, which appears to be acid/base related as reported from commercial experiences (M.R. Bedford, AB-Vista Feed Ingredients, United Kingdom, personal communication, 2007).

Xylanases: Excessive doses may result in release of free pentose sugars that are not well utilized by nonruminants (Schutte 1990, Schutte et al. 1991), and may ultimately create an osmotic problem leading to loose feces in both pigs and poultry.

Cellulases: The effects are not as well documented as for xylanases, but are presumed to be the same.

Evaluation Methods

A systematic approach to deciding what studies to conduct is provided in Figures 9-1 to 9-5 (see pages 118–122). The required studies clearly depend on the intended commercial use of the plant-derived enzyme. The studies would be designed in a similar way, regardless of enzyme source investigated (i.e., derived from a plant or through fermentation), although the comments listed in the section on general recommendations/points to consider should be reviewed before study initiation.

Considerations

Initial evaluation (see Figure 9-1) should be *in vitro* to determine whether the enzyme's characteristics are similar when delivered from a plant compared to a fermentation source if it already exists. Regardless of whether the enzyme is extracted from the plant or delivered in ground plant material, it is helpful to examine biochemical characteristics including:

- pH optima, temperature optima, kinetics (Km, Vmax, and kcat), substrate specificity, and relative susceptibility to inhibitors. These will give some insight as to whether the enzyme has characteristics suitable for the environment in which it is to perform the task at hand. In most cases, it is assumed that the enzyme can function either in the conditioner during pelleting (xylanases in poultry feed; Silversides and Bedford 1999), and/or on the feed post-application (xylanases and cellulases in ruminants; Hristov et al. 1998), and/or in the intestinal tract (xylanases, cellulases, and phytases in all species).

- Effect of variations in thermal shock (30–120 seconds) on subsequent activity. If the enzyme is to survive pelleting, then such thermostability is desirable.
- Miscibility of enzyme in feed and stability to storage over a period of time. Very low inclusion rates can lead to poor enzyme distribution within the feed. It has been shown that animal performance can suffer if an essential nutrient is poorly mixed (McCoy et al. 1994).
- Resistance to digestive enzymes. Enzymes that need to function in the digestive tract will have an advantage if they are resistant to hydrolysis up to, and including, the section of the intestine in which they are to function. An example is phytase, where activity in the animal is concentrated in the gastric phase of the intestinal tract and there is a clear difference in pepsin stability between *Aspergillus*- and *E. coli*-derived products in favor of the latter (Igbasan et al. 2000).

Methodology

Enzymes are used for several reasons and the following four are the most commonly cited. These influence the study type required to demonstrate efficacy.

(1) Enzymes are most often used to improve nutrient digestibility such that diets can be reformulated to save costs and or reduce environmental pollution. If an enzyme improves a diet's digestibility, then the best response will be achieved in diets that are marginally deficient in nutrients before adding the enzyme. In many ways, an enzyme should be viewed as a pronutrient (i.e., an additive that increases the delivery of nutrients from a diet to the host). If such an additive is supplemented to a diet that already supplies all the nutrients required for optimal growth, it will not give a beneficial response. Digestibility studies are therefore recommended as a starting point to determine the extent to which nutrient specifications can be reduced when enzymes are included without compromising performance. *In vitro* simulation models may be the first step, but these need to be considered only as an initial screen because they do not always reflect animal response to enzymes. While some cell wall degrading enzymes may first be screened in simple viscometric assays (Bedford and Classen 1993, Malathi and Devegowda 2001), there are more sophisticated *in vitro* digestibility assays that may be considered for other enzymes if there is a need to limit animal experimentation (Marteau et al. 1997, Moughan 1999). However, caution is strongly advised with respect to interpreting any *in vitro* assays. There must be a clear and unequivocal correlation between animal performance and response in such an assay before much weight is put on its outcome. This is because much of the response to enzyme addition is due to interaction with the host (Cowan et al. 2001, Geraert et al. 2001, Kocher et al. 2003, Scott et al. 2003) rather than a specific and direct effect on the diet, an outcome that is very difficult to mimic faithfully in an *in vitro* assay. In terms of animal digestibility trials, the following should be considered:

- The use of a marker for total collection is up to the individual researcher, but the animal's age and sex and the diet's ingredients should closely mimic the conditions that will be encountered in practice as closely as possible (Sales 2003a, 2003b). The intestinal site of collection also needs consideration (i.e., ileal or fecal) because the former may be more relevant to likely nutrient capture by the animal, whereas the latter takes into account hindgut fermentation, which may play a role in enzyme function. (Jagger et al. 1992).
- Inclusion of urinary excretion is required if metabolizability is an area of interest (e.g., phytase and pollution). Species physiological differences necessitate focus on metabolizable nutrients more so than digestible for poultry compared with swine or ruminants. Cannulated animals can be used for repeat measurements of dietary effects of enzymes in poultry, pigs, and ruminants. Rumen fistulated animals provide an ideal opportunity to evaluate effects of enzymes on rumen degradability characteristics. However, one must consider that the evaluation is most relevant when the ruminant is fed a diet similar to the test diet in the test polyester bag in the fistula. For example, if the animal was acclimated to a corn silage diet before placing enzyme-treated corn silage samples into the fistula, the results may have more relevance. Such a practice ensures that the microbial profiles in the rumen are relevant to the test in question. Furthermore, the researcher must consider the route of enzyme application that best describes what is planned in practice. The enzyme could be delivered via the concentrate, via a mixer wagon (sprayed onto the composite before feeding), or directly to the rumen in water. Each administration route will influence the response obtained, and it is likely that the administration route's effect will vary depending on the substrate in question (Colombatto et al. 2003, Beauchemin et al. 2004).

- Although it can be of great use in determining an enzyme's potential value, evidence collected from a digestibility trial always needs to be validated in animal growth or milk or egg production studies. If

enzyme response is mediated through modulation of endogenous losses (Cowieson et al. 2004) or microfloral profiles (Bedford and Apajalahti 2001, Sohail et al. 2003), then acute digestibility studies or those involving prior starvation periods will possibly misrepresent the enzyme's value. It is, therefore, strongly recommended that growth or milk or egg production studies be performed to validate the determined nutrient digestibility benefits.

(2) Improvement in animal performance when the enzyme is used in formulations where no nutrient adjustments are made to take its effect into account. Such sub-optimal formulations do exist in the commercial world because the optimum dietary concentration for a given nutrient in terms of performance is often not the same as the commercial optimum concentration (from an economic viewpoint). Bearing in mind the comments in the above section, the deficiency in the nutrient(s) that the enzyme can replace must be sufficiently large for a response to be observed. Phytases, for example, may be tested in diets with phosphorus, calcium, energy, and amino acids supplied at suboptimal levels. The type of performance trial recommended depends on the animal in test and the likely trait of interest, such as egg weights, milk yield, body weight gain, and bone strength. The experimentation period and age/sex of the animals used should be relevant to the enzyme's use under the proposed commercial conditions. The trait of interest should reflect the likely response to the enzyme (e.g., intestinal viscosity and feed conversion ratio for cell wall degrading enzymes, bone strength and phosphorus digestibility for phytase, rate and/or extent of digestion for ruminant enzymes). It is also essential that appropriate diet formulations are applied; thus, phosphorus-deficient diets are required for phytases, whereas energy-deficient diets are suggested for cell wall degrading enzymes. Amino acid-deficient diets can also be used if previous digestibility work suggested that the enzyme has some effect on this situation. Some effects are readily apparent after a very short time (e.g., feed conversion ratio benefits of a xylanase in a rye-based diet), whereas others require a significant period for the benefit to show (e.g., xylanase or phytase in marginal laying hen rations, cell wall degrading enzymes in marginal dairy rations); thus, the benefit may require a longer period of study to elicit the response and value of the enzyme.

(3) Reduction in wet litter/dirty eggs. The animal trial needs to be conducted over the relevant periods to show an effect when wet litter/dirty eggs need most control. It is likely that wet litter is due to only a few individuals rather than a general population effect, and, as a result, it is essential that significant numbers of animals be used to capture this effect if it exists.

(4) Improvement in gut health. Age is a key consideration along with diet formulation and relevance of challenge model if used. Some challenge models are highly artificial and may not represent the disease etiology under commercial conditions. It is recommended that the challenge model replicates the etiology of the disease in practice as faithfully as possible.

General Recommendations/Points for Consideration for Studies

Initial Enzyme Characterization

Units: Enzyme activity units are defined at a particular temperature, substrate, and pH and using a specific technique for measuring either substrate disappearance or product appearance. The user must be aware of the following:

- Such assays are useful for assuring and controlling quality and for determining enzyme stability through feed processing or the digestive tract.
- In almost all cases, these conditions differ substantially from those under which the enzyme works in the animal. As a result, enzyme source comparisons should not be based on such assays.
- When considering plant-derived enzymes, as opposed to fermentation-derived products, extraction of the enzyme may prove more difficult if pre-digestion of the cell walls is required to release the enzyme. Work may be required to optimize such extraction techniques.
- If thermal stability of a plant-derived enzyme is of interest, then it should be compared against liquid post-pellet applied products and heat-stable granulate fermentation-derived enzymes at the target pelleting temperature, preferably at several doses. Small-scale conditioning/pelleting trials are recommended. *In vitro* thermal stress tests should be used with caution because they invariably do not mimic commercial pelleting accurately. For example, a feed mill may state that conditioning and pelleting takes place at 85°C for 30-45 seconds, but this is achieved through use of steam at approximately 2-3 bars of pressure, which is almost 130°C. Thus a static thermal stress in a test-tube of 85°C is unlikely to replicate the scalding an enzyme will experience in a conditioner.
- One may account for the presence of endogenous enzyme activity by including a near isogenic control(s).

Range of Doses: All studies should look at a range of doses and establish a response curve. The

optimum biological and economic usage rates can then be determined and the effects, if any, of accidental overdosage understood.

Point of application: Enzymes can be applied pre- and post-pelleting for nonruminants. Thermostable enzymes need to be proven capable of surviving as described above before use. Thermolabile enzymes need to be applied as a liquid post-pelleting (i.e., extracted from the plant material and stabilized in a solution that is not too viscous to allow spraying). For ruminants, it is also necessary to understand whether the enzyme is most effective if applied on the forage, the compound feed, or at the mixer wagon.

Diet Formulation: It should be as close to that in which the enzyme is to be used commercially and should take into account the following:

- Phytases: Calcium and phosphorus concentrations in the diet; energy and protein concentrations in the diet; ingredient phytate content (i.e., availability of phytate as a substrate); other diet attributes (e.g., vitamin D, coccidiostats, fat content and level, feeding program)
- Cell wall degrading enzymes: energy and fat quality and content of diet; cereal type and species; processing conditions of the diet; age of animal; ambient temperature (ruminants); health status/conditions

Inclusion Rates

Enzyme Concentration: Plant-derived enzymes need to be concentrated enough so as not to need inclusion at rates that significantly change the diet composition. For example, if the enzyme is supplied in rapeseed and added to a corn-soybean meal diet but adding significant quantities of seed (e.g., >5% of the diet) is required to deliver the target dose, any observed response may be due either to the enzyme or the "carrier" plant material. In the case of rapeseed as a delivery vehicle, it may also present problems for some livestock classes (e.g., brown layers).

Site of Enzyme Deposition: Sites such as the seeds, leaves, and stems can influence the product's efficacy. The intracellular compartment in which the enzyme is secreted will influence activity and stability. If the enzyme is secreted into the aleurone layer, for example, then this may delay the release of the enzyme into the intestine because of its indigestibility in nonruminants.

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Figure 9-1. Initial steps

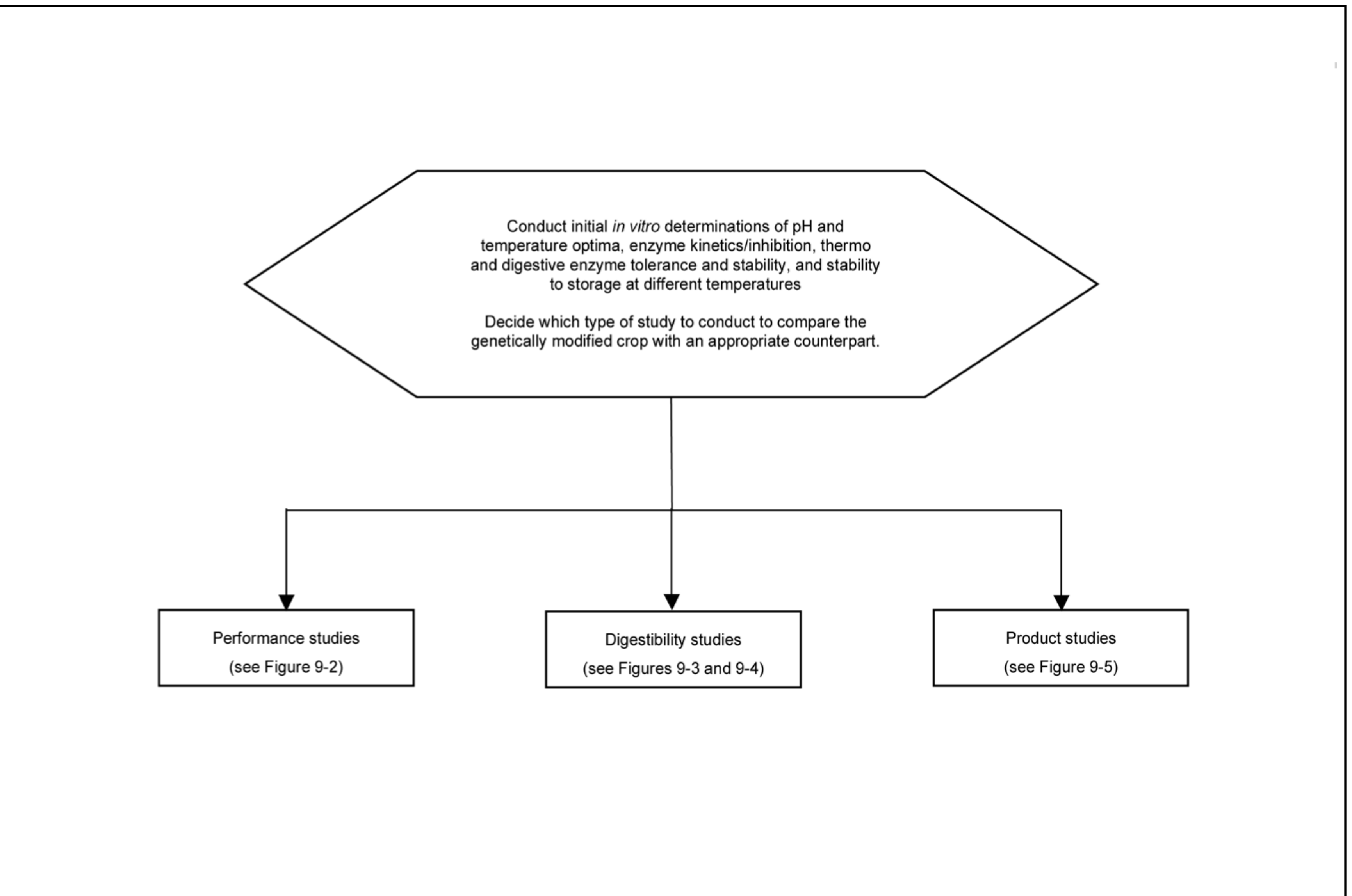


Figure 9-2. Performance studies

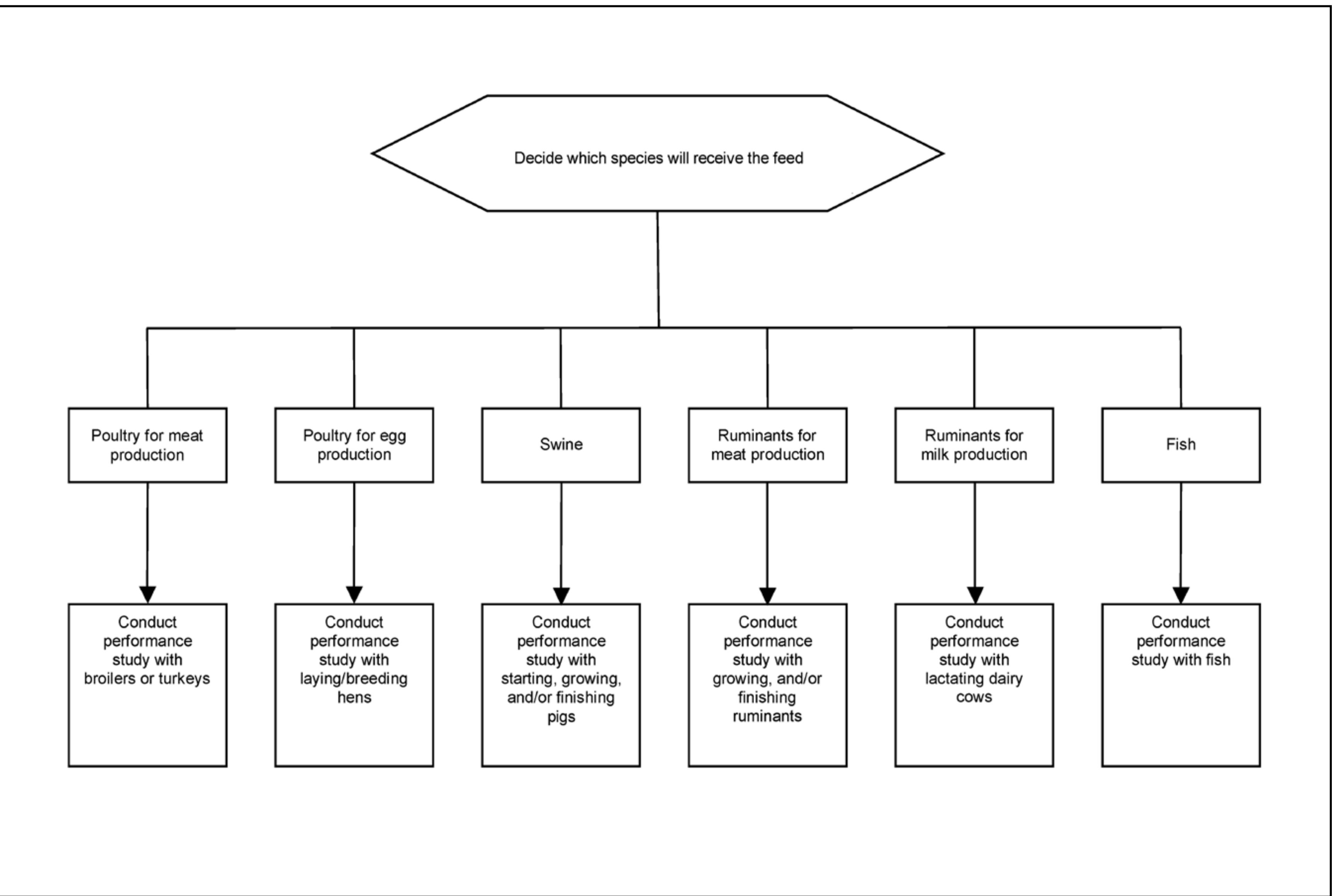


Figure 9-3. Digestibility studies

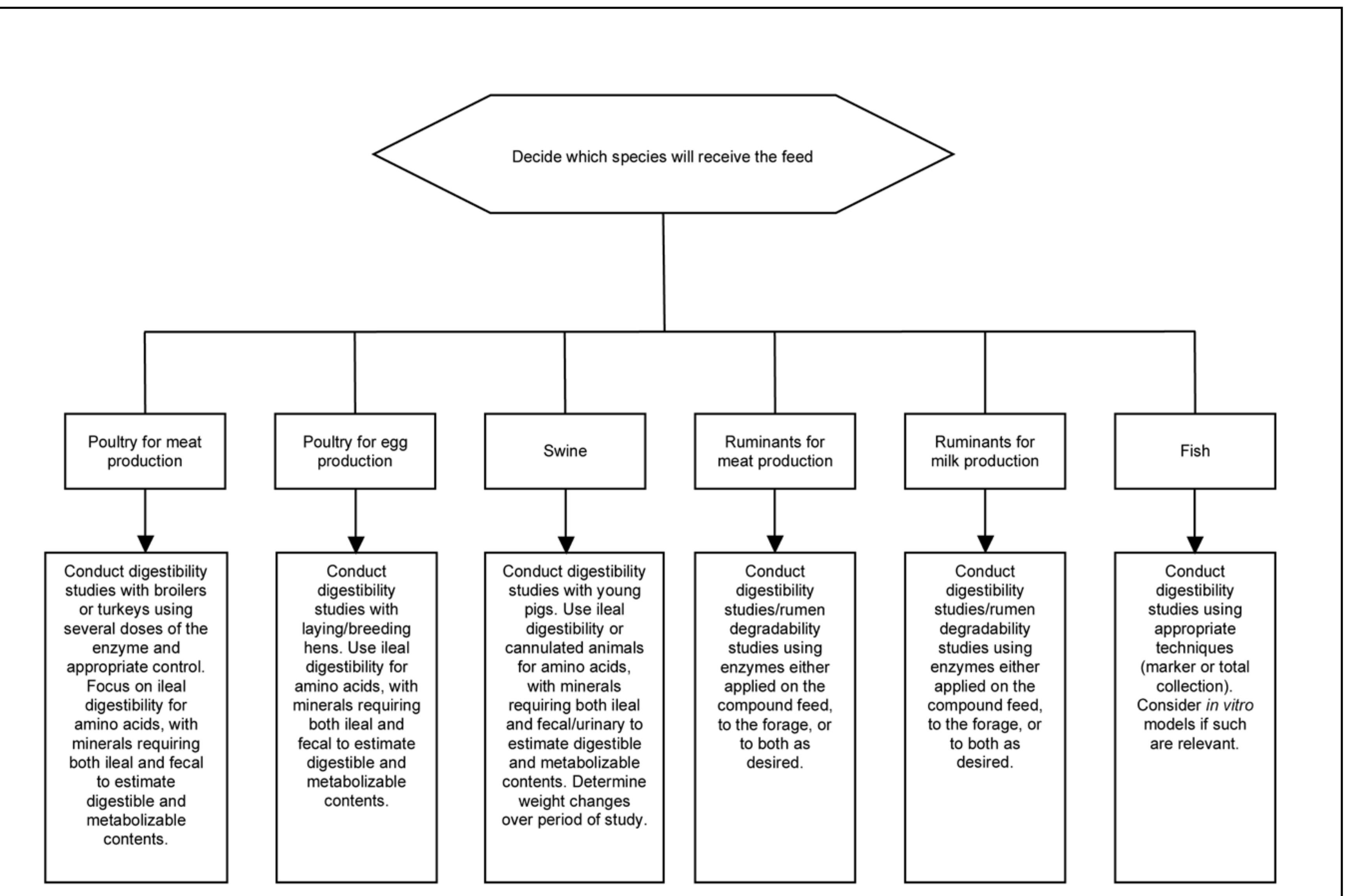


Figure 9-4. Digestibility and metabolism studies: apparent and true metabolizable energy

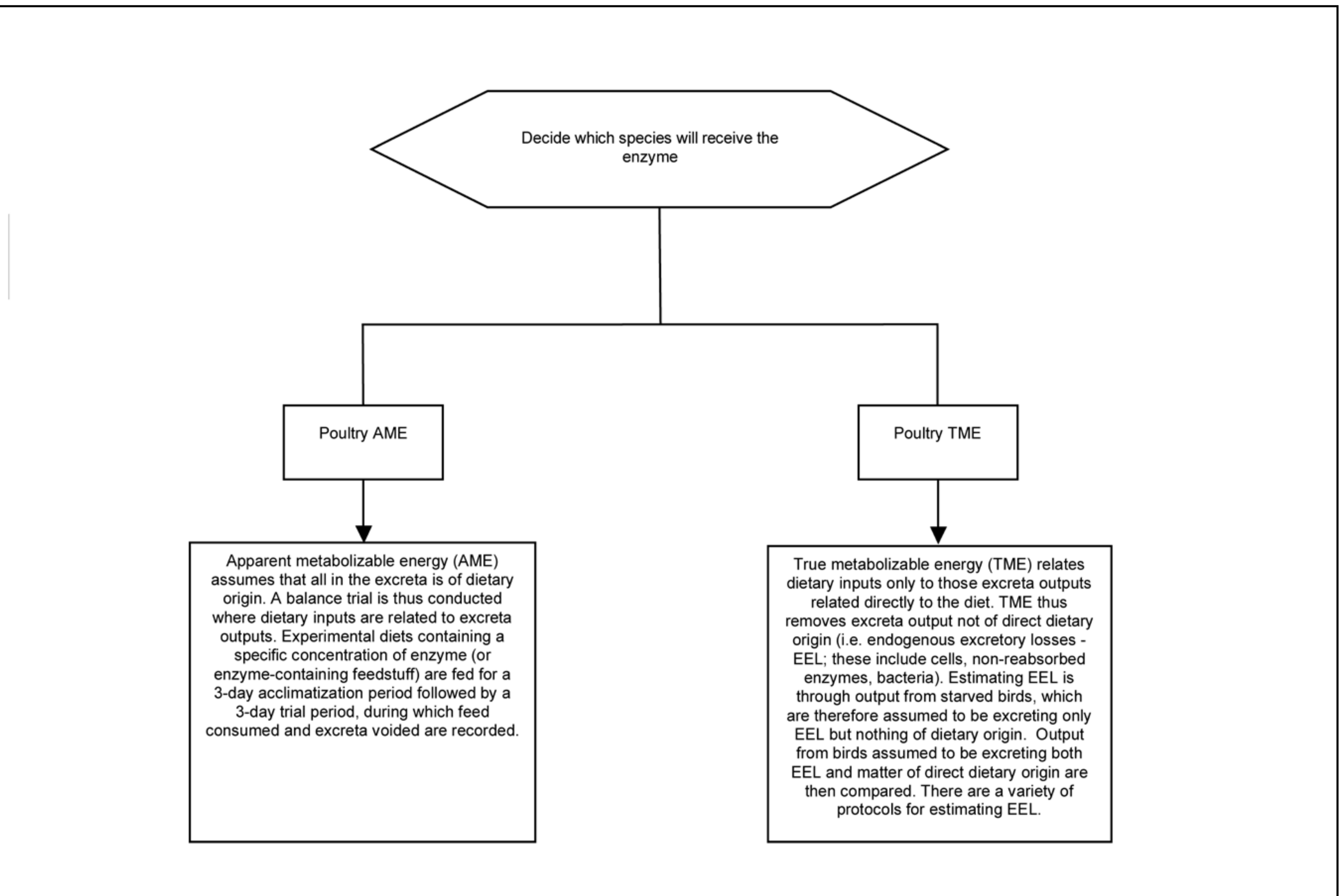
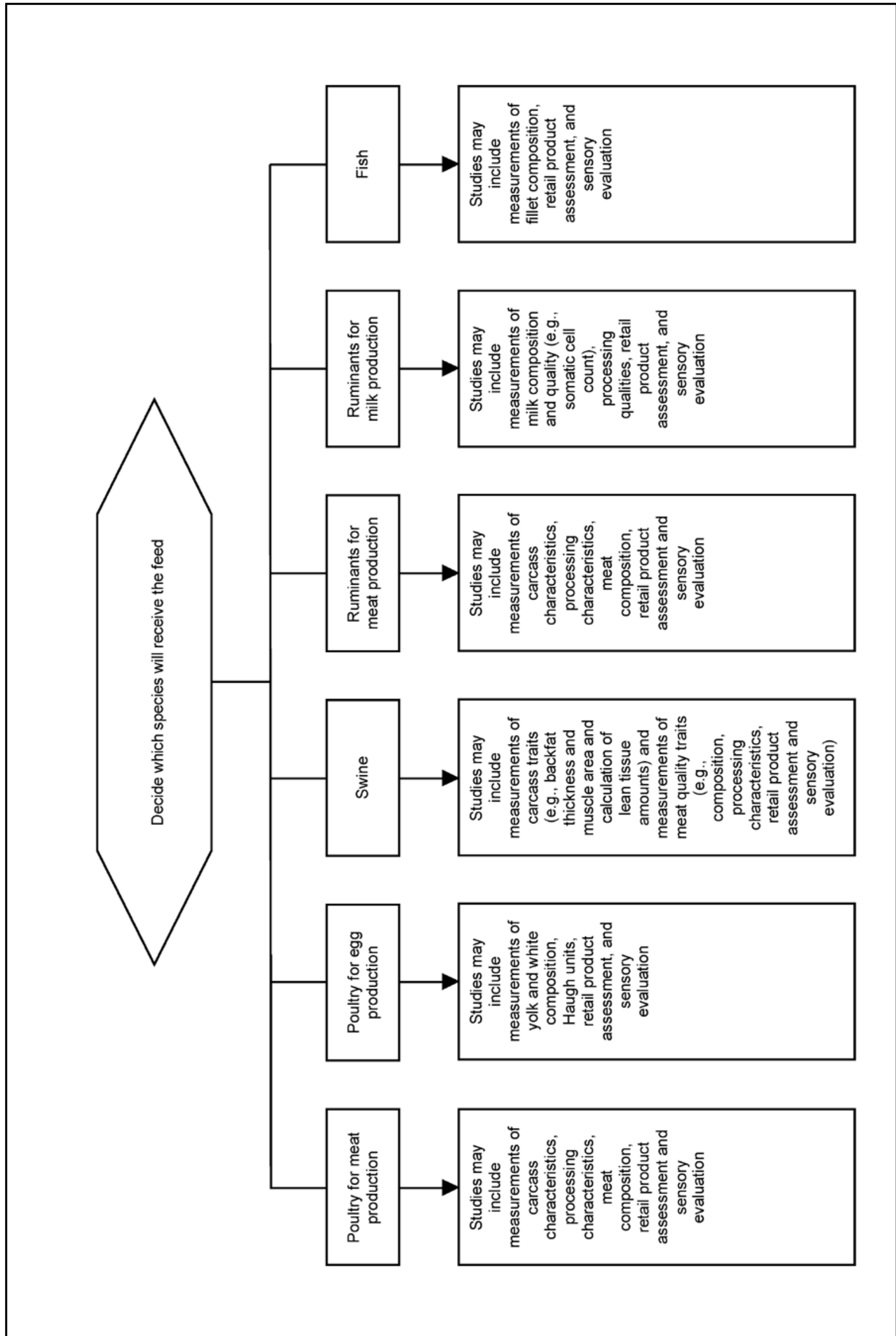


Figure 9-5. Product studies



Chapter 10

Antinutrients

A major goal in the breeding of plants grown to provide foods for animals and humans is the reduction of traits that diminish their inherent nutritional value (Mertz 1975). In many crops, this approach has resulted in greater nutritional improvements than breeding schemes aimed at increasing nutrient levels. Genetic modification of plants to change the level of antinutrients improves the efficiency of the conversion of feed into animal products as judged by either nutritional efficiency or monetary profitability. It is necessary to understand the array of antinutrients made by plants, their role in plant biology, and their effects on animal nutrition and health to rapidly realize the agricultural benefits associated with altering antinutrient levels in feedstuffs.

Antinutrients and Their Roles in Plants

Definitions

Definitions of plant toxicants and antinutrients are not harmonized across the various scientific disciplines and regulatory agencies (Novak and Haslberger 2000). Plant toxicants are usually defined as a broad group of molecules that produce adverse physiological responses in man or animals (Liener 1980); antinutrients are a specific category of toxins. Most often, antinutrients are understood to be endogenously produced plant substances that directly inhibit important pathways in the utilization of nutrients by animals. Antinutrients have their negative effects on feed intake and digestion, absorption and metabolism of specific nutrients. Reduction of economically important production traits, especially efficiency of nutrient utilization, is a common property of antinutrients due to their negative influence on nutrient assimilation. These traits distinguish antinutrients from many other plant toxins, such as carcinogens, mitogens, antimitotics, heavy metals, cytotoxins, hormone disrupters, and allergens, which act mostly via non-nutritional mechanisms.

Functions of Plant-produced Antinutrients

Plants produce antinutrients for a variety of purposes, but plants produce most antinutrients to defend their tissues against herbivorous animals. Considerable ecologic and agronomic evidence illustrates the importance of plant produced antinutrients as determinants of the food choice of herbivorous and omnivorous vertebrates (Foley and Moore 2005, Iason, 2005). Plant structures, such as nutrient-rich seeds, often have high levels of defensive antinutrients. Some antinutrients also have properties that are important for plant structure (e.g. cell wall properties) or functions (e.g. antioxidant and UV protection properties of some polyphenolics).

Antinutrient production has a cost to the plant and competes with growth and reproduction for resources (Cipollini 2004). Consequently, plants usually have mechanisms to increase antinutrient production when herbivory pressure is present. Further, herbivorous animals have developed mechanisms to protect against many antinutrients; these detoxification mechanisms allow greater consumption of plant foods (Foley and Moore 2005, Makkar 2003). Ruminants are especially tolerant due to the protective antinutrient binding proteins produced along the GI tract and due to the actions of rumen microflora. For example, the rumen microflora quickly degrade the trypsin inhibitor in soybean seeds (Hoffmann et al. 2003). Plants adapt by selecting higher levels of antinutrients and by evolving novel forms that circumvent the animal's defenses. Many omnivorous and carnivorous animals lack the extensive detoxification capacities possessed by herbivores and are more greatly affected by antinutrients (Foley and Moore 2005, Iason 2005). Thus, chickens (Leeson et al. 1995) and carnivorous fish (Francis et al. 2001) usually have lower tolerance to foods with antinutritional compounds compared to herbivores such as cattle and horses.

Antinutrients of Concern in Domestic Plants

The number of characterized antinutritional compounds is very large because some classes (e.g. phenolics, saponins) consist of hundreds of diverse molecules. Plants presumably produce this diverse array of antinutritional compounds in response to the evolution of detoxification pathways in herbivores. The major antinutrient compounds found in common foodstuffs are shown in Table 10-1. Although plant foodstuffs usually contain multiple antinutrients, a single antinutrient is often dominant in determining its nutritional value. For example, trypsin inhibitor is the primary antinutrient in soybean seeds and its removal markedly improves its nutritional value, but remaining lectins continue to

prevent their nutritional value from reaching its full potential (Ikeda et al. 1988, Liener 1975).

Table 10-1. Common plant antinutrients

Plant	Antinutrients
Alfalfa	Vitamin antagonists, protease inhibitors, saponins
Barley	lectins, NSP[a], phytate, protease inhibitors
Lupins	alkaloids, protease inhibitors, saponins
Maize	lectins, NSP, phytate, protease inhibitors
Oats	NSP, protease inhibitors, saponins
Rapeseed	glucosinolates, protease inhibitors, phytate, tannins
Rye	NSP, phytate, protease inhibitors
Sorghum	cyanogens, phytate, tannins
Soybeans	Vitamin antagonists, lectins, NSP, phytate, protease inhibitors, saponins
Sunflowers	arginase inhibitor, phytate, protease inhibitors, saponins
Wheat	lectins, NSP, phytate, protease inhibitors

[a]NSP: non-starch polysaccharides

Most unprocessed foodstuffs of plant origin contain antinutrient levels that limit the performance of highly sensitive species such as chickens and salmonids. Even maize, which is considered the best grain for supplying energy to poultry, has sufficient antinutrient levels to reduce its potential energy value (Cowieson 2005). Soybeans, which are the most common protein source fed to poultry and pigs, cannot be fed without extensive processing because of high antinutrient levels.

A variety of simple processing methods are currently used to decrease antinutrient activities in foods and feedstuffs including: dry heating, roasting, boiling, soaking in water, alkali or acid treatment, solvent extraction, ionizing radiation, germination, and fermentation (Liener 1980, Makkar and Becker 1999). A combination of processing methods is generally more effective than a single method. Currently, exogenous enzymes are added to the diet to remove antinutrients that cannot be economically removed by physical means. For example, the feed industry uses phytase to improve the availability of phosphorus in phytate, and xylanases and β-glucanases to improve the utilization of grains that are high in non-starch polysaccharides (NSP). However, none of these methods is capable of completely removing all detectable antinutrients present in feedstuffs.

Mode of Action of Antinutrients

The modes of action of antinutrients are diverse and include: affecting physical properties of the digesta, binding nutrients in an indigestible form, inhibiting digestive enzymes, inhibiting nutrient transport across the intestinal membrane, and mimicking the structure of authentic nutrients. Additionally, animals possess a large family of taste receptors that detect many antinutrients. The bitter taste imparted by many antinutrients results in avoidance of the foodstuff, and a decrease in food intake when suitable alternatives are not available. High levels of some tannins, saponins, and alkaloids can result in sufficient food refusal to cause death by starvation in sensitive species (Liener 1980).

Several excellent books are available that detail the diverse mechanisms by which antinutrients act (Liener 1975, Watson 1987). Examples relevant to the most common feedstuffs fed to poultry and livestock are provided in Table 10-2 along with typical gross pathologies induced by each antinutrient.

Wheat, rye, oats, barley and soybeans store energy in the form of nonstarch polysaccharides (NSP) that cannot be hydrolyzed by vertebrate digestive enzymes. NSP act by increasing the viscosity of the digesta and inhibiting penetration of digestive enzymes into the digesta, as well as diffusing of the digestion end-products to the epithelial membrane for absorption (Classen and Bedford 1999). Enzyme inhibitors target digestive enzymes and limit the rate and extent of digestion. This causes increased synthesis and secretion of digestive enzymes and accompanying hypertrophy of the pancreas (Hajos et al. 1995). Phenolics, such as tannins and gossypol, bind proteins and minerals in the feed and limit their digestion (Bravo 1998, Liener 1980). Some antinutrients, such as saponins and lectins, interact with the enterocytes and impair their viability and function, which is accompanied by increased replacement of enterocytes from the crypts (Casaubon-Huguenin et al. 2004, Francis et al. 2001, Pusztai et al. 1995).

Why Increase or Decrease Antinutrients?

Antinutrients in a feedstuff impair the productivity and efficiency of feed conversion of fish, poultry, and livestock. For many feedstuffs, lowering or eliminating the antinutrients results in a robust increase in the animal's productivity and health. There are two positive attributes of antinutrients that, in some situations, could compel genetic approaches to increase their levels. Antinutrients provide protection against pests

Table 10-2. Generalized actions and effects of antinutrients

Antinutrient	Mode of action	Gross Pathology[a]
Cyanogens[b]	Antagonize metabolism of B vitamins, iodine, and sulfur amino acids	enlarged thyroid and liver
Glucosinolates[b]	interfere with iodine metabolism	enlarged thyroid, liver, kidneys, and adrenals
Gossypol	interfere with protein and iron assimilation	edema, enlarged pancreas, egg abnormalities
Lectins	disrupt intestinal epithelia	Malabsorption
Non-starch polysaccharides	increase viscosity of digesta	malabsorption, sticky excreta
Nutrient antagonists	inhibit the absorption and metabolism of specific nutrients	Variable
Phytate	binds phosphorus, iron, zinc, and calcium	poor bone growth and mineralization, anemia
Protease inhibitors	impair protein digestion	enlarged pancreas, impaired muscle deposition, sticky excreta
Tannins	bind proteins and minerals, interfere with digestive enzymes	emaciation, impaired muscle and bone development

[a]All antinutrients decrease the rate of growth and efficiency of feed utilization of chick and rats.

[b]At high levels, toxic properties related to non-nutritional mechanisms are also manifested.

during production and storage of feedstuffs. There is also a developing appreciation that low levels of some antinutrients may benefit nitrogen digestion in ruminants, reduce parasitism, and slow some chronic diseases of aging (Barry and McNabb 1999, Thompson 1993, Waghorn and McNabb 2003). A thorough understanding of both the untoward and beneficial effects of antinutrients is needed to appreciate the net effect of altered antinutrient levels on crop production and animal health and well-being.

Summary of the Problems Associated with Antinutrients

When levels of antinutrients are high, their net effect on animal health is unmistakably detrimental. This is because of the antinutrient's direct effect on nutrient utilization in the feedstuff and its indirect effect on feed intake. Antinutrients in many feedstuffs are currently removed by physical or enzymatic means, which adds to the cost and variability in the nutritional value of feedstuffs. In some situations, the antinutrient is not removed, but its negative effects are ameliorated by including added levels of nutrients such as protein for protein inhibitors, vitamins for vitamin antagonists, iron for gossypol, and phosphorus for phytate. In the case of NSP, antibiotics and probiotics are sometimes added to diminish the impact of these antinutrients on intestinal microflora. Clearly, the reduction of antinutrients by genetic means would prevent the expense of neutralizing or masking antinutrients in feedstuffs.

Antinutrients Impair the Nutritional Value of Feedstuffs

By definition, all antinutrients diminish the nutritional value of feedstuffs. Quantitatively, the degree of loss in nutritional value is proportional to the antinutrient amount. For many antinutrients, but not all, there appears to be a threshold below which negative impacts on nutrient utilization are not observed. Tannins, saponins, enzyme inhibitors, and lectins demonstrate such a threshold phenomenon in animal feeding studies. For other antinutrients, like some vitamin antagonists and phytate, there is not a clear threshold effect and even low levels result in impaired utilization of vitamins and phosphorus, respectively.

Many antinutrients are detected by the family of bitter taste receptors and are perceived as a bitter flavor. This taste is associated with the presence of toxins and signals for the rejection of food items. Tannins, saponins, and alkaloids can impart sufficient bitterness to feedstuffs to cause feed refusal, and are clearly detrimental to animal welfare. Some antinutrients indirectly decrease feed consumption as a result of their effect on nutrient digestion. For example, NSP decrease the digestion rate of nutrients in the food, which, results in a decreased rate of passage of digesta through the GI tract in many species. Slowing the emptying rate of the proximal GI tract signals fullness and reduces appetite.

The antagonistic effects of antinutrients on nutrient bioavailability can result in deficiencies of specific nutrients that impinge on the animal's health and well-being. Additionally, many antinutrients have

toxic effects unrelated to nutrition that diminish animal health and well-being.

Many plant products used as feedstuffs are also used as human foods. One sustainable agricultural approach to reducing micronutrient malnutrition among people at highest risk (i.e., resource-poor women, infants, and children) is to improve the bioavailability of micronutrients in major staple food crops such as rice, wheat, maize, beans, and cassava (Welch 2002). Similarly, increasing the protein availability is likely to have nutritional benefits in many developing countries (Gilani et al. 2005).

Antinutrients Increase Loss of Nutrients to the Environment

The loss of nutrients to the environment limits animal stocking densities in many states and countries. Waste management is an escalating cost of animal production. The amount of investment in waste management depends on the nitrogen, phosphorus, and total organic matter in excreta, which are inversely proportional to the diet's digestibility. Antinutrients that impair protein digestibility (e.g. protease inhibitors, tannins, saponins, lectins) increase nitrogen excretion. The amount of phytate in the diet is the primary determinant of P in excreta of nonruminants (Feil 2001, Raboy 2001). Lott et al. (2000) estimated that 9.9 million metric tons of P is sequestered annually in phytate contained in crop seeds and fruits. Most of this is excreted by the animals and humans who consume them and becomes a major water pollutant.

Antinutrients Decrease the Use of Novel Feedstuffs

Many crops and forages that have good agronomic properties are not produced because antinutrients limit their use in animal feedstuffs and human foods. This is especially true for crops that grow well in arid or salt-stressed environments and in situations where fertilizer inputs are limited (e.g. amaranth, lupins, and many legume species). Progress toward decreasing antinutrient levels using plant breeding has been slow and has limited development of these novel crops for animal and human nutrition in many of the underdeveloped countries that suffer from food insecurity.

Positive Values of Antinutrients

Antinutrients Improve the Agronomic Properties of Crops via Pesticide Activities

Plants produce antinutrients to discourage their consumption by both vertebrate and invertebrate herbivores (as described above). Some antinutrients also protect plant products from insect damage during storage. Therefore, one should recognize that there might be new disadvantages, such as increased susceptibility to insect predation, to crops developed to have reduced antinutrient levels,.

Positive Effects of Slowed Digestion

Many antinutrients slow the rate of digestion, which can have positive implications in some situations. For example, when used under defined conditions, condensed tannins improve the efficiency of N digestion in ruminants fed fresh forage, resulting in improved productivity (Barry and McNabb 1999, McMahon et al. 2000, Ramirez-Restrepo and Barry 2005, Waghorn and McNabb 2003). Improved nutrition appears to be due to the complexing of condensed tannins to dietary protein, which protects the protein against degradation in the rumen and increases amino acid supply to the abomasum and small intestine. By complexing nutrients, tannins also shift rumen microbe populations, and this change may have positive results in some situations (Lowry et al. 1996). Changes in microbial populations and reduction of soluble nitrogen in the rumen may reduce the incidence of bloat (McMahon et al. 2000, Ramirez-Restrepo and Barry 2005, Waghorn and McNabb 2003). Ruminants also produce less methane when low levels of condensed tannins are present in their forage, which may have positive implications for lowering greenhouse gas emissions (Ramirez-Restrepo and Barry 2005). Alfalfa and other legumes have very low levels of condensed tannins and efforts are underway to increase tannin levels to improve nitrogen nutrition, reduce methane production, and reduce problems with bloat (Ramirez-Restrepo and Barry 2005).

Antinutrients that lower the rate of starch digestion, such as phytate, tannins, lectins and amylase inhibitors, also decrease the blood glucose response to a meal. In companion animal and human nutrition, this may be beneficial to health and management of diabetes and hyperlipidemia (Thompson 1988 Thompson 1993). However, positive effects in poultry and livestock have not been reported.

Antihelminthic Effects

Diets with low levels of condensed tannins decrease the intestinal parasite burden of a variety of ruminants (Barry and McNabb 1999, Min et al. 2003). It is not clear if this is due to direct toxicity towards the parasite within the GI tract (Molan et al. 2000) or improved immune defenses possibly resulting from the ability of low levels of tannins to increase the efficiency of

nitrogen digestion (Athanasiadou et al. 2001, Houdijk et al. 2005). The addition of tannin sources to forages with very low tannin concentrations has been used to reduce the severity of natural helminth infections (Barry and McNabb 1999).

Antioxidant, Hypocholesterolemic and Other Properties of Antinutrients

There is an extensive literature describing the biological properties of plant secondary compounds and their impact on human health that should be consulted for detailed information (Blaak and Saris 1995, Bravo 1998, Martinez et al. 1996, Thompson 1993, Urbano et al. 2000). In some studies, plant secondary compounds (many of which are considered antinutrients) diminish symptoms of diseases of aging including cardiovascular, cerebrovascular, and neurodegenerative diseases, cancer, and osteoporosis in rodent models and in humans. These effects are thought to be due to the antioxidant, hypocholesterolemic, estrogen-modifying, and anti-proliferative activities of various polyphenols, phytates, and saponins. Additional bioactivities attributed to various antinutrients include antibiotic, antiallergic, antidiarrheal, antiulcer, anti-inflammatory, and enzyme modulatory properties. Because there are many biological activities attributed to antinutrients, some of which could be beneficial or detrimental depending on specific circumstances, not all studies show protective effects and some show detrimental effects.

Intended Compositional Changes

Reductions in the amounts of antinutrient compounds have been an integral part of traditional plant breeding through the millennia. Examples include low tannin sorghums, low glucosinolate rapeseed, low gossypol cotton, low alkaloid lupins and low phytate grains. In the case of rapeseed, the improvement was sufficient that the commercial name was changed to canola to distinguish its superior nutritional value (Bell 1993). Modern biotechnology techniques permit faster and more direct progress toward changing the levels of antinutrients. Table 10-3 summarizes useful changes in antinutrient levels in feedstuffs based on the discussion of positive and negative attributes of antinutrients presented previously in this chapter.

Undesirable Effects of Changing Antinutrient Levels

Predictable, unpredictable, and undesirable effects may arise, regardless of the method used to change plant genetics. The targeted nature of biotechnology approaches to genetic changes reduces the likelihood of unpredictable effects relative to traditional breeding approaches. Two predictable effects that are likely to arise from decreasing antinutrient levels in plants are declines in pest resistance and loss of nutritional value in some ruminant applications.

Decreased pest resistance

There are many examples where reductions of antinutrients resulted in increased problems with insect and vertebrate pests. A classic example is the value of tannins in thwarting bird pests (Bullard and York 1996). The nutritional value of sorghums is severely limited by its high tannin levels, so a major international breeding effort was directed at decreasing tannin levels. Early efforts were unsuccessful because birds consumed the low-tannin sorghums. In many areas of Africa and South America, bird depredation is the primary form of sorghum crop loss in the field. More recent efforts have targeted lowering tannins in the ripened grain but not in the immature stages. This is advantageous to growers because birds generally inflict the most damage during the immature stages. Clearly, the manipulation of antinutrient levels in feedstuffs must be conducted with full consideration of the consequences on pest resistance of the growing plant.

Table 10-3. Examples of intended changes in antinutrient levels of plants

Targeted antinutrient	Rationale: Decreases in antinutrient levels
All	increase animal productivity
All	improve animal health and well-being
All	decrease the expense associated with detoxification
All	reduce the use of growth promoters and drugs
All	decrease environmental pollution
Tannins, gossypol	change color and processing properties
	Rationale: Increases in antinutrient levels
All	increase pest resistance of crops
Tannins	decrease GI parasites and bloat in ruminants
Tannins	increase N availability to ruminants
Tannins	reduce methane production by ruminants

Antinutrients for some species may be pronutrients for others species

There are several examples where altering the antinutrient levels to improve the nutritional value of a feedstuff for one species might reduce the value for another species. For example, tannins impair protein digestibility and feed consumption in nonruminant species, but low levels result in greater N retention, less bloat, and lower parasite loads in ruminants. Similarly, reductions in NSPs and polyphenolics in grains improve the nutritional value for fast growing pigs and chickens, but may reduce the beneficial value of these same grains in human nutrition because of the positive effects of these antinutrients on blood glucose levels, intestinal health, and cardiovascular disease. Thus, the value of changes in antinutrient levels should be evaluated on a species-specific basis, but with consideration of the effects on other species.

Evaluation Methods

Specific evaluation methods are needed to determine if the feedstuffs derived from genetically modified plants are superior to those derived from the original (isogenic) plants. The appropriate methods need to be determined on a case-by-case basis because antinutrients have widely varying modes of actions and effects on animal nutrition.

Chemical Evaluation of Feedstuff

The purpose of chemical evaluation of the feedstuff is to determine the extent to which the antinutrient was changed and to identify any unintentional changes in nutrients and other antinutrients. Antinutrients are usually concentrated in specific parts of a plant; for example, the tannins in sorghum are concentrated in the seed coat of the grain and the phytate in wheat and barley is localized predominantly in the aleurone layer. Care should be taken to include the location where the antinutrient is concentrated in the analysis. Antinutrient levels are greatly affected by the stage of maturity of the feedstuff at harvest and the agronomic conditions under which the plant was grown.

The FAO/WHO Expert Consultation on Biotechnology and Food Safety (FAO 1996, WHO 1995) concluded that a compositional comparison of the bioengineered food to its non-modified isogenic comparator is a very important step. This comparative assessment is regarded as one of the most important indicators of the safety of genetically modified foods (ILSI 2003, 2004). A minimum list of macro- and micronutrients, secondary plant constituents, inherent toxicants, and allergens should be analyzed to assess substantial equivalence (see Chapter 3). Chemical evaluation methods similar to those described for production traits are appropriate for plants modified to change antinutrient levels (see Chapter 3). However, the application of these evaluation methods to assess all crops with reduced antinutrients must be applied with good judgment.

Information on the nutritional content of modified and near isogenic comparators is crucial to properly formulating diets used in experiments designed to evaluate the efficacy of the modification in animals.

Animal Production Experiments

The ultimate value of a nutritionally improved feedstuff is its impact on animal performance and health and on loss of nutrients to the environment. These impacts are often difficult to assess by chemical analysis and carefully conducted animal experiments are essential.

Deciding What Experiments Are Needed

The required experiments must be selected and developed in a systematic science-based manner that relies on the known biology of the antinutrient that has been changed. Because there are dozens of antinutrients that act by differing mechanisms, the types of experiments must be tailored to the specific antinutrient. General guidelines are detailed in Figures 10-1 to 10-4 (see pages 133–136).

Deciding on an Experimental Design

Unlike the situation with crops that have been modified to improve agronomic traits, testing modified crops for antinutrient levels often cannot be accomplished by simply substituting the improved crop for a near isogenic control crop in a standard formulation. This is true because standard formulations currently used in animal feeding have excess levels of nutrients to compensate for the untoward actions of antinutrients. Consequently, a strict substitution approach is not likely to uncover superior nutritional value of feedstuffs with diminished antinutrients. Experiments must be designed to use diets in which the antagonized nutrient is below the animal's requirement so that any improvement in nutrition is observable. Thus, experimental design should focus on, and isolate, the nutrition of the antagonized nutrient (e.g. phosphorus for experiments examining feedstuffs with reduced phytate, and essential amino acids for experiments examining feedstuffs with reduced protease inhibitors).

Choosing Appropriate Experimental Treatments

Experiments should be designed to compare the performance of animals fed the genetically improved feedstuff (test diet) with animals fed appropriate negative and positive control diets. The negative control will show the magnitude of nutritional problems that can be attributed to the antinutrient. Positive controls will indicate the potential value of the original feedstuff when the antinutrient's effects are corrected and will also confirm that the experiment parameters are appropriate for demonstrating a positive effect of antinutrient removal. Appropriate controls depend on the biology of the antinutrient (Table 10-4). In the simplest situation, the detrimental actions of an antinutrient are known to be due to its effect on a single nutrient. In this case, the appropriate negative control might be a diet based on a near isogenic control feedstuff that has not been processed to remove the antinutrient under study. The appropriate positive control would then be this same diet supplemented with the antagonized nutrient. These control diets would be compared to a test diet that contains the genetically modified feedstuff without the supplemental nutrient. The diets should be carefully formulated so that they have identical levels of bioavailable nutrients, with the exception of the antagonized nutrient (Baker 1986).

In some situations (e.g., lectins, NSP, saponins), the antinutrient has detrimental effects on the bioavailability of nutrients that are not corrected by supplementing additional amounts of nutrients. In this situation, an appropriate positive control diet could be based on a near isogenic control feedstuff that has been physically processed or enzymatically altered to remove the antinutrient under study. This isogenic positive control should be similar to the original starting feedstuff in every way except the absence of the antinutrient. The appropriate negative control would be the isogenic positive control diet plus purified antinutrient. The purified antinutrient should be added in an amount that would be provided by the near isogenic feedstuff without processing.

Many physical techniques used to remove or inactivate the antinutrient under study are not specific and are likely to remove other antinutrients and/or change the bioavailability of other nutrients. A good example of this is heating, which is commonly employed to remove antinutrients. However, there are many antinutrients in soybeans and the heating process will not only remove the one under study, but will also inactivate others. In situations where the antinutrient cannot be specifically removed and the antinutrient has diverse effects on nutrition, a third approach is necessary. An acceptable positive control might be based on alternate feedstuffs that do not contain antinutrients (e.g. maize starch, isolated soy protein). The appropriate negative control could be based on this diet with addition of the purified antinutrient in the amount provided by the non-processed, near isogenic feedstuff. Control diets based on alternate feedstuffs should be formulated very carefully so that all nutrients are at identical levels on a bioavailable basis as those in the test diets containing the modified feedstuff.

Testing the nutritional value of a genetically modified feedstuff is most sensitive when it is included in the diet at high levels. However, the inclusion levels should not be so high that other antinutrients or toxicants become a limiting factor.

Selection of Dependent Variables

The appropriate dependent (response) variables selected for study should be tailored to the known biology of the specific antinutrient being studied. By definition, each antinutrient affects feed intake and nutrient digestion, absorption, or metabolism (see Table 10-2). Consequently, dependent variables should be selected to measure the predicted disruptions in nutrition. For most antinutrients, the most important response variables for measuring productivity include growth, yield of meat, milk or eggs, feed intake, and feed efficiency.

The intake, digestibility, or bioavailability of the antagonized nutrient may, in some cases, be a more sensitive indicator of the value of a modified feedstuff than performance criteria. This is especially true for antinutrients that antagonize vitamins and minerals. In these cases, marginal deficiencies may not result in marked changes in productivity, especially in well-managed research facilities. The specific procedure that is appropriate for measuring digestibility or bioavailability depends on the nutrient and the species under consideration (Ammerman et al. 1995) and should be selected accordingly.

Additional specific response variables should be tailored to the antinutrient in question. That antinutrient's mechanism of action should guide the selection of specific response variables (Table 10-2). Possibilities include: size of relevant organs (e.g. pancreas, thyroid), activity of digestive enzymes in the lumen of the intestines, and morphology of intestines. Finally, many antinutrients have additional negative attributes, beyond the antagonism of nutrients, that impinge on specific measures of animal health and these should be evaluated when appropriate.

Table 10-4. Examples of control and test diets that might be used to test feedstuffs modified to remove antinutrients

Indications for use	Example	Experimental diets
The antinutrient has a specific nutrient that it antagonizes	Phytate, amino acid analogues	– Control: Isogenic feedstuff[a]
		+ Control: Isogenic feedstuff + nutrient
		Test diet: Modified feedstuff
The antinutrient can be removed from the feedstuff without affecting the nutrient profile	NSP, phytate	– Control: Isogenic feedstuff
		+ Control: Processed isogenic feedstuff lacking antinutrient
		Test diet: Modified feedstuff
The antinutrient cannot be removed without changing the nutrient profile and it antagonizes more than one nutrient.	Saponins, tannins	– Control: Diet based on alternative feedstuffs + antinutrient
		+ Control: Diet based on alternative feedstuffs[b]
		Test diet: Modified feedstuff

[a]Near isogenic feedstuff that has not been processed to remove antinutrient.

[b]Alternative feedstuffs should lack antinutrients and provide bioavailable nutrients in amounts identical to those provided by the test feedstuff.

Product Quality

In general, changes in antinutrient levels are not expected to influence the quality of products derived from animals. Currently, diets using traditional feed ingredients containing antinutrients are formulated to correct the reduced bioavailability of the antagonized nutrient. Diets that will be formulated with feedstuffs modified to contain fewer antinutrients will be prepared with lower levels of supplementation of the antagonized nutrient. For this reason, the actual amount of the nutrient available to the animal and incorporated into edible products is not likely to change when genetically modified feedstuffs replace near isogenic feedstuffs. Some antinutrients (e.g. saponins, lectins) are relatively general in their effects on nutrients; they antagonize the bioavailability of many nutrients. In these specific instances, the changes in nutrient absorption and deposition that are not normally supplemented to diets may increase. This situation exists for some vitamins and minerals, but not usually for energy or protein. Though it is unlikely that increased levels of vitamins or minerals in edible tissues would diminish their quality characteristics, experiments designed to validate this assumption could be useful.

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Figure 10-1. Initial steps

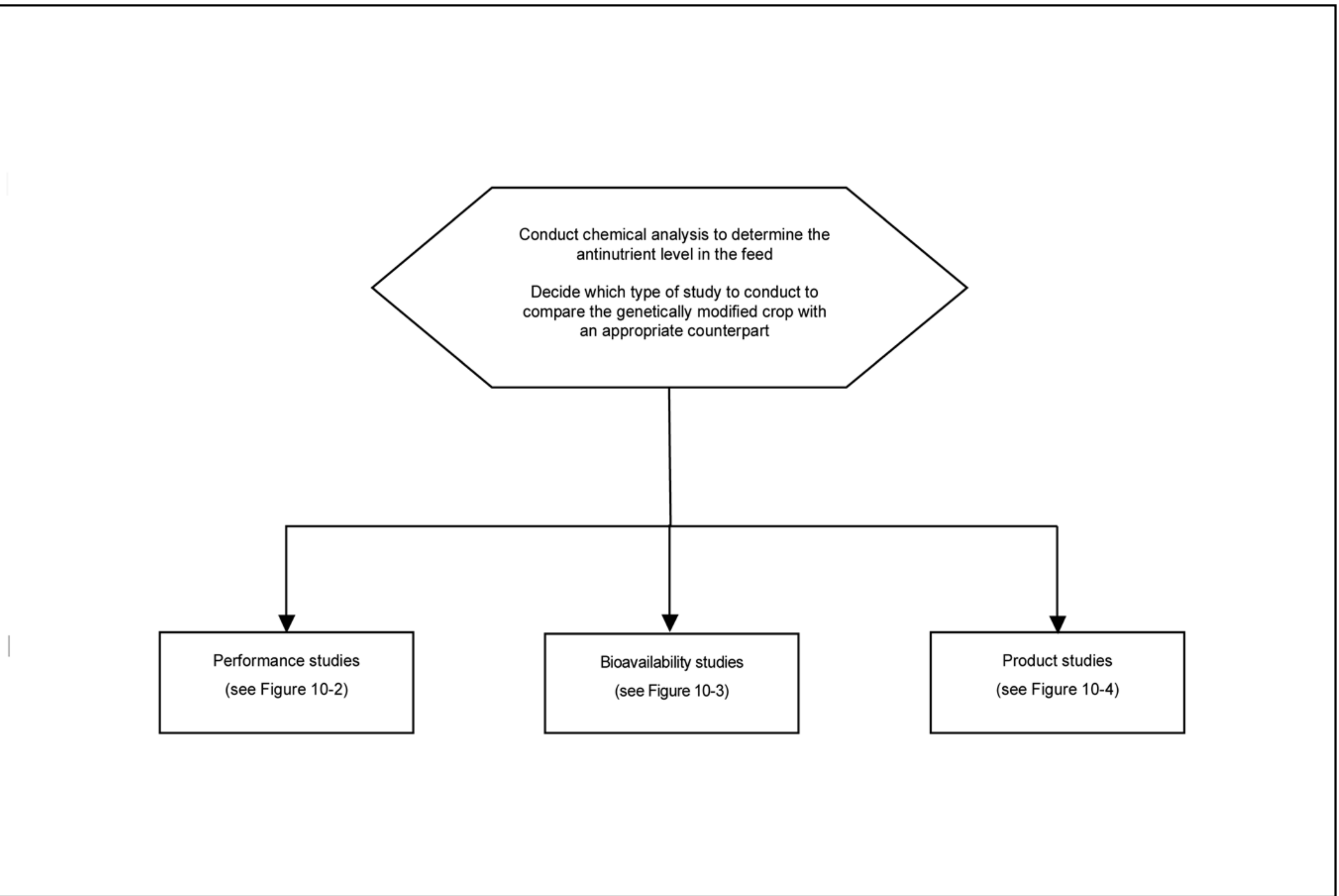


Figure 10-2. Performance studies

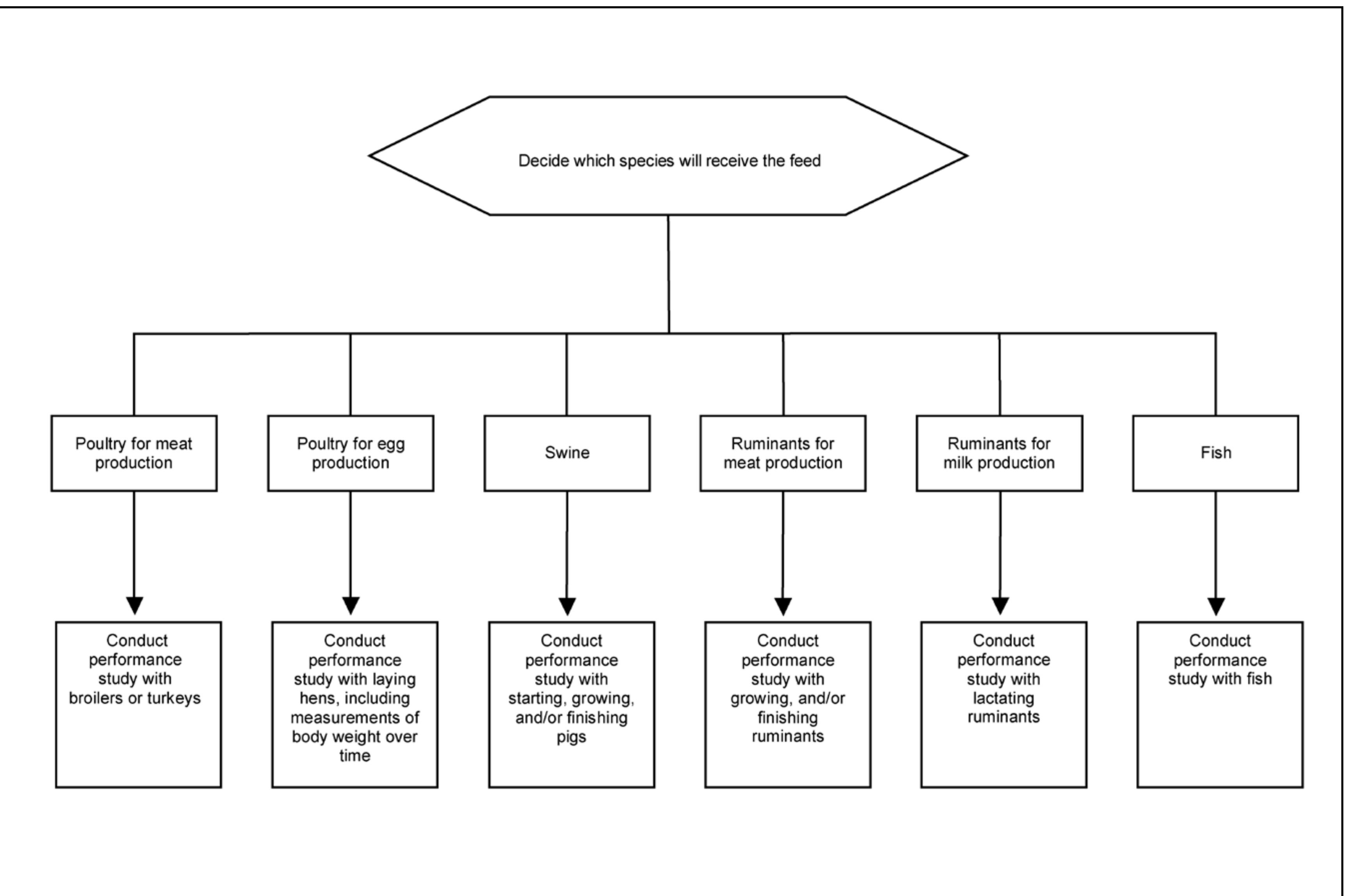


Figure 10-3. Bioavailability studies

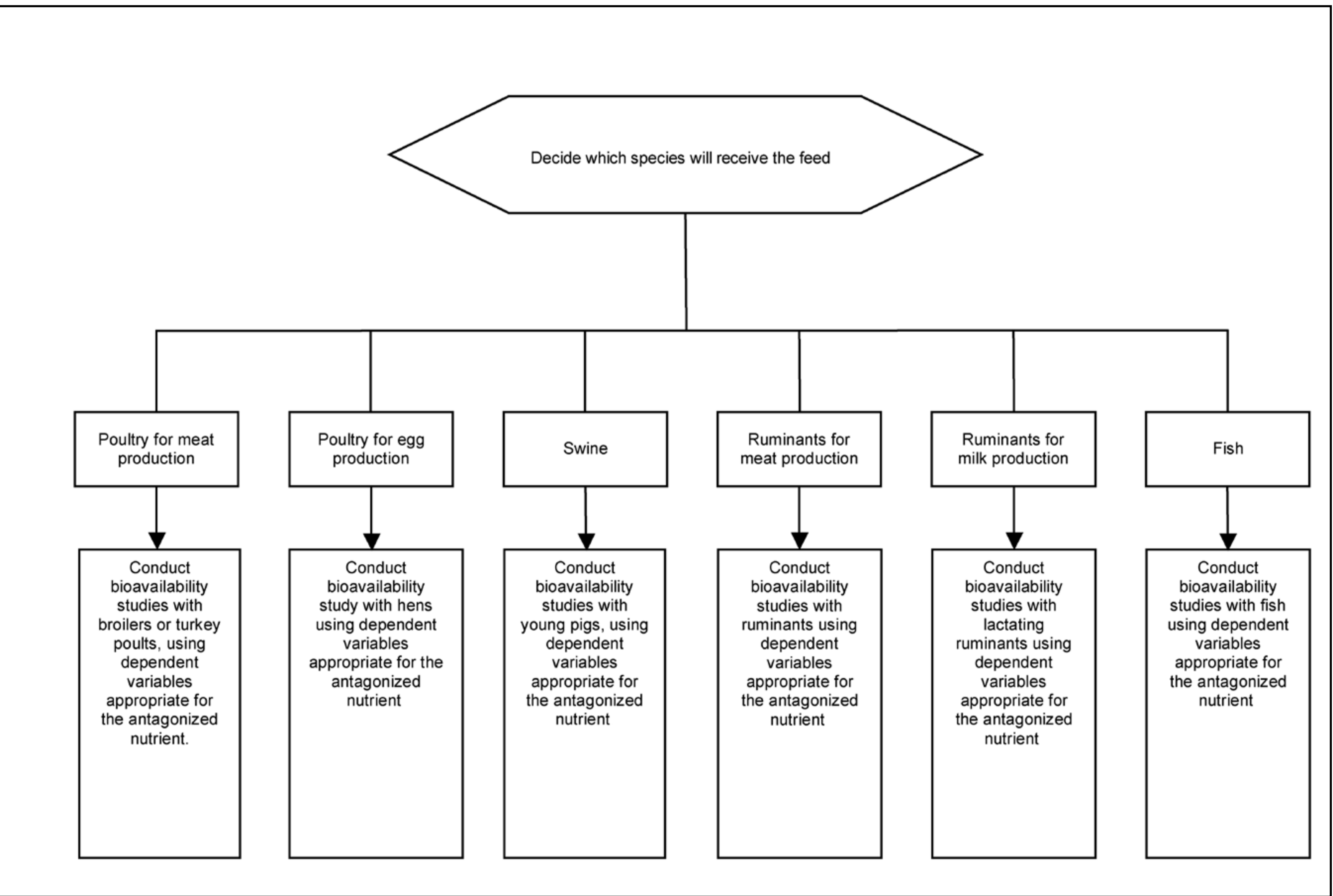
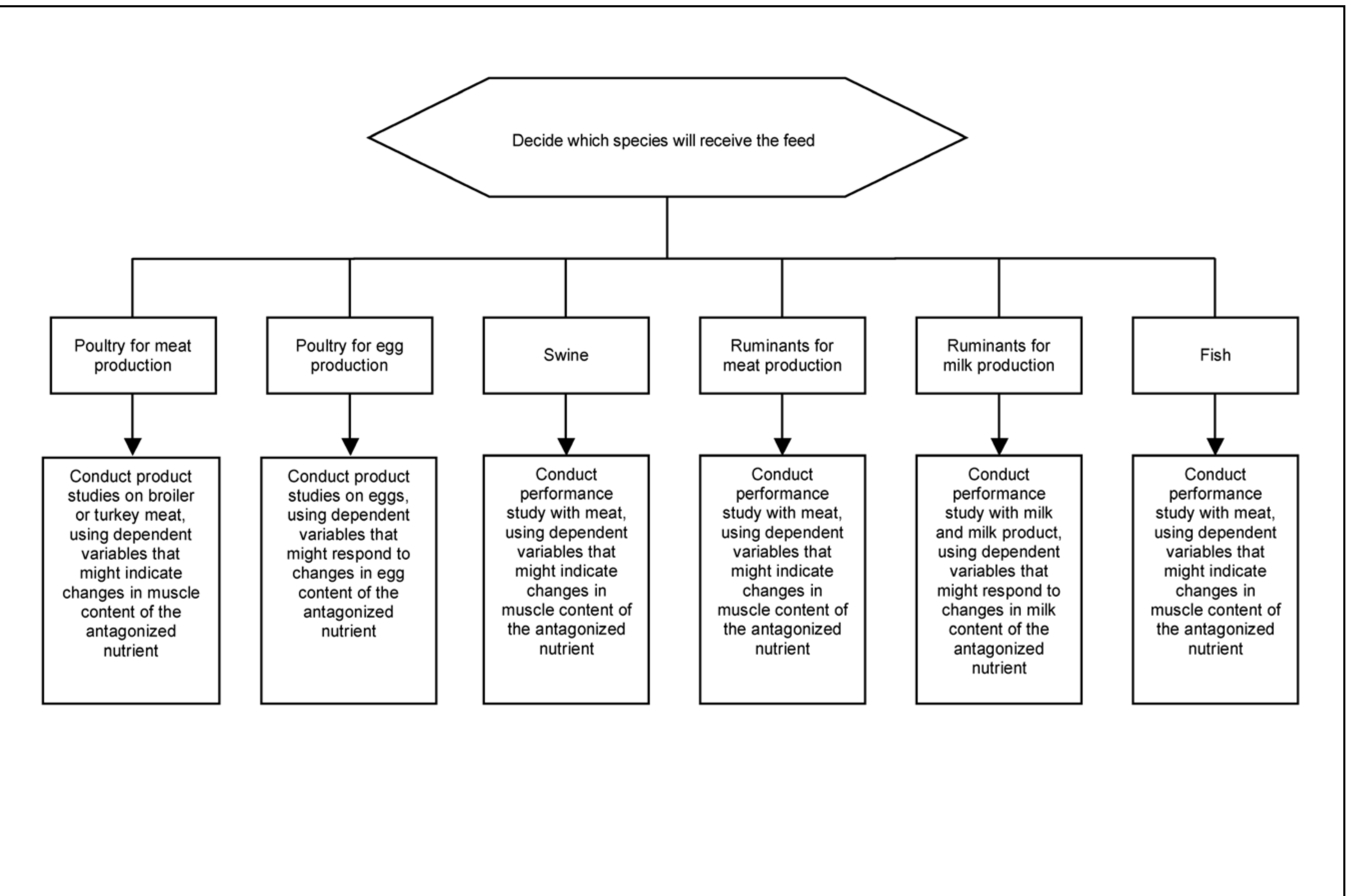


Figure 10-4. Product studies



Chapter 11

Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Poultry Meat Production

Poultry are found all over the world. Poultry that are produced for their meat (e.g., broilers, turkeys, etc.) have increased in popularity during the past several decades. Countries all over the world are major producers of broiler meat including United States (16.23 M metric tons), China (10.35 M metric tons), Brazil (7.54 M metric tons), European Union (7.54 M metric tons), Mexico, (2.64 M metric tons), India (2.20 M metric tons), Argentina (1.18 M metric tons), Japan, (1.15 M metric tons), Thailand (1.10 M metric tons), Canada (1.02 M metric tons), Malaysia (0.92 M metric tons), and others (6.54 M metric tons) (USDA-FAS 2006). Consumer demand has caused increased broiler meat production, with many of the highest producing countries also being the highest consumers (USDA-FAS 2006).

The demand for chicken meat has risen dramatically in some countries. For example, the present per capita consumption of chicken in the US (38.4 kg) has more than doubled in the past 25 years, such that it is now the most widely consumed meat in the US (Anon 2005). Similarly, turkey meat consumption has more than doubled during the same period. The top consuming broiler countries on a kilograms per capita basis include United Arab Emirates (68.0 kg/capita) and Kuwait (60.8 kg/capita), which consume almost twice as much chicken meat per capita as the next group of countries including Australia, Brazil, Canada, Hong Kong, Malaysia, Saudi Arabia, Taiwan, and the United States (USDA-FAS 2006). The consumption of turkey is < 1 kg/capita in most countries except for the United States (7.4 kg/capita), Canada (4.4 kg/capita), European Union (3.9 kg/capita), Mexico (1.9 kg/capita), and Taiwan (1.0 kg/capita) (USDA-FAS 2006).

Like other nonruminant animals, the simple digestive tract of poultry necessitates that they be fed diets in which the energy sources are readily digested and the protein sources have a desirable profile of amino acids. In addition, because most of the diet will generally consist of cereal grain, many of the essential minerals and vitamins must be supplemented to attain optimal growth rate and feed utilization efficiency. Maize containing higher levels of free lysine (Taylor et al. 2004) has recently been developed. In the future, feed ingredients that improve protein and amino acid characteristics, mineral availability, vitamin and antioxidant content, fatty acid profiles and content, decreased antinutrients, and enhanced enzymatic activity will be developed (Hartnell 2004). With changes in the nutrient profile of the dietary ingredients, the effects on bird performance, carcass traits, meat quality, and organoleptic attributes of the meat will need to be evaluated.

This chapter focuses on guidelines for conducting nutritional evaluations of GM crops and their byproducts with improved nutritional characteristics as measured by: (1) growth performance of broiler chickens for meat production, (2) digestibility of specific nutrients by chickens, (3) bioavailability of nutrients, and (4) assessment of meat quality including sensory evaluation; all of which could be affected by a GM output trait. Background information regarding the various nutrient groups (amino acids, carbohydrates, lipids, minerals, and vitamins) as well as comments on antioxidants, enzymes, and antinutrients are found in Chapters 4–10. A GM output trait in a cereal grain will be used as an example.

Nutrient Requirements

In designing nutrition studies to evaluate grains with enhanced nutrient composition and/or their byproducts, the nutrient requirements of the bird are needed. In the United States, the National Research Council has published nutrient requirements of poultry including chickens, turkeys, geese, ducks, ring-necked pheasants, Japanese quail, and Bobwhite quail (NRC 1994). Other country-specific feeding standards may be considered. One should have an understanding of nutrient digestion and metabolism, intermediary metabolism, nutritional physiology, pathology, and nutrient flow and retention in the bird in order to develop appropriate strategies for evaluating specific GM-enhanced feed ingredients. Klasing (2000) has provided an excellent discourse on these topics with respect to the avian species and D'Mello (2000) has provided information on farm animal metabolism and nutrition. Additional resources are available that pertain to amino acids in animal nutrition (D'Mello 2003), secondary plant metabolites in poultry nutrition (Smithard 2002), vitamins in feedstuffs (Whitehead 2002), fats and fatty acids (Zornig et al. 2001, Palmquist

2002), carbohydrate chemistry (Carré 2002), and calcium and phosphorus availability (Coon et al. 2002).

Management

Management of broilers is important in realizing optimum growth and feed efficiency as well as low mortality and morbidity. It is not within the scope of this document to provide detailed management guidelines. However, specifics such as feeding practices, feed handling, processing and storage, lighting, and housing need to be considered. *The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999), breeder company recommendations, or accepted local guidelines will provide guidance on the proper management of the birds.

Climatic conditions and water quality can affect bird performance. Therefore, 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. Grains should be analyzed for mycotoxins before use.

Removal of Birds

Mortality will be recorded daily and dead or moribund birds will be removed. Weight of dead or cull 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. One method is to divide the total feed consumed by the total weight gain of surviving and dead or culled birds per pen.

Production, handling, storage, and processing are described in Chapter 2 of this document, while sampling and analysis are described in Chapter 3.

Diet Formulation and Processing

Diet formulation and processing will be dictated by the trait to be evaluated, the grain that will contain the trait, and the bird's age, sex, and species. For example, if the trait is an enzyme that is heat sensitive to pelleting temperatures, one would not pellet the meal, or if the trait involved increased polyunsaturated fatty acids, one should consider adding an antioxidant to the mixture to stabilize the feed. If the trait involved a change in the starch matrix in maize, one may consider comparing pelleting at various temperatures to see whether there was an effect on the gelatinization of the starch. If the trait involved an enhanced level of an amino acid, the diet needs to be formulated such that the amino acid in question is below the bird's requirement and all other nutrients are not limiting. The diets should be formulated such that the bird will be responsive to a change in the amount or bioavailability of test nutrient. Inclusion levels of the test grain should represent the maximum amounts that would be used in practice.

The cereal grain should be ground to a consistent geometric mean particle size (ASAE 2003) 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 unless the form of the feed is part of the experimental design. Enzyme supplements may be added to the feed to enhance digestion and/or absorption of feed components, remove antinutritional factors, and supplement the activity of endogenous enzymes. Bedford and Partridge (2001) have provided a resource in this area. Broz and Beardsworth (2002) have discussed recent trends and future developments in the use of feed enzymes in poultry diets. Diets may also include growth promoters, coccidiostats, and organic acids at the discretion of the investigator, based on the study's objectives and according to local best practices and regulations. The inclusion rate for additives should be the same for each treatment diet. 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. In some cases where the composition of the grain sources being evaluated varies considerably, it may be necessary to include low levels of ingredients such as powdered cellulose (e.g., Solka-Floc®) or corn starch to formulate diets to near identical nutrient specifications.

Growth Performance Studies

Depending on the trait of interest, performance studies may be conducted with birds in battery cages or floor pens up to 21 days of age and continuing in floor pens up to the targeted commercial weight, which may vary from five to eight weeks. Most studies should have the test grain, its near isogenic counterpart (control), and preferably four or more conventional reference varieties to help explain any unexpected differences or confirm any expected differences observed between the test and the control.

Design and Allotment

The goal is to ensure that the number of replications (number of pens per treatment) will be adequate to detect at least 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. Blocks of pens will typically be arranged by location, and the number of pens within a block will be equal to two times the number of treatments, allowing one pen of each sex per treatment. Birds will be randomly assigned to 10 to 12 pens per treatment, holding nine to 12 birds of the same sex per pen. In some cases, a factorial arrangement of treatments may be used.

Termination of Experiment

The experiment will be terminated when birds reach a desired market weight (at 5 weeks of age or older). Depending on the trait, carcass and meat quality data should be collected on all or a subgroup of birds from each treatment group.

Statistical Analysis of Data

Performance and carcass 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 17, Statistical Analysis and Interpretation of Results.

Literature Examples

Researchers have used various experimental approaches, depending on the trait and research objective(s). Investigators have conducted performance studies in broilers with several different GM crops with improved nutritional characteristics. These include maize containing higher levels of lysine (Taylor et al. 2004), maize with different kernel characteristics (Collins et al. 2001), different levels of polyunsaturated fatty acids (Cortinas et al. 2004), high-oil maize (Daghir et al. 2003), high-oleic acid sunflower seed (Rodriguez et al. 2005), high-oil sunflower meal (Senkoylu and Dale 2006), L-carnitine (Xu et al. 2003), source and levels of vitamin D (Fritts and Waldroup 2003), zinc and selenium supplements (Bou et al. 2005), and isomalto-oligosaccharides (Zhang et al. 2003). Numerous researchers have published performance studies that evaluated dietary enzymes in broilers. Performance studies have been conducted with α-galactosidase (Kidd et al. 2001a, 2001b; Waldroup et al. 2006), carbohydrase (Meng et al. 2005), α-amylase (Onderci et al. 2006), β-mannase (Ouhida et al. 2002), cellulases and xylanases (Ponte et al. 2004), and phytase (Yan et al. 2003, Silversides et al. 2004, Wu et al. 2004, Shelton and Southern 2006). As an example of a case study, see Box 11-1 for more details of lysine maize.

Digestibility Studies

Digestibility is classically defined as the difference between the amount of nutrient consumed and that excreted, divided by the amount consumed. In some instances, assessing digestibility of a specific nutrient or nutrient groups is warranted. For example, this may be needed when evaluating a GM feed that has an altered nutrient composition (such as a reduced amount of cellulose or hemicellulose or increased amounts of amino acids) or one with a specific enzyme incorporated that is intended to increase digestibility.

Fuller (1991) reviewed digestibility studies in poultry and Parsons (2002) discussed digestibility of protein and amino acids. Digestibility is usually determined by: (1) classical fecal collection method, or (2) collection of ileal contents from surgically modified or killed animals. For poultry, the total excreta (feces and urine) are usually collected (birds excrete feces and urine together via a cloaca and they are difficult to separate). Hence, using classical definitions, it is the metabolizable nutrient and not the digestible nutrient that is measured because the urinary nutrient is added. However, the term "digestibility" will be used when referring to excreta assays for protein and amino acids because the term "metabolizable amino acids" is uncommon and the amount excreted in the urine is small (Parsons 2002). For energy, one has (1) apparent digestible energy (DE), which is the feed energy consumed minus the gross energy of the feces; (2) apparent metabolizable energy (AME), which is the gross energy of the feed consumed minus the gross energy of the feces, urine, and gaseous products of digestion (these are negligible); (3) true metabolizable energy (TME), which is the gross energy of the feed consumed minus the gross energy of the excreta corrected for metabolic fecal and endogenous urinary energy; and (4) net energy (NE), which is the metabolizable energy minus the energy lost as the heat increment (NRC 1994). A correction for nitrogen retained in the body may be applied to yield a nitrogen-corrected AME (AMEn) and TME (TMEn). AMEn is the most common measurement used in formulating poultry feeds; NE is seldom used (NRC 1994).

Digestion assays measure only nutrient digestion and absorption, not utilization. Apparent metabolizable nutrient is most commonly determined by obtaining

Box 11-1
Case Study: Lysine Maize Growth Performance Study

A pertinent example is maize that has been enhanced with free lysine (Taylor et al. 2004). First, a dose titration study should be conducted with the target strain and sex of the bird for each feeding phase to determine the response in terms of growth and/or feed efficiency to the addition of free lysine (L-lysine•HCl). This step may be omitted if the same data can be gleaned from appropriate studies in the literature. In this study, lysine must be the only limiting nutrient. Therefore, the ratio of the other amino acids to lysine should exceed the ideal amino acid ratio to lysine (Baker 2003). From the response profile (dietary lysine concentration plotted against the performance trait such as gain or gain:feed), select the dietary lysine concentration from the portion of the response curve in which the bird are highly sensitive in terms of the desired performance response (a small change in dietary lysine results in a large performance response). The dietary lysine value at the high end of the sensitive range should be selected. This value will be used in formulating the diets containing the test grain (lysine maize).

In formulating the diets for the performance study, all amino acids will be formulated such that they are in excess (~105% of the ideal lysine to the respective amino acid ratio). Assume the test grain expresses an additional 0.15% lysine (total lysine is 0.40%) as compared to the control (total lysine is 0.25%). The performance study will be designed with two objectives: (1) to determine the efficacy of the additional lysine expressed in the test grain, and (2) to determine if there are any unexpected effects on performance and health. Therefore, the treatments will include the test diet containing the test grain at the high commercial inclusion level formulated for the predetermined lysine level. There will be two control diets: one containing the near isogenic maize at the same inclusion level as the test grain with added L-lysine•HCl similar to the amount that is expressed in the test grain, and a second diet containing the near isogenic maize at the same inclusion without the added L-lysine•HCl. The control diet without the added lysine will have a dietary lysine level less than the test diet. Eight additional diets will be formulated containing conventional reference maize at the same inclusion level as the test diet. Four of these diets (four different reference maize varieties) will contain added L-lysine•HCl such that the dietary lysine level is the same as the test diet, and the other four diets (same four reference maize varieties) without the added crystalline lysine will have a lower dietary lysine content, similar to the control diet without the added lysine.

Ten pens of nine to 12 birds per treatment group would be used. To show that the birds in the study were responsive to lysine supplementation, feed efficiency would be compared between the birds fed the control and reference diets that were supplemented with lysine and those that were not. There should be a significant difference because the dietary lysine levels were different. Secondly, the performance of the birds fed the test diet would be compared with the unsupplemented lysine control and references to show the bioavailability of the free lysine in the test material. Then, the performance of the birds fed the test diet will be compared with the performance of the birds fed the control and reference diets supplemented with lysine. The results of this comparison would be expected to show no differences because the dietary lysine levels were the same (assuming no difference in bioavailability) and no negative effects were expected from the genetic modification.

a total collection of feces, analyzing the feces for the nutrient in question, and subtracting the total amount of nutrient excreted from the amount of the nutrient consumed, generally during a 3-day period. The difference in these two values is the amount of the nutrient digested. Another method is to include an indigestible marker in the feed, and take a series of fecal samples after several days. By determining the ratio between the indigestible marker and the nutrient in question in the feed and feces, one can determine the apparent digestibility of the nutrient. Fuller (1991), NRC (1994), and McNab (2000) describe AME methodology and TME measurements of feedstuffs. Parsons (2002) and Lemme et al. (2004) have published reviews on methodology for assessing amino acid digestibility in poultry. Sales and Janssens (2003) have provided a discussion on the use of markers to determine energy metabolizability and nutrient digestibility in poultry when total collection is not desired.

Literature Examples

Recent publications have reported the use of the precision-fed cockerel assay in the evaluating the digestibility of distillers dried grains (Lumpkins and Batal 2005, Batal and Dale 2006), the classical

digestibility study in evaluating soybean lectins in turkey poults (Fasina et al. 2004), and the ileal digestibility assays to evaluate amino acids in feed ingredients (Huang et al. 2006). Apparent metabolizable energy bioassays were used to evaluate enzyme combinations in broilers (Kocher et al. 2003). Meng et al. (2005) evaluated combinations of carbohydrase enzymes using enzyme evaluation, and determined AME and TME and nutrient digestibilities in broiler studies. Others measured the effects of β-mannose (Ouhida et al. 2002) and phytase (Silversides et al. 2004) on digestibility in broilers.

Bioavailability Studies

Bioavailability can be distinguished from digestibility in that bioavailability includes all processes of digestion, absorption, and metabolism or utilization (Parsons 2002). The growth assay or slope-ratio assay is considered the absolute method for determining bioavailability (Parsons 2002). The assay measures the ability of a feedstuff or nutrient to replace a specific limiting nutrient. A basal diet deficient in the test nutrient is supplemented with increasing amounts of the test nutrient or the test feedstuff to produce a linear growth response curve. Bioavailability is then calculated by regression analysis and from the ratio of the slopes of the growth response lines for the test nutrient and ingredient.

Details on conducting bioavailability studies are covered in excellent reviews by Ammerman et al. (1995), Gabert et al. (2001), and Parsons (2002) and an example is described in Box 11-2.

Literature Examples

Lumpkins and Batal (2005) measured the bioavailability of lysine and phosphorus in distillers dried grains with solubles (DDGS) and Amezcua et al. (2004) measured the bioavailability of phosphorus in DDGS. Miles et al. (2003) measured the bioavailability of amino acid chelates of manganese and copper in chickens.

Product Quality

Some GM traits can affect the carcass and meat quality, including the weight of the various portions of the carcass (e.g., breast, legs, thighs, and organs [heart, gizzard, liver], internal fat), the distribution of fat and lean in the carcass, the fatty acid composition of the meat, or the level and kind of pigment in the meat and skin. For example, meat color and skin pigmentation can be modified by feeding astaxanthin (Akiba et al. 2001, An et al. 2004) and xanthophylls (Castaneda et al. 2005). Specific carcass and meat quality measurements to consider depend on those that are commercially important and may include: chill weight; fat pad weight; breast meat weight; thigh, drumsticks, and wing weights; shear force; and breast and thigh moisture, protein, and fat content. In certain instances, the GM trait could affect the tenderness, flavor, or other characteristics

Box 11-2
Case Study: Phosphorus Bioavailability Studies

Studies on the bioavailability of a nutrient would be conducted to determine whether a nutrient's bioavailability in a GM feedstuff differs from that same nutrient in a conventional feedstuff. An example would be an evaluation of two cereal grains with the same amount of phosphorus, but with the GM grain having less phosphorus in the form of phytic acid (e.g., low-phytate corn) and more phosphorus in inorganic form than the phosphorus in a conventional grain. For an assessment of such a trait, one would feed a low phosphorus basal diet consisting of a purified carbohydrate (e.g., cornstarch and/or dextrose) and an amino acid source, vitamins, and minerals (except phosphorus) to meet the chick's nutrient requirements. Additional diets would be the basal diet with the addition of the GM grain (substituted for the purified carbohydrate source), a conventional grain, and a highly bioavailable phosphorus source (such as monosodium phosphate) for the standard. The GM grain, the conventional grain, and the standard should ideally be added at three or more incremented levels, but the highest level of phosphorus from the standard and the grains must be below the bird's requirement so that the response is linear. Slopes of the responses are determined and a ratio of the linear slopes of the two grains compared with the slope of the standard gives the relative bioavailability of phosphorus in the two grains. The standard is considered 100% bioavailable.

In studies of this type, it is paramount that the response be one that is sensitive to the nutrient under investigation. For example, with phosphorus, measures of bone mineralization (bone ash, bone strength, etc.) are best. For an assessment of iron, blood hemoglobin concentration would be an appropriate measurement. Growth responses are generally not sensitive enough to determine bioavailability of nutrients other than energy, protein, or amino acids.

of the final cooked poultry product. Northcut (2006) discussed factors that affect poultry meat appearance (color), texture, and flavor. Standard methods have been described for assessing carcass merit and for instrumental measurements of tenderness and sensory evaluation of tenderness, flavor, and other measures of consumer satisfaction (Mountney 1989, Sams 2000, Mead 2004).

Sensory (Organoleptic) Evaluation

If a new feedstuff that is fed to poultry has the potential to affect the organoleptic quality of the retail product offered to the consumer, a sensory evaluation should be done. It is not within the scope of this chapter to delve into the specifics of sensory studies. Specific details on conducting sensory studies have been published (Johnsen and Kelly 1990, Meilgaard et al. 1999, Stone and Sidel 2004). Meilgaard et al. (1999) discussed in detail sensory attributes and human perception; controls for the test room, product, and panel; physiological and psychological factors affecting sensory verdicts; measuring responses; Triangle Test; Duo-Trio Test; Two-out-of-Five Test; Simple Difference test; "A" – not "A" Test; Difference-from-Control Test; Sequential Tests; Paired Comparison Designs; Directional Difference Test; Pairwise Ranking Test; numerous Multi-sample Difference Tests; determining thresholds; selection and training of panel members; descriptive analysis techniques; the Spectrum™ Descriptive Analysis Method; Affective Tests, including consumer tests and in-house panel acceptance tests; statistical methods; guidelines for choice of technique; and guidelines for reporting results. Stone and Sidel (2004) provided additional information on organizing and operating a sensory evaluation program; measurements, test strategy, and experimental design; discrimination testing; descriptive analysis; affective testing; and dealing with special problems.

Literature Examples

Broiler studies evaluating the effects of dietary fat sources (Sirri et al. 2003, Bou et al. 2004, Cortinas et al. 2004, Bou et al. 2005), amino acid addition (Fatufe et al. 2004), overfeeding energy (Chartrin et al. 2006), mineral supplementation (Bou et al. 2004, 2005), additives such as L-carnitine (Xu et al. 2003), diet and feed withdrawal (Lyon et al. 2004), and strain and deboning time (Mehaffey et al. 2006) on carcass composition and/or consumer acceptability of chicken meat have been published.

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Chapter 12

Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Poultry Egg Production

Poultry are raised throughout the world for meat and egg production, with almost 58 million metric tons produced in 2004 (ThePoultrySite 2005). Chicken egg production comprises up to 96% of the worldwide egg production (Taha 2003). The remaining egg production is mainly from ducks, geese, and quail in China (83%), Thailand (7%), Indonesia (3%), and the Philippines (2%) (Taha,2003). Eggs are an excellent source of protein and are widely consumed around the globe.

Like other nonruminant animals, the simple digestive tract of laying hens necessitates that they be fed diets in which the energy sources are readily digested and the protein sources have a desirable profile of amino acids for maintaining their body functions and for efficiently producing eggs. In addition, because most of the diet will generally consist of cereal grain, many of the essential minerals and vitamins must be supplemented to attain optimal egg production and feed utilization efficiency. Calcium is especially important in laying hens because of the large amount of calcium carbonate deposited in the egg shell. In the future, feed ingredients will be developed that have improved protein and amino acid characteristics, mineral availability, vitamin and antioxidant content, and fatty acid profiles and content; decreased antinutrients; and enhanced enzymatic activity (Hartnell 2004). Recently, efforts have been made to produce "designer" or "specialty" eggs that contain higher levels of vitamins; lower amounts of cholesterol; higher concentrations of omega-3 fatty acids; lowered saturated to unsaturated fatty acid ratios; higher levels of minerals such as selenium, iodine, and chromium; pigmented yolks; and pharmaceuticals (Sims 1998, Jacob and Miles 2000). With changes in the nutrient profile of the dietary ingredients, the effects on egg production, egg quality, and egg organoleptic attributes will need to be evaluated.

This chapter focuses on guidelines for conducting nutritional evaluations of GM crops and their byproducts containing nutritionally enhanced traits as measured by (1) egg production of laying hens, (2) digestibility of specific nutrients by layers, (3) bioavailability of nutrients for layers, and (4) assessment of egg quality, all of which could be affected by a new trait. Background information regarding the various nutrient groups (amino acids, carbohydrates, lipids, minerals, and vitamins) as well as information on antioxidants, enzymes, and antinutrients are found in Chapters 4-10. A nutritionally improved cereal grain will be used as an example.

Nutrient Requirements

In designing nutrition studies to evaluate nutrient-enhanced grains and/or their byproducts, the bird's nutrient requirements must be known. In the United States, the National Research Council has published nutrient requirements of poultry including chickens, turkeys, geese, ducks, ring-necked pheasants, Japanese quail, and Bobwhite quail (NRC 1994). Other country-specific feeding standards may be considered. One should have an understanding of nutrient digestion and metabolism, intermediary metabolism, nutritional physiology, pathology, and nutrient flow and retention in the bird for developing appropriate strategies for the evaluation of specific GM-enhanced feed ingredients. Klasing (2000) has provided a discourse on these topics with respect to the avian species, and D'Mello (2000) has provided information on farm animal metabolism and nutrition. Additional resources are available that pertain to amino acids in animal nutrition (D'Mello 2003), secondary plant metabolites in poultry nutrition (Smithard 2002), vitamins in feedstuffs (Whitehead 2002), fats and fatty acids (Zornig et al. 2001, Palmquist 2002), carbohydrate chemistry (Carré 2002), and calcium and phosphorus availability (Coon et al. 2002).

Management

Management of layers is important in realizing optimum egg production as well as achieving low mortality and morbidity. It is not within the scope of this document to provide detailed management guidelines. However, specifics such as feeding practices, feed handling, processing and storage, lighting, and housing need to be considered. *The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999), breeder company recommendations, or accepted local guidelines will provide guidance on the proper management of the birds.

Climatic conditions and water quality can affect bird performance. Therefore, experiments conducted

under outdoor 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. Grains should be analyzed for mycotoxin before use.

Removal of Birds

Mortality will be recorded daily and dead or moribund birds will be removed. Weight of dead or cull birds should be recorded. A qualified veterinarian should perform or supervise a diagnostic necropsy on all dead birds. The final egg production data should not include data from any birds removed from the experiment. Adjusted feed conversion should be calculated. One method is to divide the total egg mass production (grams of egg produced per day or egg production multiplied by egg weight) from all birds including dead or culled layers by the total feed consumed by the surviving and dead or culled birds per pen.

Maize production, handling, storage, and processing is described in Chapter 2 of this document, while maize sampling and analysis for mycotoxins and chemical components is described in Chapter 3.

Diet Formulation and Processing

Diet formulation and processing will be dictated by the trait to be evaluated, the grain that will contain the trait, and the age and strain of the bird. For example, if the trait is an enzyme that is heat sensitive to pelleting temperatures, one would not pellet the meal; or if the trait involved increased polyunsaturated fatty acids, one may consider adding an antioxidant to the mixture to stabilize the feed. If the trait involved the starch matrix in maize, one may consider comparing pelleting at various temperatures to see whether there was an effect on the gelatinization of the starch. If the trait involved an enhanced level of an amino acid, the diet needs to be formulated such that the amino acid in question is below the requirement of the bird and all other nutrients are not limiting. The diets should be formulated such that the bird will be responsive to a change in the amount or bioavailability of test nutrient. Inclusion levels of the test grain should represent the maximum amounts that would be used in practice.

The cereal grain 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 unless the form of the feed is part of the experimental design. Enzyme supplements may be added to the feed to enhance digestion and/or absorption of feed components, remove antinutritional factors, and supplement the activity of endogenous enzymes. Bedford and Partridge (2001) have provided a resource in this area. Broz and Beardsworth (2002) have discussed recent trends and future developments in the use of feed enzymes in poultry diets. Diets may also include coccidiostats at the discretion of the investigator, based on the study's objectives and according to local best practices and regulations. The inclusion rate for additives should be the same for each treatment diet. In some cases where the composition of the grain sources being evaluated varies considerably, it may be necessary to include low levels of ingredients such as powdered cellulose (e.g., Solka-Floc®) or corn starch to formulate diets to near identical nutrient specifications.

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 50 weeks of age and possibly throughout the entire laying cycle. Generally, the study should include a minimum of three treatments. For example, an evaluation of a GM grain with a greater amino acid content or superior amino acid profile would require the feeding of a low-protein (amino acid) diet containing the GM grain, a similar low-protein (amino acid) diet with a conventional grain, and an adequate-protein (amino acid) diet with the conventional grain (positive control). The low-protein diet is necessary to allow the expression of the superior amino acid content or profile of the GM grain. The concentration of the amino acid of interest should be such that it is the first limiting nutrient in the diet and should be at a level that is below the requirement of the bird. It is preferable that the conventional grain be near-isogenic to the GM grain, or at least as similar as possible to the GM grain. If possible, additional commercial conventional varieties typically produced in the test region should be included in the study.

Layers

Healthy pullets of defined genetic background should be used in the study. From hatch to age 16 weeks, all birds should be fed the same diets formulated to meet the nutritional needs of developing pullets. At approximately 16 to 18 weeks of age, birds should be

randomly placed in cages. The actual starting time of the study will depend on the trait to be evaluated. For traits affecting shell quality, a start time of about 20 weeks of age is desirable. For traits affecting production, starting at post-peak production (approximately 32 weeks of age) is preferred. Generally there should be three to five hens per cage, but fewer or more birds per cage can be used if necessary. Birds should be allowed space in accordance with approved guidelines as described in *The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999) or local regulations. Feed consumption should be measured at the end of every feeding phase and egg production should be determined daily. Hens should 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, egg quality (shell, albumen, yolk), and other data should be kept as appropriate for good management practices.

Design and Allotment

A complete randomized block design should be used. Twelve to 15 cages holding three to five layers per cage should be randomly assigned to treatments. Cages should be randomly assigned within the research facility to eliminate any bias due to location in the building. Other cage sizes may be utilized depending on the question to be addressed and the variability previously observed.

Number of Replications

The number of replications (number of cages per treatment) should be adequate to detect 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 (NRC 1994) requirements (except for the nutrient of interest, which may be formulated to be at and below the requirement) or accepted local nutrient requirements with most of the energy requirement of the test species being met by inclusion of the cereal grain. Diets should be based on the GM or conventional grain and soybean meal (or other protein source commonly used for poultry in the region) and supplemented with calcium, phosphorus, salt, trace minerals, and vitamins as necessary.

Diets should be formulated on the basis of total or digestible amino acids. The amount of cereal grain in the two low-protein (at least the test amino acid should be below requirements) diets should be the same. Likewise, the amount of soybean meal in these 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. The positive control diets should be formulated to meet or slightly exceed the amino acid requirements with the test amino acid being the first limiting in relationship to the other amino acids. Crystalline amino acids may be included in the diets as appropriate to ensure that the test amino acid is the first limiting in the diet and that the ratio of this amino acid to the other amino acids is similar among control and reference diets. In countries where commercial diets normally contain added fat, all diets should contain similar levels of 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 rejecting of specific fractions 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 reformulated at the beginning of each phase as described above.

Removal of Birds

Mortality will be recorded daily, and the weight of dead or removed birds should be recorded. A qualified veterinarian should perform or supervise a diagnostic necropsy to the extent where a definitive diagnosis can be made on all dead birds.

Termination of Experiment

The experiment will be terminated after the last designated phase has been completed. The minimum duration should be about 12 weeks for the study.

Statistical Analysis of Data

Performance data (egg production, feed intake, body weight changes, egg quality traits [i.e., egg weight, eggshell quality, albumen quality, and yolk quality], 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 17, Statistical Analysis

and Interpretation of Results.

Digestibility Studies

Digestibility is classically defined as the difference between the amount of nutrient consumed and that excreted, divided by the amount consumed. In some instances, assessing digestibility of a specific nutrient or nutrient groups is warranted. For example, this may be needed when one is evaluating a GM feed that has an altered nutrient composition (such as a reduced amount of cellulose or hemicellulose or increased amounts of amino acids) or one with a specific enzyme incorporated that is intended to increase digestibility.

Fuller (1991) reviewed digestibility studies in poultry and Parsons (2002) discussed digestibility of protein and amino acids. Digestibility is usually determined by: (1) classical fecal collection method or (2) collection of ileal contents from surgically modified or killed animals. For poultry, the total excreta (feces and urine) are usually collected (birds excrete feces and urine together via a cloaca and they are difficult to separate). Hence, using classical definitions, it is the metabolizable nutrient and not the digestible nutrient that is measured because the urinary nutrient is added. However, the term "digestibility" will be used when referring to excreta assays for protein and amino acids because the term "metabolizable amino acids" is uncommon and the amount excreted in the urine is small (Parsons 2002). For energy, one can consider (1) apparent digestible energy (DE), which is the feed energy consumed minus the gross energy of the feces; (2) apparent metabolizable energy (AME), which is the gross energy of the feed consumed minus the gross energy of the feces, urine and gaseous products of digestion (these are negligible); (3) true metabolizable energy (TME), which the gross energy of the feed consumed minus the gross energy of the excreta corrected for metabolic fecal and endogenous urinary energy; and (4) net energy (NE), which is the metabolizable energy minus the energy lost as the heat increment (NRC 1994). A correction for nitrogen retained in the body may be applied to yield a nitrogen-corrected AME (AMEn) and TME (TMEn). AMEn is the most common measurement used in formulating poultry feeds; NE is seldom used (NRC 1994).

Digestion assays measure only nutrient digestion and absorption, not utilization. Apparent metabolizable nutrient is most commonly determined by obtaining a total collection of feces, analyzing the feces for the nutrient in question, and subtracting the total amount of nutrient excreted from the amount of the nutrient consumed, generally during a 3-day period. The difference in these two values is the amount of the nutrient digested. Another method is to include an indigestible marker in the feed (such as 0.25% of chromic oxide), and take a series of fecal samples after several days. By determining the ratio between the indigestible marker and the nutrient in question in the feed and feces, one can determine the apparent digestibility of the nutrient. Fuller (1991), NRC (1994), and McNab (2000) describe AME methodology and TME measurements of feedstuffs. Parsons (2002) and Lemme et al. (2004) have published reviews on methodology for assessing amino acid digestibility in poultry. Sales and Janssens (2003) have provided a discussion on the use of markers to determine energy metabolizability and nutrient digestibility in poultry when total collection is not desired.

Literature Examples

Recent publications have reported the use of the precision-fed cockerel assay in evaluating the digestibility of distillers dried grains (Lumpkins and Batal 2005, Batal and Dale 2006), the classical digestibility study in evaluating soybean lectins in turkey poults (Fasina et al. 2004), and the ileal digestibility assays to evaluate amino acids in feed ingredients (Huang et al. 2006).

Bioavailability Studies

Bioavailability studies are not commonly conducted in laying hens. See Chapter 11, Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Poultry Meat Production, for an explanation of bioavailability studies.

Product Quality

Certain GM traits could affect different portions of the egg's weight, grade, fatty acid composition, vitamin composition, yolk color, or other product quality measures. In certain instances, the GM trait could affect the flavor or other characteristics of the cooked egg. Standard methods have been described for the physical measurements of egg traits and for measuring sensory evaluation by a trained taste panel (Mountney 1989, The Poultry Site 2005).

Sensory (Organoleptic) Evaluation

If a new feedstuff that is fed to poultry has the potential to affect the organoleptic quality of the retail product offered to the consumer, a sensory evaluation should be done. It is not within the scope of this paper to delve into the specifics of sensory studies. Specific details on conducting sensory studies have

been published (Johnsen and Kelly 1990, Lawless and Heymann 1999, Meilgaard et al. 1999, Stone and Sidel 2004). Meilgaard et al. (1999) discussed in detail sensory attributes and human perception; controls for the test room, product, and panel; physiological and psychological factors affecting sensory verdicts; measuring responses; Triangle Test; Duo-Trio Test; Two-out-of-Five Test; Simple Difference test; "A" – not "A" Test; Difference-from-Control Test; Sequential Tests; Paired Comparison Designs; Directional Difference Test; Pairwise Ranking Test; numerous Multi-sample Difference Tests; determining thresholds; selection and training of panel members; descriptive analysis techniques; the Spectrum™ Descriptive Analysis Method; Affective Tests including consumer tests and in-house panel acceptance tests; statistical methods; guidelines for choice of technique; and guidelines for reporting results. Stone and Sidel (2004) provided additional information on organizing and operating a sensory evaluation program; measurements, test strategy, and experimental design; discrimination testing; descriptive analysis; affective testing; and dealing with special problems.

Literature Examples

Laying hen studies have been published on evaluating the long-term effects of feeding flaxseed on performance and egg fatty acid composition (Bean and Leeson 2003), influence of different fat sources on egg quality and lipid profile of egg yolks (Filardi et al. 2005), and enrichment of eggs with lutein (Leeson and Caston 2004) and vitamins (Leeson and Caston 2003, Mori et al. 2003).

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Chapter 13

Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Swine

Swine are produced in every country of the world, and pork (pig meat) is the most widely consumed meat by mankind. More than one billion pigs are slaughtered annually for fresh or processed meat that is consumed by approximately five billion people, resulting in a per capita consumption of nearly 15 kg of pork (carcass weight basis) worldwide. In the US, per capita pork consumption has remained relatively consistent since the 1970s. In 2004, per capita consumption averaged 30.5 kg (or 23.2 kg, retail weight basis), which is similar to the per capita consumption 25 years ago (Anon 2005).

Swine are omnivorous and can obtain nutrients from a variety of feedstuffs. However, they most efficiently utilize energy sources that are easily digested and that do not require microbes to ferment the more complex energy sources. In addition, swine require amino acids rather than protein per se, so the profile of amino acids in the protein of feedstuffs that are fed is important. Also, because they are concentrate consumers and are generally raised in confinement, swine are susceptible to numerous mineral and vitamin deficiencies. Therefore, diets must be supplemented with adequate levels of these nutrients for swine to grow, reproduce, and lactate efficiently. Nutrient standards for swine are outlined in the NRC (1998) publication, *Nutrient Requirements of Swine*.

This chapter focuses on guidelines for conducting nutritional evaluations of GM crops and their byproducts containing output traits as measured by: (1) growth performance, (2) digestibility of specific nutrients, (3) bioavailability of nutrients, and (4) assessment of pork quality, all of which could be affected by a GM output trait. Background information regarding the various nutrient groups (amino acids, carbohydrates, lipids, minerals, and vitamins) as well as background on antioxidants, enzymes, and antinutrients is found in Chapters 4-10. A GM output trait in a cereal grain will be used as an example.

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 animals should have *ad libitum* access. 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.

Growth Performance Studies

This protocol describes a study to evaluate the nutritional value of a 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). Generally, the study will include a minimum of three treatments. For example, an evaluation of a GM grain with a greater amino acid content or superior amino acid profile would require the feeding of a low-protein diet containing the GM grain, a similar low-protein diet with a conventional grain, and an adequate-protein diet with the conventional grain (positive control). The low-protein diet is necessary to allow the expression of the superior amino acid content or profile of the GM grain.

Production, handling, storage, and processing of the grain are described in Chapter 2 of this document, while sampling and analysis of the grain for mycotoxins and chemical components are described in Chapter 3.

Pigs

Healthy pigs of similar genetic background that have been fed a common diet for at least one 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. In these cases, the pen of pigs is the experimental unit. 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 *The 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 to treatment 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 regular 2- or 3-week intervals during the experiment. Periodic weighing and feed intake measurements, rather than only initial and final measurements, enable 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. Whenever possible, pigs should be weighed on the same day of the week and 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 kept to a minimum so that feed disappearance can be equated to feed consumption. 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. If possible, littermates should be distributed evenly across treatments. For example, in a study with three treatments, the first outcome group may be the three heaviest males randomly assigned to three pens in the first block; the second outcome group is the next three heaviest males, etc. In this example, the three pens in the first block (each consisting of four to eight outcome groups) will have similar average starting weights and will be randomly allotted to the three treatments. If possible, blocks should consist of the same sex. If not possible, blocks may consist of mixed sexes, but the sex ratio must be constant within each block. Having the same sex within a pen/block will reduce the variation in growth performance of pigs within the pen and it will reduce any effects of sexual maturity within the pen near slaughter. Also, 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 the types of differences anticipated (see Chapter 17, Statistical Analysis and Interpretation of Results). For a coefficient of variation of 5–7% (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 the cereal grain and soybean meal (or other high-quality protein source commonly used for swine in the region) fortified with highly bioavailable sources 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.

Diets should be formulated on the basis of total or, preferably, digestible or available amino acids. The diet containing GM grain, although having less protein content, should be formulated to meet 105% of the lysine requirement of pigs, similar to that in the positive control diet. The negative control diet containing the conventional grain will contain less lysine and amino acids, but should contain similar amounts of grain and protein supplements as the GM grain diet. 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. In countries where commercial diets normally contain added fat, all diets should contain 1–3% added fat, with adjustments made so that all diets are isoenergetic.

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

The cereal grain should be ground to a consistent geometric mean particle size (600–900 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. If antimicrobial growth promoters or other additives are used, the inclusion rates 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), 10th rib backfat, 10th 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 17, Statistical Analysis and Interpretation of Results). Either the 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.

Short-term Grower Studies

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 3–5 weeks (7–12 kg body weight) and will last 4–6 weeks. "Grower" experiments to evaluate new crops will generally involve pigs with an initial average body weight of 15–25 kg and will last 6–8 weeks. The protocols will be similar to those described for growing-finishing pigs. However, pigs may be weighed and feed consumption may 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.

Digestibility Studies

In some instances, assessing digestibility of a specific nutrient or nutrient groups is warranted. For example, this may be needed when one is evaluating a GM feed that has an altered carbohydrate (such as a reduced amount of cellulose or hemicellulose) or one with a specific enzyme incorporated that is intended to increase digestibility. In digestibility studies, a minimum of four to six replications is desirable.

Digestibility studies in pigs are based on either total tract digestibility or ileal digestibility; the latter assesses the nutrient digestibility at the terminal ileum before ingesta are exposed to the microflora in the pig's hind gut. Ileal digestibility is particularly important for determining digestibility of individual amino acids in a protein source. Digestibility coefficients can be determined on the basis of either apparent digestibility or true digestibility. For true digestibility, the investigator must obtain an estimate of endogenous (non-dietary) excretion of nutrients, which will require additional treatments so that results can be extrapolated back to zero endogenous.

Digestibility of nutrients can be determined by obtaining a total collection of feces, analyzing it for the nutrient in question, and subtracting the total amount of nutrient excreted from the amount of the nutrient consumed, generally during a 5-day collection period after at least a 7-day acclimation period during which the experimental diets have been offered. The difference between these two values is the amount of the nutrient digested. Another method is to include an indigestible marker in the feed, and take a series of fecal samples after several days. By determining the ratio of the indigestible marker in the feed and feces and the ratio of the nutrient in question in the feed and feces, one can determine the apparent digestibility of the nutrient. This approach is used in ileal digestibility studies because it is not practical to do a complete collection of digesta.

Collection, quantification, and analysis of urine

can also be done in digestibility studies. This procedure would allow one to determine retention of a specific nutrient. In some instances, this measurement may be useful, but generally total tract or ileal digestibility gives a good estimate of the feedstuff's value.

Details on procedures for conducting digestibility studies were reviewed by Fuller (1991) and Adeola (2001).

Bioavailability Studies

Studies of a nutrient's bioavailability would be conducted to determine whether a nutrient in a GM feedstuff is more bioavailable to the animal than the same nutrient in a conventional feedstuff. An example would be an evaluation of two cereal grains with the same amount of phosphorus, but with the GM grain having less phosphorus in the form of phytic acid (e.g., low-phytate corn) and more inorganic phosphorus than in a conventional grain. To assess such a trait, one would feed a low-phosphorus basal diet consisting a purified carbohydrate (e.g., corn starch and/or dextrose) and an amino acid source, vitamins, and minerals (except phosphorus) to meet the pig's nutrient requirements. Additional diets would be the basal diet including the GM grain (substituted for the purified carbohydrate source), a conventional grain, and a highly bioavailable phosphorus source (such as monosodium phosphate) as the standard. Ideally the GM grain, the conventional grain, and the standard should be added at incremented levels (minimum of two, but preferable three or more levels), but the highest level of phosphorus from the standard and the grains must still be below the requirement to ensure the response is linear. Slopes of the responses are determined and a ratio of the linear slopes of the two grains compared with the slope of the standard gives the relative bioavailability of phosphorus in the two grains. The standard is considered 100% bioavailable.

In studies of this type, it is paramount that the response be one that is sensitive to the nutrient under investigation. For example, with phosphorus, measures of bone mineralization (bone ash, bone strength, etc.) are best. For an iron assessment, blood hemoglobin concentration is an appropriate measurement. Growth responses are generally not sensitive enough to determine bioavailability of most nutrients, but are useful in determining bioavailability of amino acids.

Details on conducting bioavailability studies are covered in reviews by Ammerman et al. (1995) and Gabert et al. (2001).

Pork Quality

Certain GM traits could affect distribution of fat and lean in the carcass, or the trait could affect the tenderness, flavor, or other characteristics of the final cooked pork product. The NPPC (2000) publication describes standard methods for determining the amount of backfat, longissimus area, carcass lean percentage, and other measures of overall carcass quality. Procedures for instrumental measurements of tenderness and sensory evaluation of tenderness, flavor, and other measures of consumer satisfaction with respect to cooked pork were reported by the AMSA (1995). Aberle et al. (2001) reviewed meat color and other assessments of meat quality.

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Chapter 14

Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Lactating Ruminants

Lactating ruminants are found worldwide with more than 239 million milking cows, 187 million milking sheep, 151 million milking goats, and 54 million milking buffalo producing 520, 8, 12, and 77 million metric tons of milk, respectively (FAOSTAT data 2005). Lactating ruminants (i.e., dairy cows, sheep, goats, and buffalo) have the ability to convert forages; roughages; grains; and coproducts of the milling industry, oilseed industries, fruit and nut industries, citrus industries, and other food industries into milk for human consumption. Ruminants have a fermentation vat (reticulo-rumen) near the beginning of the digestive tract that connects to the mouth via the esophagus. It is this fermentation system that enables them to process feed ingredients that are of poor nutritional value to nonruminants into highly nutritious products for humans. The animal relies on the endproducts of fermentation and the undigested dietary residues leaving this compartment to meet its nutrient requirements for maintenance, growth, reproduction, and lactation. One goal of the ruminant nutritionist is to provide an economical blend of dietary ingredients that optimizes ruminal fermentation as well as maximizes the available nutrient flow to the small intestine for the health and productive performance of the animal. Environmental considerations, including minimizing animal wastes such as biomass, nitrogen, phosphorus, and methane, are coming more to the forefront. In addition, recognition that consuming healthy foods and functional foods may enhance our lifespan is resulting in the development of healthier milk products. Examples of these products may include milk containing more antioxidants or omega-3 fatty acids, milk with better fatty acid profiles, milk containing known potent anticancer agents, and milk with reduced levels of antigenic proteins.

In the future, there will be development of forages, roughages, grains, and oilseeds with improved protein and amino acid characteristics, fiber digestibility, mineral availability, vitamin and antioxidant content, fatty acid profiles and content; decreased antinutrients; enhanced enzymatic activity to augment the digestive capacity of the animal; and compounds that improve the ruminal-reticulo fermentation and reduce the endproducts of digestion that have potential negative health effects on the animal (Owens et al. 2002, Hartnell 2004, Hartnell et al. 2005). Feed ingredients will be developed that contain components that escape ruminal fermentation, are absorbed in the bloodstream, and deposited in the milk. With changes in milk composition, the organoleptic attributes of milk and dairy products derived from milk will need to be evaluated.

This chapter focuses on guidelines for conducting nutritional evaluation studies of GM crops and their coproducts containing output (nutritionally enhanced) traits when fed to lactating ruminants. Factors such as nutrient requirements; management of the feed, animal, and facility; and diet formulation and processing are covered. Studies to evaluate the nutritionally enhanced traits in the crops or their coproducts that are discussed include digestibility of specific nutrients; bioavailability of a nutrient(s); animal performance; milk quality, such as composition and retail product assessment; and sensory evaluation of dairy products destined for human consumption.

Nutrient Requirements

In designing nutrition experiments to evaluate nutrient enhanced grains or forages, knowledge of the animal's nutrient requirements is needed. In the United States, the National Research Council has published nutrient requirements for lactating dairy cattle (NRC 2001), lactating goats (NRC 2007), and lactating sheep (NRC 2007). Other country-specific feeding standards may be considered. An understanding of nutrient digestion and metabolism, intermediary metabolism, nutritional physiology and pathology, and nutrient flow and retention in lactating ruminants will be important in developing strategies for evaluating specific nutritional enhancements (D'Mello 2000).

The intent of this section is not to cover the specific requirements of all lactating ruminants but to use the lactating dairy cow as an example. A discussion on each nutrient category will not be provided here because the Nutrient Requirements of Dairy Cattle (NRC 2001) work has covered this material in sufficient detail. Topics include: (1) dry matter intake, including factors that affect intake and methods of predicting it; (2) energy, including definitions of energy terms, measurement of energy, requirements for maintenance, lactation, activity, and pregnancy, tissue

mobilization, body condition scoring, digestibility, effects of fat on rumen fermentation, and use of fat in lactating dairy cow diets; (3) carbohydrates, including nonstructural (sugars, starches, organic acids, etc.) and structural (cellulose, hemicellulose, and lignin) with a focus on neutral detergent fiber (NDF) and acid detergent fiber (ADF); (4) protein and amino acids, including ruminal protein degradation and synchronization with carbohydrate digestion, rumen degradable and undegradable protein, animal and microbial requirements, lactational responses, limiting amino acids, effects on reproduction; (5) minerals, including macro- and trace minerals, bioavailability, and requirements; (6) vitamins, including fat-soluble and water-soluble vitamins, bioavailability, and requirements; and (7) other, including feed chemistry and processing, water, environmental effects, etc. Relevant information has been published on nitrogen metabolism in the rumen (Bach et al. 2005) and amino acid and protein metabolism in the mammary gland (Bequette et al. 1998, Doepel et al. 2004).

Management

Management of lactating dairy cattle is important in realizing optimum milk yield, feed efficiency, and health; however, it is not within the scope of this document to provide the detailed management guidelines used in dairy cattle husbandry. Specifics on issues such as feeding practices and practical diets, grazing management, feed formulation, feed types and uses, feed handling and storage, and feeding methods are available in other publications (Bath et al. 1985, Broster et al. 1986, Ensminger et al. 1990, Speedy and Sansoury 1991, Van Horn and Wilcox 1992, Jurgens 2002, Kellems and Church 2002). Lactating dairy cows used in experiments to evaluate GM crops should be housed and managed in a similar fashion.

Diet Formulation and Processing

Numerous publications discuss diet formulation and processing in detail (Ensminger et al. 1990, Jurgens 2002, Kellems and Church 2002). Many factors must be considered in formulating feed for lactating animals. Important points to consider include: feeding total mixed rations versus offering forage separately from the concentrate, proper mixing, homogeneity testing, fat/oil application, and amino acid and fatty acid protection in the rumen. Nutritional considerations include: body weight, stage of lactation, reproduction, parity, nutrient requirements, available feed ingredients, form of the feed required for optimal consumption, and feed processing. Non-nutritional factors that need to be considered include pasture growth, logistics of acquiring and storing ingredients, and capabilities for mixing feed, such as equipment that can handle long forage for blending total mixed rations. The feed manufacturing process also needs to be considered because if the grain or concentrate mix will be fed in the milking parlor, the product needs to flow freely out of the bins with minimal dust. This will result in better feed consumption by the animals, especially when they have a limited amount of time to consume the feed (e.g., eating in the milking parlor). Also, with a pelleted feed there is less worry about ingredient separation occurring in storage. However, if fat/oil needs to be added, one may have to consider spraying it on the pellet to maintain good pellet durability. Binding agents help prevent poor pellet quality.

Consideration should be given to environmental conditions (i.e., temperature and humidity) that can affect ingredient handling. Enzyme supplements are added to feed to enhance digestion of feed components, remove antinutritional factors, and supplement the activity of endogenous enzymes. The review by McAllister et al. (2001) discusses production responses to supplementary exogenous enzymes in ruminant diets, including possible mechanisms. With enzyme(s) addition, one must consider the temperature effect of the pelleting process. In many cases, temperature-sensitive ingredients will have to be sprayed onto the outside of the pellet or, as is the case for vitamins, be added in excess to account for the destruction that occurs during the manufacturing process. Alternatively, the concentrate mixture is not pelleted but is incorporated directly into a total mixed ration or the concentrate mixture is fed in a feed manager where the animal has time to consume the meal feed. The mixture of ingredients should be assessed along with the storage conditions and duration to determine whether antioxidants need to be added to preserve the feed. The advantage of a total mixed ration is that each bite of feed contains the same concentration of nutrients so the researcher knows each animal has received the same ratio of nutrients. When concentrate and forage are offered separately, different animals will eat different amounts of forage and concentrate, so the ratios and amounts of nutrients may vary significantly from one animal to another. In studies designed to evaluate dietary ingredients and nutrition, the researcher needs to remove as much variation from the study as possible to improve the sensitivity of the test system. Therefore, a total mixed ration is preferred whenever possible or the proportion of concentrate and forage offered needs to be fixed.

Animal Studies

Biological evaluation of feed ingredients and diets containing the trait of interest involves feeding lactating cows and measuring endpoints such as digestibility, bioavailability, milk yield, feed efficiency, body weight, body condition, milk composition, and sensory evaluation of the dairy products. The benefit of feeding studies is that a direct evaluation of the trait of interest can be made in the target species. *In vivo* evaluation of feedstuffs with nutritionally enhanced traits must be conducted in a controlled environment to remove as many biases and potentially confounding variables as possible. This helps to provide clarity in the interpretation of the results. For any experiment, the investigator must try to control as many variables as possible such that the test variable is the main effect. Proper controls or comparators should be utilized based on the trait of interest.

Factors that might affect the study results include animal characteristics (i.e., breed, age, weight, parity, stage of lactation or days-in-milk, milk yield, physiological state, health), water quality (i.e., temperature, salinity, dissolved oxygen, pH, bacterial load, suspended solids, nitrates, heavy metals), milking routine and frequency, feed quality (i.e., ingredient quality, homogeneity of the mix, diet consumption), and the amount and timing (time of day and number of hours per day) of feed offered. In an experiment, treatment groups of lactating dairy cows should be of the same breed, body condition, stage of lactation, milk yield, and milk composition. Depending on the experimental design, initial differences in milk yield may be adjusted by using a covariate. The desired stage of lactation will depend on the nutritional trait to be evaluated, the objective, and end points of the study. For digestibility studies, lactating cows should be beyond their peak milk yield in lactation period. To evaluate amino acids, protein, and energy sources, the most responsive period is in early lactation.

Experimental Design Considerations

Experimental design and statistical power (Cochran and Cox 1957, Montgomery 2001, Tempelman 2004) are key components in planning lactating cow studies. Determining the number of replicates to use in a study is crucial to making sure the experimental design has sufficient power to detect the minimum differences between treatments. The number of replications required depends on the experimental design employed, the desired difference from the mean that one would like to be able to detect, and a specific probability level and type II error. Crossover, switchback, or Latin square designs are typically used because digestion studies are labor intensive and require specific facilities. Generally, the periods range from 21 to 28 days with most having at least a 10 to 14 day adaptation period before the collection phase begins. Digestibility studies using lactating cows in these types of experimental designs have been published for the evaluation of fats and fatty acids (Canale et al. 1990;,Grummer et al. 1996), protein and amino acids (Grummer et al. 1996, Pruekvimolphan and Grummer 2001, Korhonen et al. 2002), enzymes (Sutton et al. 2003, Kincaid et al. 2005), minerals (Ben-Ghedalia et al. 1996, Weiss 2004, Kincaid et al. 2005), carbohydrates (Batajoo and Shaver 1994, Dado and Allen 1996, Beauchemin et al. 1997, Oba and Allen 2000, Harvatine et al. 2002, Qiu et al. 2003, Beckman and Weiss 2005, Ivan et al. 2005, Charbonneau et al. 2006), and pastures (Stobbs and Sandland 1972, Berzaghi et al. 1996). Many of the studies used replicated squares in the Latin square designs, so a study using a 4 × 4 Latin square design would need eight animals. The crossover and switchback designs typically incorporated six animals per treatment group. Randomized block designs (Sullivan et al. 1993, Hammond et al. 1996, Yang et al. 2000), factorial arrangement of treatments (Chen et al. 1995, Ouellet et al. 2003), and split-plot designs (Blackwelder et al. 1998) may also be used for digestibility studies where the measurement of digestibility is part of a performance study. In these designs, typically 12 to 15 replicates per treatment were used. However, to determine the precise number of replicates to use (based on the predetermined difference likely to be detected for the targeted traits), one should use data from a previous study at the same facility to calculate the required statistical power. For specific experimental designs and statistics, see the Chapter 17 on statistics in this document and publications such as those by Cochran and Cox (1957) and Montgomery (2001).

Bioavailability: Digestibility Studies

Nutrient bioavailability can be determined in digestibility studies that measure the percentage of nutrients absorbed and used by dairy cows. Digestibility describes the quantity of nutrients or energy from an ingested dietary component that is not excreted in the feces, whereas metabolizability also includes the quantity of nutrients or energy that is not excreted in the urine. Digestibility studies are time consuming, expensive, and require large amounts of the test and control materials (Weiss 1994). In addition, variability of the results can be quite large with procedural variability among investigators being an important factor (Cochran and Galyean 1994). Using an accepted, standardized methodology is important in obtaining reliable data.

Cochran and Galyean (1994) provided a review of the information on procedures used to conduct digestion trials. They discussed housing, restraint and collection methods, animal considerations (i.e., animal selection and training, number of animals, schedules and records), digestion trial conduction methods (i.e., period structure and length, feeding practices and measurement of nutrients consumed), excreta output measurement (i.e., total fecal collection, external markers, internal markers, processing fecal samples), calculation of digestion coefficients, and sources of error and design considerations (i.e., analytical and technical errors, animal variation, experimental design considerations). Cochran and Galyean (1994) also provided general protocols for digestion experiments conducted in confinement and under grazing conditions.

A mathematical analysis of the digestion process has been discussed in detail (Van Soest 1982). Examples of digestibility studies for lactating dairy cows include protein sources (Sullivan et al. 1993, Van Straalen et al. 1993, Grummer et al. 1996, Korhonen et al. 2002), fat sources (Palmquist 1991, Elliott et al. 1996, Pantoja et al. 1996, Weiss and Wyatt 2000, Gonthier et al. 2004), pasture (Bargo et al. 2003), carbohydrates (Bernard et al. 1991, Santini et al. 1992, Dado and Allen 1996, Batajoo and Shaver 1994, Schmidely et al. 1999, Leiva et al. 2000, Oba and Allen 2000, Miron et al. 2002, Qiu et al. 2003, Beckman and Weiss 2005), vitamins (Doreau and Ottou 1996), minerals (Ben-Ghedalia et al. 1996, Weiss 2004, Kincaid et al. 2005), and enzymes (Chen et al. 1995, Yang et al. 2000, Bowman et al. 2002, Knowlton et al. 2002, Sutton et al. 2003, Eun and Beauchemin 2005, Kincaid et al. 2005). Most digestibility studies used a Latin square design with 21 to 28 day periods. Sample collection took place during the last five to seven days of the period.

Because *in vivo* measurement of nutrient digestion is time-consuming, labor-intensive, and expensive, alternative techniques that are simple and reliable in their prediction of nutrient digestion in the rumen and small intestine have been developed (Stern et al. 1997, Weiss 1994). Weiss (1994) discussed the different methods of estimating digestibility of forages (i.e., *in vitro* disappearance, two-stage *in vitro* method); factors that affect the accuracy, precision, and reproducibility of estimated values and how these factors can be controlled (i.e., donor animal effects, statistical considerations, *in vitro* digestion kinetics); various methods for estimating digestibility (i.e., enzymatic methods, *in situ* disappearance [bag characteristics, sample characteristics, dietary effects, analytical techniques, correction for microbial contamination, statistical considerations, accuracy]); and chemical composition and statistical models. More recently Stern et al. (1997) reviewed the use of methods for measuring ruminal digestion of nutrients (i.e., *in vitro*, *in situ*) and intestinal protein digestion (i.e., bioassays, *in situ* mobile-bag technique, and *in vitro* [such as lysine availability test, intestinal fluid, enzymatic methods]).

Performance

In vivo performance trials provide a more absolute evaluation of a nutrient's bioavailability in a feed ingredient than digestibility trials do. A digestibility trial may show two ingredients having equal digestibilities, but an *in vivo* performance trial may show a difference in milk yield due to differences in how the absorbed nutrient(s) were metabolized. In an *in vivo* performance trial, test animals are fed the test nutrient or a standard reference nutrient in a basal/control diet that is deficient in the test nutrient but otherwise nutritionally adequate. Similar concentrations of the test nutrient from a standard source that is traditionally used and of high bioavailability should also be used in the reference diet. The animals should be in a nutritional state such that they are responsive to the test nutrient in question. Cows should be given *ad libitum* access to the test diet, a diet that is deficient with the nutrient of concern, and the reference diet containing a similar amount of the nutrient in question from a highly bioavailable source for a specified period of time.

The choice of what experimental design to select depends on whether one expects to see a significant carry-over effect from feeding one treatment to another. If significant carry-over effects are not expected, crossover, switchback or Latin square designs may be used. Typically, these designs use a 21 to 28 day period with the first 10 to 14 days for adjustment and the last 7 to 14 days of the period for data collection. Latin square designs have been used to evaluate protein and amino acids (Canale et al. 1990, Grummer et al. 1996, Pruekvimolphan and Grummer 2001, Korhonen et al. 2002, Sannes et al. 2002), enzymes (Bowman et al. 2002, 2003; Knowlton et al. 2002; Sutton et al. 2003), fat and fatty acid sources (Jones et al. 2000, Drackley et al. 2001, Ward et al. 2002, Ruppert et al. 2003), vitamins and antioxidants (Atwal et al. 1990, Doreau and Ottou 1996, Majee et al. 2003, Al-Mabruk et al. 2004), minerals (Weiss 2004, Kincaid et al. 2005), and carbohydrates (Feng et al. 1993, Batajoo and Shaver 1994, Friggens et al. 1995, Beauchemin et al. 1997, Oba and Allen 2000, Drackley et al. 2001, Sannes et al. 2002, Qiu et al. 2003, Beckman and Weiss 2005, Ivan et al. 2005, Taylor and Allen 2005, Charbonneau et al. 2006).

If there is an anticipated trait effect on intake or milk yield, a 28-day period should be adequate. However, if the effect may be subtler, a 10- to 13-week

period should be used. The treatment period is usually preceded by a 2-week pretreatment phase that can be used as a covariate. The covariate adjusts the treatment groups for any differences in milk yield among the treatments at the initiation of the study. Randomized block designs have been used to evaluate protein and amino acids (Sullivan et al. 1993, Park et al. 2002, Ouellet et al. 2003), enzymes (Schingoethe et al. 1999; Kung et al. 2000, 2002; Yang et al. 2000; Vicini et al. 2003), fat and fatty acid sources (Teh et al. 1994, Pantoja et al. 1996, Weiss and Wyatt 2000, Ramaswamy et al. 2001), vitamins and antioxidants (Brzezinska-Slebodzinska et al. 1994, Weiss 2001, Weiss and Wyatt 2003), minerals (Ben-Ghedalia et al. 1996), and carbohydrates (Bernard et al. 1991, Santini et al. 1992, Minor et al. 1998, Schmidely et al. 1999, Broderick et al. 2002, Miron et al. 2002). Factorial treatment arrangements (Casper et al. 1990, Chen et al. 1995) or split-plot designs (Adams et al. 1995) have been used when multiple traits are being evaluated within a study.

Milk production, composition, and component yields are usually the most important criteria for measuring lactating cow response to experimental diets. However, feed or milk production efficiency (milk yield/unit of dry matter consumed) is becoming an important tool (Hutjens 2005). Besides milk yield, its composition (i.e., protein, fat, lactose, solids nonfat) is important in producing dairy products (i.e., butter, cheese, ice cream, yogurt, etc.). For feed traits that are known to affect milk characteristics, those specific milk components should be measured. An example is feeding lactating cows sources of unsaturated fat that may escape biohydrogenation in the rumen. The increase in the level of these polyunsaturated fatty acids postruminally could affect the fatty acid composition of the milk (Grummer 1991, Baer et al. 2001). Alternative endpoints such as enzymes, mineral, and vitamin levels in serum and milk may be used when evaluating dietary minerals and vitamins (Brzezinska-Slebodzinska et al. 1994). In the longer-term studies, changes in body weight and body condition should be measured.

Dairy Product Quality

Dairy product quality may be defined as microbial quality, shelf life, flavor, or a combination of these (Drake 2004). Specific response measurements will need to be included in the experimental design based on expected effects of the target traits. For example, changing dietary fatty acid consumption could affect the fat content, fatty acid composition, and peroxidation state of milk (Jones et al. 2000, Baer et al. 2001). Changes in milk composition may affect the dairy product flavor, shelf life, and functionality. Nutritional factors have been shown to influence milk composition (Grummer 1991, DePeters and Cant 1992, Palmquist et al. 1993, Santos et al. 1998, Chilliard et al. 2003). Alteration in the amount and type of proteins in milk may affect cheese yield and flavor (Coulon et al. 2004).

Retail Product Assessment

For traits such as dietary oils and fatty acids, antioxidants, and others that may result in appearance and shelf life differences in the retail product, an assessment of the retail products derived from those treatments should be considered.

Sensory (Organoleptic) Evaluation

If a dietary trait has the potential to affect the organoleptic quality of the retail product offered to the consumer, a sensory evaluation should be done. It is not within the scope of this publication to delve into the specifics of sensory studies or into the specific feed and animal factors affecting sensory characteristics. The relationship between ruminant management and sensory characteristics of dairy products has been reviewed recently (Coulon et al. 2004, 2005; Martin et al. 2005). The authors reported that the amount of carotene in the diet affects the yellow coloration of cheese. Concentrated levels of terpenes found in certain dicotyledons species can invoke discernable aromatic properties. In addition, these terpenes may have an indirect effect on the sensory properties of cheese by modifying the dynamics of the microbial ecosystem during the cheese making and ripening process. Modifications in protein and fat composition can affect dairy product sensory properties. Endogenous enzymes such as plasmin, which affects cheese texture and ripening, could be increased as the result of the animal consuming certain plant species (e.g. buttercups [Ranunculus spp.]).

Sensory analysis is a compilation of different tools or tests that can be used for subjective or objective evaluation of food sensory properties (Drake 2004). Sensory character of cheese and its evaluation (Delahunty and Drake 2004), flavor lexicons (Drake and Civille 2003), sensory attributes of dried milk powders and dairy ingredients (Drake et al. 2003), and an extensive discussion of quality judging for dairy products (Bodyfelt et al. 1988) have been reported. Specific details on conducting sensory studies have been published and will serve well as references (Lawless and Heymann 1999, Meilgaard et al. 1999, Stone and Sidel 2004). Lawless and Heymann (1999) provided background on the physiological and psychological foundations of sensory function, discrimination testing

and theories, measurement of sensory thresholds, scaling, time-intensity methods, biases in sensory judgment, descriptive analysis, texture evaluation, color and appearance, acceptance and preference testing, consumer field tests and questionnaire design, qualitative consumer research methods, and statistics. Meilgaard et al. (1999) discussed in detail sensory attributes and human perception; controls for the test room, product, and panel; physiological and psychological factors affecting sensory verdicts; measuring responses; Triangle Test; Duo-Trio Test; Two-out-of-Five Test; Simple Difference test; "A" – not "A" Test; Difference-from-Control Test; Sequential Tests; Paired Comparison Designs; Directional Difference Test; Pairwise Ranking Test; numerous Multi-sample Difference Tests; determining thresholds; selecting and training of panel members; descriptive analysis techniques; the Spectrum™ Descriptive Analysis Method; Affective Tests including consumer tests and in-house panel acceptance tests; statistical methods; guidelines for choice of technique; and guidelines for reporting results. Stone and Sidel (2004) provided additional information on organizing and operating a sensory evaluation program, measurements, test strategy and experimental design, discrimination testing, descriptive analysis, affective testing, and dealing with special problems.

General Guidelines

Lactating ruminants must be healthy, show no signs of mastitis, and have fully functional mammary glands for valid conclusions to be drawn from the data. A large enough pool of animals within the desired selection criteria should be available to select a uniform population for the study. The desired difference among treatment means for target endpoints must be determined a priori so an adequate number of replicates can be used. All treatments groups should be housed, fed, milked, and managed in a manner that avoids any bias. The amount of test material to be included in the diet should be at the maximum level that would be considered commercially viable. Each dietary component (maize, maize silage, soybean meal, etc.) should be prepared in the same way (ground, rolled, chopped, etc.) for each dietary treatment so that all diets are similar in particle size, forage content, etc. If any animal exhibits morbidity, its data collected during that period of morbidity should be removed from the analysis. If the animal's milk production does not return to normal and a qualified veterinarian deems the animal unhealthy to finish the study, it should be removed from the study. Any animal that dies during the study should be necropsied to the extent necessary to obtain a definitive cause of death. Data should be statistically analyzed using accepted variance methodology. Either the 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 HP 1 1ES, UK; (http://www.vsn-intl.com/genstat/) is recommended. When feed is given to individual cows, the cow is the experimental unit; when cows are fed as a group, the pen will be the experimental unit. See detailed guidelines in Chapter 17, Statistical Analysis and Interpretation of Results.

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Chapter 15

Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Growing and Finishing Ruminants

Ruminants are produced worldwide under range conditions where inputs and costs are relatively low. Uniquely equipped with the capacity to digest large quantities of fiber, ruminants occupy an ecological niche not shared by nonruminant animals. With 64% of the world's land area being unsuitable for cultivation, ruminants can both harvest and utilize forages from a vast expanse of non-tillable land as well as forage from arable land produced during crop rotations. In addition, their capacity for fiber digestion permits ruminants to utilize numerous and widely prevalent coproducts of both agricultural (e.g., crop residues) and industrial production (e.g., distillation and feed and food processing wastes). By harvesting and digesting fibrous feeds, ruminants convert inedible and underutilized resources and waste products into work, food, and fiber valued by humans. Worldwide, excluding ruminants maintained for commercial milk production, the number of ruminants is immense, with 1.1 billion cattle, 994 million sheep, 650 million goats, and 120 million buffalo (FAOSTAT data 2005). In the US, with its abundant grasslands, ruminants typically thrive largely on grazed or harvested forages, but for finishing, ruminants are usually fed grains and grain coproducts for two to five months.

The US beef industry developed independently from its dairy sector. With the largest fed-cattle industry in the world, the US is the world's largest producer of beef, primarily high-quality, grain-fed beef that is consumed domestically and exported. In the US, the beef industry includes two production sectors: cow-calf operations and cattle feeding. Beef production is cyclic in terms of herd size and production with peaks in population every eight to 12 years. Production of fed beef cattle is affected by grain availability and price because beef cattle are a major user of feed grains.

Cow-calf and ewe-lamb operations are scattered throughout the US, typically on land not suited or needed for crop production. Individual operations rely on range and pasture conditions that vary with rainfall and temperature. Beef cows and ewes graze forage from grasslands to maintain themselves and raise their young with little, if any, supplemental grain. The beef cow or ewe is usually maintained on forage (grazed pasture during the summer and harvested pasture during winter), as is the calf or lamb until it is weaned. Depending on forage supply and cattle prices, weaned calves or lambs may be retained for an additional period of grazing or be fed harvested forage until either being marketed or moved to a feedlot. An average beef cow herd consists of 40 cows, but operations with ≥100 cows, though they comprise only 9 % of all beef operations, account for over half of the beef cow inventory. Herds of < 40, are usually part of multicrop enterprises or are supplemental enterprises for owners with off-farm employment.

Although concentrated in the Great Plains, cattle and lamb feeding (finishing) is a viable enterprise in the Corn Belt, Southwest, and Pacific Northwest. Feedlots produce high-quality beef or lamb. Beef, typically of a USDA Choice or Select grade, is obtained by feeding grain and other concentrates for 90–300 days depending on weight at placement, feeding conditions, and the desired degree of finish. During finishing, daily gains will range from 1–2 kg with 5–6 kg of feed dry matter per kg of weight gained. Although most nutrients and energy come from forage until calves or lambs are weaned, feedlot rations generally contain 70–80% grain with 5–15% roughage and 2–5% of a supplement that provides protein, minerals, and vitamins. Nearly 50% of the corn grain produced in the US is fed to beef and dairy cattle. Coproducts of oilseeds (e.g., soybean meal, cottonseed meal) and manufacturing (e.g., distillation products) are included as sources of supplemental protein and energy for lactating cows and for growing beef cattle and lambs. Young growing and lactating ruminants are used to evaluate protein supplements because protein requirements are higher during early growth and lactation than during the finishing phase of growth. 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. Nutrients that influence carcass and meat quality also are evaluated with finishing ruminants. Further overview of commercial ruminant operations in the US and around the world can be gleaned from texts that address beef production (Field and Taylor 2002) and lamb production (Croston and Pollet 1994). Nutrient or energy requirements at various stages of production for growing and finishing ruminants also are available for beef cattle (NRC 2000), small ruminants (NRC 2007), and ruminants in general (Ferrell 2004).

This chapter outlines guidelines for conducting nutritional evaluation of crops and coproducts from GM crops containing specific output traits. In contrast to research on GM crops with modified input traits where equality in nutrient composition and animal productivity is anticipated, GM products with altered output traits can be expected to differ in nutrient composition or availability and to alter animal productivity relative to the unaltered product. This chapter will focus on the use of growing and finishing ruminants to evaluate GM grains and grain products and the use of growing ruminants to evaluate GM protein supplements. This chapter also addresses the use of growing ruminants to evaluate GM forages or forage products and crop residues from GM forages.

Numerous feed additives (nutrients that are not required but are added to feed to improve feed utilization, acceptance, health, or metabolism/productivity of the animal) are included in commercial diets for ruminants. These include feed stabilizers (e.g., antifungals, antioxidants, pellet binders, wetting agents), feed flavors, digestion modifiers (e.g., enzymes, buffers, ionophores, probiotics, acidifiers, defaunating agents), growth promoters (e.g., antibiotics, feed hormones, chemotherapeutics, saponins), environmentally active substances (e.g., zeolites, saponins), and health modifiers (e.g., anthelmintics, antimicrobial drugs, immunomodulators). Only factors present in GM products related to nutrient or energy supply and to feed acceptance and palatability will be discussed in this chapter. Certainly, health and metabolism of ruminants can be altered by GM products (e.g., reduced mycotoxin or enhanced antioxidant concentrations in feeds; specific fatty acids that alter microbial activity in the rumen). However, advertising claims that a specific GM product can alter health or metabolism of ruminants may require clearance as New Animal Drugs through the Food and Drug Administration in the US or its equivalent regulatory agency in other countries.

When conducting nutritional evaluation studies, special attention must be devoted to nutrient and energy composition of the diet and management of feed, animals, and the facility; each can influence results. The response criterion for both growing and finishing ruminants that is typically measured is performance (feed intake, rate of gain, and gain:feed ratio as an index of efficiency of growth). However, with GM crops, digestibility and bioavailability of nutrients and product composition and quality also may be of special interest and serve as response criteria.

Nutrient Requirements

To appraise grains and forages genetically altered to increase nutrient or energy content or nutrient availability, composition of the basal and test diets are of special interest. Published nutrient or energy requirements developed on a regional or national basis such as the National Research Council Publications for Beef Cattle (2000) or Small Ruminants (2007) or parallel publications from other countries should serve as the basis for diet formulation when combined with current understanding of digestion and metabolism. Specific chapters in this publication and in other publications that relate to the specific nutrient that has been modified in the GM product (energy, protein, minerals, and vitamins) will require particular attention. Additional information from published reviews and research papers may also provide useful information on proper diet formulation.

Animal Management

Because feeding experiments may be conducted under diverse environmental conditions (e.g., open-front buildings, pastures, dry lots), reports should include a summary of housing and climatic conditions and specific dates that the trial was conducted. Water is a key nutrient for all animals. An available supply of potable water must be provided and research locations should test their water source periodically for microbial contamination and toxicants that could affect animal performance and health.

Management practices involving the animal (e.g., transport, isolation, housing, veterinary practices), the diet (e.g., feeding frequency, bunk management, source and level of roughage and grain, grain processing), and response measurements (e.g., weighing conditions, sampling of digesta, tissue, or blood) shall follow standard industry practices, meet all humane treatment specifications (FASS 2004), and be clearly outlined in publications and reports. Specific exceptions to standard industry practices may be required for testing GM crops with modified output traits. One exception to the specification that all diets must contain the minimum amount of every required nutrient is that negative control diets (deficient in the test nutrient being studied) may need to be included in the study so that availability of and animal response to the test nutrient can be quantified. Also, for measuring bioavailability, a nutrient depletion phase may be needed to increase an animal's sensitivity to detect a difference in nutrient availability. In negative control and nutrient depletion diets, adequate intakes of all other unrelated nutrients still must be maintained, humane animal treatment specifications still must be followed, and the local Institutional Animal Care and Use Committee shall review the treatment regimen.

With the exception of the diet modification that

becomes necessary to assess nutrient availability, all animals within an experiment should be subjected to identical dietary and management practices. Proper animal practices shall be verified through review of protocols and facilities by Institutional Animal Care and Use Committees as outlined by FASS (2004). Individual health and performance records should clearly specify all preventative health measures (e.g., vaccinations and antibiotic therapy) as well the incidence of specific health problems (e.g., respiratory disease incidence and recurrence). The cause for removing any animal from a trial should be certified by a veterinarian with a diagnostic necropsy for all animals that die during a study. Any unapproved GM crops and animals fed such crops must be handled and disposed in accordance with regulations in place for each country.

Evaluation of Grains and Grain Products in Experiments with Finishing Ruminants

The following protocol shall be used to evaluate the nutritional value of GM grains with modified output traits (e.g., barley, maize, sorghum grain, millet, oats, rice, triticale, wheat) or products produced by extracting or processing of these grains (e.g., wet or dry milled products, fermentation residues) for finishing ruminants (e.g., beef cattle and sheep). In all experiments, an appropriate control consisting of grain or product—preferably the near-isogenic cultivar of the same hybrid that lacks the output trait being studied—must be included. Other controls outlined below and additional test diets described in Table 15-1 may be needed for full and proper interpretation or application of results of animal trials.

If the output trait of the GM grain being tested is intended to alter nutrient or energy content or nutrient availability, in addition to the negative control diet noted above, a positive control diet is required as noted in Table 15-1. The negative control diet shall consist of a near-isogenic conventional (control) grain. In cases where a near-isogenic cultivar or byproduct is not available, the nearest facsimile available shall be substituted. The positive control diet shall contain this control grain supplemented with the nutrient being evaluated to equal precisely the absolute concentration or the presumed nutrient availability of the diet containing the nutritionally altered GM product.

Though not required to evaluate a nutritionally altered GM product, additional diets may be needed to answer questions as noted in Table 15-1. For example, a "diluted GM diet" consists of the nutritionally altered product diluted with other diet components so that the concentration or availability of the altered nutrient in the diluted diet shall be equal to that of the negative control diet; this can be used to examine if the supplement amount can be reduced when the GM product is substituted for the typical supplement or whether other nutrients or factors become limiting. A "limit fed GM" treatment, in which the quantity of the GM diet is limited so that supply or availability of the test nutrient is equal to that of the negative control diet, can be used to determine if substituting the GM product for the control product can decrease the total feed or supplement needed by animals. In addition, multiple levels of supplementation are required to develop response curves and assess bioavailability of test nutrients more precisely. In summary, each study with output traits must include a minimum of three treatments, a negative control, the GM product, and a positive control.

Other treatments involving one or more other types of conventional grain, typically produced in the region of the investigator, may be included to obtain the spectrum of production or performance to be expected from additional cultivars or hybrids. Production, handling, storage, and processing of the grain shall follow procedures described in Chapter 2 of this document, while sampling and analysis of the grain for mycotoxins and chemical components shall follow procedures outlined in Chapter 3.

Test Animals

Intact males, castrates, or female ruminants of similar breed or type can be used. All animals used shall be healthy, free of parasites, and ideally share a similar genetic and nutritional history. Each animal will be individually identified with a unique ear tag, ear notch, or brand. Animals may be fed individually or in groups in accordance with guidelines described in *The Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS 1999).

At least five different types of studies can be used to evaluate the nutritive value of GM products for growing and finishing ruminants. These types include palatability measurements, feed intake studies, digestibility/bioavailability studies, performance studies, and carcass and meat quality trials. Several of these trial types may be combined into a single experiment. The specific type of study to be conducted will differ depending on the nutrient altered in the GM product and availability of resources (e.g., feed, animals, facilities, time, labor). However, an experiment should not be conducted unless it has adequate statistical sensitivity to detect differences specified below.

Table 15-1. Potential treatments for evaluating nutritional value of GM products with altered output traits in performance trials with ruminants

Treatment	Definition	Essentiality	Feed intake	Example	Expectation from useful GM product	Relevance of experiment
Negative control	Isogenic or basal diet	Yes	Free choice	Typical soybean meal (2.8% lysine) mixed with corn silage at 10% of diet dry matter	Equal to commercial products	Checks whether experimental conditions match those of the industry
GM product	Test material	Yes	Free choice	Lysine-rich GM soybean meal (e.g., 3.6% lysine) at 10% of DM mixed with corn silage	Statistically superior to Negative control	Tests value of GM product relative to current industry standard
Positive control	Negative control supplemented with nutrient modified in GM product	Desirable	Free choice	Typical soybean meal at 10% of diet with lysine added [e. g., negative control diet with 0.8% (3.6%-2.8%) lysine added to diet dry matter]	Not statistically superior to GM product	Checks veracity of experiment (whether a response SHOULD be expected from GM product)
Diluted GM product	GM product diluted with other diet components to provide the same PERCENTAGE of nutrient as negative control	Varies with target of GM product	Free choice	Lysine-rich GM soybean meal diluted with starch to match lysine of negative control diet [e.g., Lysine-rich GM soy meal at 7.8% (10%*2.8/3.6) of diet DM plus 2.2% (17-13.2) corn starch in a corn silage diet]	Not statistically inferior to Negative control	Examines potential for feeding less GM product in a supplement
Limit fed GM product	GM product limit fed to provide the same AMOUNT of nutrient modified as provided by negative control treatment	Varies with target of GM product	Equal to Negative control	Lysine-rich GM soybean meal 7.8% of diet dry matter mixed with corn silage with no additional supplement	Not statistically inferior to Negative control	Checks possibility of feeding less total supplement with GM product

Palatability Studies with Animals

Preference or feed palatability can be altered by feed flavors and odors (Provenza 1996, Damron 2003, Goff and Klee 2006). Preference trials test the ability of individual animals to select and prefer a specific feed when given a choice among two or more feeds; these trials are used extensively to evaluate feed components and additives for horses and companion animals. Specific treatment arrangements, rotation of feed locations, and experimental designs suited to quantify animal preference have been devised and should be followed to permit robust statistical analyses (Preston 1986). In addition, specific compounds with objectionable flavors (e.g., quinine, aluminum sulfate) can be added to a feed to quantitatively examine the degree of preference or rejection. Application of results of palatability trials is typically limited to conditions where animals have a choice among feeds (e.g., grazing trials with either pastures or crop residues). Results should be applicable to predict whether specific GM products will be sorted from other diet components in a compiled ration and either preferred or rejected. However, palatability trial results have little if any meaning for ruminants fed a single compiled diet because voluntary feed intake is not well correlated with preference for a feed (Larson 1995).

Feed Intake Studies

In contrast to palatability studies in which multiple feeds are provided to a single animal, animals in feed intake trials are offered only their specified test diet. With alteration of a feed's fiber components, voluntary feed intake and consequent animal productivity can be increased or decreased. Because individual animals differ in voluntary feed intake, precision of feed intake trials can be improved through use of crossover, switchback, or Latin square experiments in which the animal is provided with different diets during different time periods so that it statistically serves as its own control. An excess supply of fresh feed must be provided and extreme care taken to quantify dry matter and nutrient content both of the feed offered and the feed refused, while reducing feed sorting and waste are essential components of feed intake trials. A minimum time period for animals to adapt to the amount of grain in their diet (as long as a month for transfer from high-roughage to high-concentrate diets) and to measure intake (typically one week of each diet within a roughage level) is required to obtain repeatable measurements. Because numerous details, such as time of day that forage is harvested, can influence voluntary feed intake (Mayland 2002), special care must be exercised to equalize harvest time and feed preparation conditions as well as other dietary and environmental factors in feed intake trials. Furthermore, researchers must not confuse the quantity of feed delivered to an animal or group of animals (where excess feed remains some days but not others) with trials designed to specifically measure voluntary feed intake. Nevertheless, results from feed intake trials should accurately predict the feed intake by animals in feeding trials that do not limit the supply of feed available to animals.

Digestion Studies

To detect whether GM products with altered nutrient or energy content or bioavailability have value for ruminants, digestion or metabolism trials can be conducted. Most digestion trials measure nutrient or energy disappearance through the total digestive tract and involve collection of feces with or without collection of urine (Merchen 1988). Site and rate of digestion also can be measured. Extent of ruminal digestion for specific durations can be determined *in situ* (e.g., with incubation of samples enclosed within small-pore size polyester bags inside the rumen of cannulated animals) as described by Vanzant et al. (1998), *in vitro* (e.g., incubation of samples with digestive fluids from the rumen or intestines) as reviewed by Stern et al. (1997), or with mobile polyester bags (e.g., samples placed in bags that traverse the digestive tract with digesta) as outlined by Arieli et al. (1999). In addition, site of digestion (ruminal, prececal, postcecal) can be measured using ruminants equipped with intestinal ports (usually at the start and end of the small intestine) as discussed by Merchen (1988). When feed is suspended in bags or digestive fluids, filtration is typically used to recover undigested feed. Compounds that become soluble, plus the particles small enough to pass through pores of polyester bags or through a filter, are considered "digested" although the term "disappeared" is more realistic. In addition, within the rumen and large intestine, feed nutrients can be incorporated into the microbial mass; hence, some index or marker of microbial mass combined with composition of microbes is needed to quantify true digestion of feed components in both laboratory and animal measurements. Broderick and Merchen (1992) discussed these issues.

With total tract measurements, total excreta can be collected or the amount of feces can be estimated using an indigestible marker either inherent to a feed (e.g., lignin, acid insoluble ash, long chain fatty acids) or added to a diet (e.g., chromic oxide, titanium dioxide) as discussed by Owens and Hanson (1992). Disappearance of specific nutrients or energy from a diet can be calculated from the relative concentrations of the indigestible marker compound in the diet and in feces. Because total collection often proves difficult in site of digestion studies, estimates of digestion in site of digestion trials are typically based on markers.

Feed intake is typically limited during digestion trials to avoid feed sorting and to reduce the variability in digestibility that is introduced by fluctuations in feed intake. Yet, restricting feed intake increases the extent of digestion of slowly digested components (neutral detergent fiber [NDF], acid detergent fiber [ADF]) of coarse forages (e.g., long hay). Thus, restricting intake can lead to an increased extent of digestion of ground forages and of less extensively processed grain. This inverse relationship between intake level and digestion rate or extent complicates direct application of digestion trial results, particularly with forages. For example, when feed intake is limited, forage processing (e.g., grinding of alfalfa; ammoniation of low-quality forages) typically increases digestibility of fiber (NDF). But when feed intake is not restricted, ruminants typically consume a larger amount of forage after it has been processed, and NDF digestibility will be no greater for the processed forage than the unprocessed. Failure to increase digestibility when intake of processed forage is not restricted has been attributed to an increased rate of passage reducing the time for digestion of the processed forage.

Performance Studies

Because the target for nutritionally modified crops and crop coproducts is ruminants at high rates of production, performance trials are extensively used to evaluate such products. For data to be directly applicable to commercial production conditions, whether it is pasture or feedlot, animals and feeding conditions should be similar to those used in commercial practice. Steers (bull castrates) shall be fed from ≥300 kg until finished, heifers will be fed from ≥270 kg until finished, and lambs will be fed from ≥20 kg until finished. 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 trial's start and end shall be used. These procedures can include weighing on two consecutive days with feed intake being either restricted or equalized during the interval, or weighing after an overnight period during which 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.

Carcass and Meat Quality Studies

Consumers in developed countries exhibit a preference for meat and meat products that are tender, flavorful, and juicy with minimal external fat. Although animal genetics and feeding duration (harvest weight) largely dictate carcass and meat quality traits, the digestion site and products can influence flavor and color of meat products. In addition, one can alter the quality of milk and meat from ruminants by feeding crops or crop products with altered concentrations of fatty acids and antioxidants. Of specific interest are altered lipid compositions purported to have health benefits (e.g., conjugated linoleic acid, substituting oleic acid for palmitic and stearic acids) or to increase shelf life and visual appeal of red meats (e.g., antioxidants, oleic acid). Although carcass measurements (longissimus area [rib eye], fat thickness, prevalence of internal fat, intramuscular [marbling] fat that provides flavor) are routinely measured at the end of performance trials, meat quality measurements are rare. Typical meat quality evaluations include consumer acceptability based on visual or instrument color as well as rancidity indices (e.g., concentrations of thiobarbituric acid-like reactive compounds) following exposure under simulated retail display conditions for several days. Tenderness can be based on shear force of cores from various muscle groups or taste panel appraisal. Flavor of both fresh and reheated meat products is based on scoring by trained taste panels and, to a limited degree, with electronic devices (e.g., the artificial nose). For specifics regarding carcass and meat quality evaluation, researchers should examine procedures outlined in published articles and texts (e.g., Aberle et al. 2001).

Design and Allotment

An experimental design appropriate for statistical testing of effects will be used. For palatability, intake, and digestion trials, animals can be rotated among treatments over time in an attempt to statistically remove animal effects. For growth or performance measurements (i.e., when the animals will be fed the GM or the control grain for the full trial), as well as for meat quality studies, the design will typically be a randomized complete block (preferably with blocking by initial body weight, breed, or gender and by pen location). Animals of different genders will be placed in different blocks or balanced within pen among test diets. If gender is balanced within pen among test diets, then the gender and gender × treatment effects cannot be statistically evaluated. For intake or digestibility measurements, experiments that provide increased statistical power or reduced animal numbers (crossover, switchback, Latin square treatment arrangements) can be used. Different blocks can be housed in different locations or buildings, but the environment within each block must be as similar as possible to avoid interactions that decrease statistical power of the experiment. Treatments shall be assigned randomly to pens or to 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) shall be adequate to detect the types of differences anticipated. With a 5–7.5% coefficient of variation for the primary trait of interest, six 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 is 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 and forages will be measured,

recorded, and reported. 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 test's power. Therefore, diets shall consist of ≥60% of the diet dry matter as the test grain (≥55% for lambs) with addition of appropriate amounts of protein, roughage, mineral, vitamin, and feed additives so that nutrient or energy requirements specified by the National Research Council (NRC 2000, 2007) or accepted local requirements for the species used for testing are supplied and tolerance limits are not exceeded. Exceptions to this requirement may exist for the test nutrient in the negative control diet, for which various supplemental concentrations of the test nutrient may be provided.

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 potentially adverse effects on animal health. For example, feeding ground or rolled wheat may lead to acute indigestion. Thus, wheat should not exceed 50% 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 after animals adapt to their diet. Except when depletion-repletion studies are being conducted, 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 or the high concentrate diet can be limit-fed. The concentration of roughage will be sequentially decreased or the amount of feed provided for limit-fed animals will be altered for all dietary treatments simultaneously except when an equal-feeding approach is employed; in this case, the amount of feed provided on a daily or weekly basis shall be proportional to the negative control diet. All dietary ingredients will be mixed before delivery to livestock. Sorting and rejecting specific fractions shall be monitored and recorded.

Removal of Test Animals

Any animal that exhibits morbidity, loses weight, or gains much less weight than contemporary animals during two consecutive periods of the trial will be removed from the experiment and the reason(s) 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 plus 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 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 proportional gain or 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 either all animals in the pen or all animals fed the same diet. 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 death and date and cause of death should be recorded.

Termination of the Experiment

The experiment will be terminated for each block 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; preferred trial lengths are 112 and 56 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. Harvest data for cattle shall 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 11th 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 shall 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

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 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 for continuous variables with other analyses as appropriate for discontinuous variables. The pen shall be the experimental unit for all analyses, although treatment effects on variation among animals within a block also can be evaluated. For evaluating carcass traits, covariance adjustment based on carcass weight of individual animals may be used. See detailed protocols in Chapter 17, Statistical Analysis and Interpretation of Results. Levels of sensitivity for both alpha and beta errors shall be stated so that precision of the test is apparent to readers.

Evaluation of Crop Protein Supplements in Experiments with Growing Ruminants

The following protocol shall be used to evaluate the nutritional value of crop protein supplements prepared from GM crops with altered nutritional concentrations (e.g., soybeans, canola [rapeseed], cottonseed, sunflower, safflower, lentils, or lupins or meals produced from these crops) for growing ruminants (beef and dairy cattle, sheep and goats) grown from weaning to the end of the growing period. In all experiments, an appropriate control crop or product (preferably the near-isogenic variety of the same variety that lacks the output trait being studied) must be included with or without supplementation of the test nutrient being studied (positive and negative control). The nature of such diets is outlined in Table 15-1 and discussed below.

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

Production, handling, storage, and processing of the oilseeds or oilseeds products will be as described in Chapter 2 of this document, while sampling and analysis of the oilseeds or oilseeds products for mycotoxins and chemical components will be as described in Chapter 3.

Test Animals

Males, castrates, or female ruminants can be used. To test responses during 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 until finished, heifers will be fed from ≥270 kg until finished, and lambs will be fed from ≥20 kg until finished. All animals used should be healthy with similar genetic and nutritional 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 *The 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 or equalized during the interval or weighing after an overnight period during which 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 Evaluation of Grains and Grain Products in Experiments with Finishing Ruminants on page 167.

Number of Replications

See Evaluation of Grains and Grain Products in Experiments with Finishing Ruminants on page 167.

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 being 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. When included, the positive control diet and the diet containing the GM altered nutrient shall be isonitrogenous and isoenergetic. Except for the negative control, all diets should contain appropriate amounts of protein, energy, minerals, vitamins, and feed additives along with roughage so that nutrient or energy requirements specified by NRC (2000, 2007) or locally accepted standards for the species being used

are supplied and tolerance limits are not exceeded. The diets should be formulated such that the animals are responsive to only the nutrient of interest. This may preclude meeting the requirements as specified by NRC or locally accepted standards. The amount of oilseed or oilseed product and other ingredients in each treatment diets should be equal. 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, or intake can be restricted with the restriction being sequentially removed. If feed intake is restricted, feed supply shall be proportional to that of the negative control diet on a daily or weekly basis for the full duration of the experiment. All dietary ingredients will be mixed before delivery to livestock with any sorting and rejecting specific fractions being monitored and recorded. If certain 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; feeding of diet ingredients separately invalidates interpretation of performance results but effects of supplementation on quality of animal products still can be assessed.

Removal of Test Animals

See Evaluation of Grains and Grain Products in Experiments with Finishing Ruminants on page 167.

Termination of the Experiment

Experiments with growing animals will be terminated on a block basis when the mean of animals within a 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; preferred trial length is 100 and 50 days, respectively. If carcass data are obtained at the end of the growing period, the same number of animals per pen within a block will be harvested at the same location on the same date. Data for cattle shall 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 11th 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 shall 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 Evaluation of Grains and Grain Products in Experiments with Finishing Ruminants on page 167.

Evaluation of Forages or Forage Products (Crop Residues) with Growing Ruminants

The following protocol shall 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 output trait being studied—must be included in similar physical form. Other controls may be included as outlined in Table 15-1.

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. These include the altered GM product and a negative control product with all other dietary ingredients being held constant. A positive control diet consisting of the negative control diet supplemented with the nutrient of interest may be included if feasible. Additional treatments that may include one or more types of conventional forage or forage product of the same genus typically produced or used in the region may also be fed so that comparisons with more diverse hybrids, varieties or strains can be drawn.

The sponsoring organization will normally provide the investigator with the two types of forage seed (GM and control) for some studies or the two types of forage or forage product (GM and control) for other studies. The investigator may produce and (or) process both types of forages separately under identical processing conditions (e.g., chopping, ensiling) depending on the experiment's objectives. Responses of the control and the GM product to processing (particle size measurements; fermentation product concentrations) shall be monitored and reported.

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 the same soil type using agronomic practices as similar to that used 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. If the forage is to be harvested with or without processing before feeding, the GM and the 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. The yield difference between the GM and control crops (fresh and dry matter basis) will be recorded. Crop residues may be harvested to avoid differences in field losses, but harvest of the crop residues from test and control crops should be at the same time interval following grain harvest. Bales should be wrapped in plastic or ensiled to avoid mold. Care must be taken to clearly identify each forage or forage product and prevent cross-mixing of forages or forage products of different types. If the forage or forage residue is to be grazed, subdivisions that form paddocks around replicate groups of animals will be installed with paddocks being assigned randomly to block. For experiments designed to evaluate growing forage, a "put and take" system that adds or removes animals depending on the measured amount of available forage mass is preferred.

Analysis

If the forage is to be grazed, the amount of available forage will be quantified before grazing 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. Regardless of whether forage or forage products are grazed or harvested for feeding, representative samples will be analyzed for dry matter, crude protein, crude fat, ADF and NDF, and ash in a laboratory certified to produce high-quality, consistent results. For pre-ensiled forage, fermentation quality indices such as water-soluble carbohydrates and pH should be measured in addition to the proximate components. 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 (concentrations of lactic and volatile fatty acids, ethanol, pH, ammonia, and water-soluble protein; aerobic stability) should be taken and reported.

Test Animals

Intact males, castrates, 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 gender or gender 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 harvested forage or they may graze either individually (in separate paddock or tethered) or as a member of a group in accordance with guidelines described in *The 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 or equalized during the interval or weighing after an overnight period during which animals have no access to feed or water. Animal weights and other health and feeding data will be recorded and maintained as appropriate following good management practices.

Design and Allotment

An experimental design appropriate for statistically testing effects will be used. For growth or performance measurements, when the animals graze or are 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 gender so that all animals within the block are similar; paddocks should have similar agronomic and environmental properties. For intake or digestibility measurements, indigestible markers (e.g., acid-insoluble ash, n-alkanes, chromic oxide, titanium dioxide) can be fed with a supplement. To increase statistical power when obtaining ruminal samples from animals fed GM or control forage to evaluate ruminal responses, animals can be rotated among paddocks in crossover, switchback, or Latin square experiments. An adjustment period should be used that is sufficiently long for transition of the ruminant microbial population. The duration of adaptation will depend on the extent of change in

dietary ingredients. Different blocks can be in different locations, but the environment within each block must be as similar as possible to avoid bias. Treatments shall 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 the types of differences anticipated. With a coefficient of variation of 5–7.5%, six to 10 replications per treatment will be required. The number of replicates required will increase 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 or equalized as appropriate for the positive control and GM diets. Nutrient or energy intakes should meet or exceed requirements specified by the National Research Council (NRC 2000, 2007) or accepted local nutrient or energy requirements for growing ruminants of the species of interest except as necessary with the negative control diet to evaluate responses to the nutrient altered by GM. In addition, tolerance limits for nutrients 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 except as necessary for specific control diets. Supplements shall be analyzed for the same nutrients as the forage.

Removal of Test Animals

Any animal that exhibits morbidity, loses weight, or gains much less weight than contemporary animals during two consecutive periods will be removed from the experiment. Final performance data shall not include information from animals removed from the experiment. A qualified veterinarian should perform or supervise a diagnostic necropsy of animals that die during the experiment and the cause of death shall 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; preferred trial lengths are 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 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, UK; http://www.vsn-intl.com/genstat/) is recommended for continuous variables. For discontinuous variables, statistical analysis will follow accepted procedures. The mean for all animals in a paddock shall be used as the experimental unit for all analyses, although treatment effects on variation among animals within a block also can be evaluated. Levels of sensitivity for both alpha and beta errors shall be stated so that precision of the test is apparent for readers.

General Guidelines

Growing and finishing ruminants used for evaluation of GM products must be healthy. Ruminants used in performance trials must have performance levels similar to those of commercial feedlot animals. Animals selected for experiments shall be selected for uniformity from a larger group with similar genetic and nutritional history. Measurements must be selected a priori so that the number of animals per treatment is sufficient to statistically detect the desired difference among treatments. Precision in maintaining identity of all feeds and for sampling and analyzing these feeds is essential. Animals shall be housed, fed, managed, and harvested in ways to avoid any bias.

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Chapter 16

Protocols for Evaluating Feedstuffs with Genetically Modified Output Traits: Aquaculture

Aquaculture, the water farming of fish and shellfish (Meyer 1991), is a rapidly growing sector with an average growth rate of 8.9% per year since 1970, compared to 2.8% for terrestrial farmed meat-production systems (FAO 2004). In 2002, aquaculture contributed 29.9% of the global supply of fish, crustaceans, and mollusks, of which China reportedly produced 71.2% of the total quantity (FAO 2004). The growth rate is such that it is predicted that aquaculture will contribute 41% of global fish production by 2020 (Delgado et al. 2003). Much of the global aquaculture production is low cost and involves the herbivorous species (i.e., grass carp, silver carp, milkfish, and some tilapia) and the omnivorous species (i.e., common carp, channel catfish, some tilapia) that are grown for local consumption (Seaweb 2004). However, there is an increasing industry trend toward producing more carnivores (e.g., Atlantic salmon and expanding into Atlantic cod, barramundi, cobia, halibut, sablefish/black cod, southern hake, tuna, and yellowtail/kingfish; Seaweb 2004). In 2002, the top 10 species groups in terms of quantity produced according to FAO (2004) were carp and other cyprinids (41.9%); oysters (10.8%); miscellaneous marine mollusks (9.4%); clams, cockles, and arkshells (8.6%); salmon, trout, and smelt (4.5%); tilapia and other cichlids (3.8%); mussels (3.6%); miscellaneous marine mollusks (3.4%); shrimp and prawns (3.2%); and scallops and pectens (3.1%). Nearly 200 species of fish and crustaceans are grown in some form of aquaculture production throughout the world with the range of nutritional information on the majority of these species being scant at best. However, there are 20 or 30 species for which nutritional information is complete enough to conduct reasonably controlled studies. For some species, notably the salmonids, catfish, carp, tilapia, and several marine species, the research base is extensive, and these species are used as surrogates for related species for which less is known (R. Hardy, University of Idaho Aquaculture Research Institute, personal communication, 2007).

In the future, development of feeds that are less dependent on wild fisheries for providing a source of fishmeal will be important due to the limited harvest of wild fish. As a result, the industry is investing more in developing vegetable-based feeds that are lower in cost and that replace a portion of the fishmeal while maintaining growth and health of the fish. Soy protein is the major vegetable protein that has been tested and is used commercially to replace part of the fishmeal in diets of omnivorous fish such as catfish, carp and tilapia and to partially replace fishmeal in diets of carnivorous fish and shellfish such as salmon, trout, grouper, and seabass and shrimp (Gomes et al. 1995, Seaweb 2004).

This chapter focuses on guidelines for conducting nutritional evaluation studies of GM crops and their coproducts containing output (nutritionally enhanced) traits when fed to fish or shrimp. Factors such as nutrient requirements; management of the feed, animal, and facility; and diet formulation and processing are covered. Studies to evaluate the nutritionally enhanced traits in the crops or their coproducts that are discussed include: digestibility of specific nutrients; bioavailability of a nutrient(s); animal performance; animal processing; fish meat quality, such as composition and retail product assessment; and sensory evaluation of fish meat destined for human consumption.

Nutrient Requirements

Most fish grown in intensive aquaculture are carnivorous, whereas most fish grown in extensive and semiextensive systems are omnivorous and herbivorous (Goddard 1996). Regardless of the natural diet, one must provide a diet that meets the nutrient requirements of the particular species of interest. There are a number of publications on the nutrient requirements of fish and shellfish (Goddard 1996, Lovell 1998g, NRC 1993) as well as for individual species (Webster and Lim 2002b) such as salmon (Hardy 1998, Storebakken 2002), channel catfish (Robinson 1998, Robinson and Li 2002), rainbow trout (Hardy 1998, 2002), tilapias (Lovell 1998d, Shiau 2002), and shrimp (Lim 1998).

Understanding nutrient digestion and metabolism (Lovell 1998e, NRC 1993), intermediary metabolism (Dabrowski and Guderley 2002), nutritional physiology (Rust 2002) and pathology (Roberts 2002), and nutrient flow and retention (Halver and Hardy 2002) in fish is important in developing strategies to evaluate specific nutritional enhancements.

Protein/Amino Acids

Proteins make up between 65–75% of the fish on a dry matter basis (Wilson 2002). Therefore, fish need a continual supply of amino acids for growth, reproduction, and maintenance. Fish require the same 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) as most nonruminants (Lovell 1998b). There are several publications that discuss protein/amino acid requirements and utilization in fish in general (NRC 1993; Webster and Lim 2002a, 2002b; Wilson 2002) and in single species of fish (Lovell 1998e, Webster and Lim 2002b).

Energy

Publications on bioenergetics and energy metabolism in fish include Bureau et al. (2002), Lovell (1998h), NRC (1993), and Webster and Lim (2002a), and there are discussions of energy requirements of individual species of fish (Lovell 1998e, Webster and Lim 2002b). Nutritional energetics of fish, with an emphasis on determining dietary energy allowances for captive fish, have been previously reviewed (Cho and Kaushik 1990, Smith 1989). Digestible energy (DE) and metabolizable energy (ME) values of feedstuffs for fish are available (NRC 1993). Practically, ME offers little advantage of DE in evaluating energy in feedstuffs for fish because fecal energy accounts for most of the excretory losses (NRC 1993).

Lipids

Fish require dietary lipids to meet their essential fatty acid requirements. Cold-water fish require highly unsaturated fatty acids of the n-3 (omega-3) class of lipids, whereas warm-water fish require highly unsaturated fatty acids from either n-3 or n-6 (omega-6) classes of lipids or a mixture of both (Webster and Lim 2002a). Also, dietary additions of EPA and DHA to penaeid prawn diets have shown growth-promoting activity (Glencross and Smith 2001). Lipid metabolism, functions, requirements, optimal levels and ratios of omega-3 and omega-6 fatty acids, and sources have been reviewed previously (NRC 1993, Sargent et al. 2002, Webster and Lim 2002a, Torstensen et al. 2004) as well as lipid peroxidation (Benzie 1996). Webster and Lim (2002b) have provided additional discussion on dietary lipid by fish species.

Carbohydrates

In general, carbohydrates provide energy and spare protein in some fish species. Fiber sources (cellulose, gums, pectins and hemicelluloses) have been used as diluents and binders in experimental fish diets. Most fish can tolerate up to 8% crude fiber in the diets but higher amounts may decrease growth (NRC 1993). Cold-water and marine fish cannot use as much dietary carbohydrate as warm-blooded fish (NRC 1993). Even though a specific carbohydrate requirement has not been determined, elimination of carbohydrate from the diet results in catabolism of protein and lipid (NRC 1993). Several authors have reported the nutritional value of carbohydrate for fish (NRC 1993, Lovell 1998b, Webster and Lim 2002a). Webster and Lim (2002b) have provided a discussion on carbohydrate by individual fish species. Work has been published on carbohydrate utilization in salmon; in this species, 9% of the diet seemed to be optimal (Hemre et al. 1995).

Minerals

All forms of aquatic life require inorganic minerals. Fish have the ability to absorb minerals from their diets as well as their external environment, and thus it is difficult to demonstrate mineral deficiency in fish. Most research has been confined to osmoregulation, toxicity, and related physiological functions (Lall 2002). Reviews on mineral needs and metabolism in fish have been published (NRC 1993; Lall 2002; Webster and Lim 2002a, 2002b).

Vitamins/Antioxidants

Essentiality of all of the 15 vitamins has been established for fish, although all fish species do not seem to have a dietary requirement for all 15 vitamins (Lovell 1998b). The quantitative requirements for most of the vitamins have been established for Chinook salmon, rainbow trout, common carp, channel catfish, and yellowtail, whereas only some of the requirements have been determined for red sea bream and tilapia (NRC 1993). Vitamin needs of fish have been discussed in several publications (NRC 1993; Lovell 1998b; Halver 2002; Webster and Lim 2002a, 2002b).

Antioxidants are commonly used in fish feeds that contain high concentrations of polyunsaturated fatty acids to prevent lipid peroxidation (NRC 1993). Adding vitamin E to the diet has been shown to protect the fillet against iron ascorbate-stimulated oxidation with no effect on the other variables (Hamre et al. 2004). Others have shown that Atlantic salmon can tolerate a large range of dietary antioxidants (above the requirement and below the known level of toxicity) under normal growing conditions (Lygren et al. 1999).

Antinutrients

Antinutrient factors such as protease inhibitors, phytates, glucosinolates, erucic acid, saponins, tannins, lectins, oligosaccharides and nonstarch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids such as sterculic and malvalic acid in cottonseed oil and meal, canavanine, antivitamin factors, phorbol esters, and oxycarotenoids have been reviewed with respect to their effects on fish (NRC 1993, Lovell 1998f, Francis et al. 2001, Roberts 2002).

Enzymes

Enzyme supplements are added to feed to enhance the digestion of feed components that are inefficiently utilized by fish. Phytate is an example of a phosphorus source that is not digested by fish (Rodehutscord and Pfeffer 1995, Olivia-Teles et al. 1998) and has negative effects on protein digestibility (Spinelli et al. 1983) and availability of other minerals (Sugiura et al. 2001). Phytase is an example of an enzyme that, when added to feed, hydrolyzes phytate to an available phosphorus source. Phytase has been shown to neutralize the effect of phytic acid on protein digestibility, resulting in enhanced growth in salmon (Sajjadi and Carter 2004). Others have reported phytase–treated soy concentrate to be equivalent to fish meal as a source of dietary protein for Atlantic salmon with phytase supplementation resulting in improved protein digestibility, feed conversion, protein retention, and improved apparent digestibility coefficients of calcium, magnesium, and zinc (Storebakken et al. 1998b). However, others have shown no effect of phytase supplementation on protein digestibility in striped bass, although a 23% improvement in phosphorus digestibility was observed (Papatryphon and Soares 2001).

Pigments

Astaxanthin, a carotenoid pigment, has been shown to be an essential nutrient for Atlantic salmon to produce viable offspring. Essentiality for other salmonid species has not been demonstrated because studies have not been conducted, but essentiality is almost assured (Torrissen and Christiansen 1995). Pigmentation is important in the flesh of some fish (i.e., salmon, trout), in the skin of others (i.e., red sea bream, shrimp), and is not desired in the flesh of fish in which the consumer expects to see a white flesh (Lovell 1998f). Astaxanthin and canthaxanthin are the two main carotenoid pigments responsible for the red to orange coloring in salmonids (NRC 1993). However, these marine carotenoids will not be deposited if they were to be fed to fish, such as catfish or tilapia, although they will deposit xanthophyll from corn products or other sources under some circumstances (R. Hardy, University of Idaho Aquaculture Research Institute, personal communication, 2007). The red tissue pigment for wild salmon is derived from the consumption of marine algae via zooplankton or insects. Consumption of pigments of plant origin cannot produce the desired color in salmonids. In farmed salmon, supplemental astaxanthin and/or canthaxanthin may be incorporated into the diet to achieve the flesh color of the consumer expects. Studies have been conducted evaluating these pigment sources in rainbow trout (Torrissen 1986) and in Pacific and Atlantic salmon (Bjerkeng et al. 1999).

Management

Management of fish and shellfish is important in realizing optimum growth, feed efficiency, and health. It is not within the scope of this document to provide the detailed management guidelines used in aquaculture. However, specifics such as feeding practices and practical diets, feed formulation, feeds and feeding for various fish species (NRC 1993, Lovell 1998g, Webster and Lim 2002b), temperature and water quality, feed types and uses, feed handling and storage, feeding methods, feed rations and schedules (Goddard 1996) are available. For example, chapters have been written on feeding catfish (Robinson 1998, Robinson and Li 2002), salmon (Hardy 1998, Storebakken 2002), trout (Hardy 1998, 2002), hybrid striped bass (Webster 1998, 2002), tilapia (Lovell 1998g, Shiau 2002), penaeid shrimp (Lim 1998), and others (Webster and Lim 2002b). Discourses on nutrition's effects on fish health have also been published (Meyer 1991, Lovell 1998h, Roberts 2002).

Diet Formulation and Processing

Numerous sources that discuss formulating diets and processing for aquaculture in detail are available (NRC 1993, Goddard 1996, Lovell 1998g, Hardy and Barrows 2002). Hardy and Barrows (2002) described it well by stating, "Together, formulation and manufacture involve the selection and combination of feed ingredients to form a mixture that can be manufactured into a product that delivers the nutrients needed to meet the production goals in animal and fish husbandry." Many factors must be considered in formulating feed for aquaculture. Nutritional considerations include fish species, stage of growth and/or reproduction, nutrient requirements, available feed ingredients, form of the feed required for optimal consumption (i.e., meal, crumbs, pellet, extruded product), feed processing, and

whether all nutrition is supplied by the manufactured diet (e.g., when catfish are raised in tanks or trout in raceways) or supplemental nutrition is needed because natural foods are present in the environment.

Several non-nutritional factors should also be considered when formulating diets for aquaculture. The raw materials for the diet need to be free of contaminants. This is very difficult to achieve in the case of fish oil owing to contamination with dioxins. The existing decontamination methods are expensive, but should be done to reduce dioxin levels below 4 ng/kg. Additional nonnutritional factors that should be considered include logistics of acquiring and storing ingredients (Li 1998). Furthermore, the desired feed characteristics, such as sinking or floating in water and pellet size, should be taken into account. The feed manufacturing process also warrants consideration because extrusion generally requires that at least 25% of the diet be composed of grain or coproducts from the milling industry for proper starch gelatinization to enable the expansion necessary for the pellet to float (Li 1998). If a floating characteristic is desired, consideration should be given to ingredients that result in better water stability or pellets that take longer to disintegrate in the water. Environmental conditions such as temperature and humidity can affect whether certain ingredients pose handling problems. If pellets are desired, then fiber content must be restricted and the amount of fat included in the pellet must be limited to maintain a high-quality pellet. Binding agents are available that enhance the pellet quality (Li 1998). If additional fat is required, it can be sprayed onto the outside of the pellet. With enzyme(s) addition, one must consider the temperature effect of the pelleting and extruding processes. In many cases, temperature sensitive ingredients must be sprayed onto the outside of the pellet or, as is the case with vitamins, be added in excess to account for the destruction that occurs during the manufacturing process. The mixture of ingredients should be assessed along with the storage conditions and duration to determine whether antioxidants need to be added to preserve the feed.

Hardy and Barrows (2002) have provided a review on diet formulation and manufacture, including topics on aims and strategy of fish feed production, feed ingredients, diet formulation, grinding, mixing, conditioning and expansion, pelleting, cooling and drying, crumbling and screening, coating, shipping and storage, semipurified diets, microdiets, and ingredient and diet evaluation. Examples of production-type reference diets and purified reference diets for salmon and trout (NRC 1993, Lovell 1998e, Hardy and Barrows 2002), catfish (NRC 1993, Robinson and Li 2002), and other species (Webster and Lim 2002b) have been published.

Worldwide, about one million tons of fish oil are used mainly for aquaculture; one-third of the oil contains more than 4 ng WHO TEQ dioxins per kg (4 ng/kg is the limit for dioxins in the EU, Korsager 2004; Robb 2004).

Animal Studies

Biological evaluation of feed ingredients and finished diets containing the trait of interest involves feeding fish and measuring some endpoint such as digestibility, bioavailability, growth rate, feed efficiency, survival rate, meat composition, and sensory evaluation of the meat (Hardy and Barrows 2002). The benefit of feeding studies is that a direct evaluation of the trait of interest can be made in the target species. *In vivo* evaluation of feedstuffs with nutritionally enhanced traits must be conducted in a controlled environment such as aquaria or tanks. Water temperature, oxygen levels, water quality, and feed source can be controlled in these facilities. If more practical conditions such as experimental ponds, raceways, pens, or cages are desired, one must realize that fewer variables can be controlled and the amount of test material required will be significantly increased. Size of the aquaria or tanks should be large enough to accommodate the originally stocked population after a 500% to 1,000% weight increase (Lovell 1998e). Containers should have continuous flowing good quality, nutrient-free water with sufficient dissolved oxygen and at a temperature accepted for that species of fish.

For any experiment, the investigator must try to keep as many variables as possible equal among the treatment groups except for the test variable. Factors that might affect the study results include animal (i.e., species, age, size, weight, physiological state, molting, health), water quality (i.e., temperature, salinity, dissolved oxygen, pH, bacterial load, suspended solids, soluble proteins), photoperiod, and feed quality (i.e., ingredient quality, homogeneity of the mix, water stability, diet consumption). The target fish species should be full siblings of a fast-growing genetic strain. Young small fish respond faster than older large fish to nutritional variables and are more sensitive to nutritional changes. More small fish can be reared in the container, which will help in negating any effects due to unequal sex ratio because males grow faster and will also help to prevent hierarchical feeding patterns (Lovell 1998e).

Depending on the experimental purpose and the fish size distribution, fish may be blocked by size. In cases where a nutrient requirement is very small and has a long tissue residual time, such as with essential fatty acids, body stores may need to be depleted before the start of the experiment. Thus, the previous dietary composition and intake must be taken into account.

Experimental Design Considerations

Determining the number of replicates to use in a study is crucial to making sure the experimental design has sufficient power to detect the minimum differences between treatments as desired. Experimental design (Shearer 2000, Torstensen et al. 2001, Ruohonen and Kettunen 2004) and statistical power (Ling and Cotter 2003) are topics of interest by researchers conducting aquaculture studies. Many aquaculture studies utilize a completely randomized design when working primarily with individual traits. Factorial treatment arrangements have been used by investigators who are evaluating combinations of traits such as minerals (Gatlin and Phillips 1989); fatty acids (Glencross and Smith 2001); vitamins and energy (Brønstad et al. 2002); or a combination of vitamins, antioxidants, minerals, astaxanthins, and lipids (Lygren et al. 1999, Hamre et al. 2004). Many published studies have used three replicates per treatment (range of one to six replicates/treatment group) and yet based on the variation among aquaria and among treatment groups, the number of replicates may have been insufficient to detect a difference of 10% from the mean of the variables of interest. Therefore, when using variability data from previous studies that were conducted in a similar fashion to the study that is being planned, one should calculate the power to make sure an adequate number of replicates are provided in order to detect the level of difference desired for the targeted traits. One pair of investigators did report using a power analysis for a prawn study where it was noted that their experimental design using four replicates per treatment group was sufficient to detect a 10% difference in the growth of the prawns after six weeks (Glencross and Smith 2001). For specific experimental designs and statistical analyses, see Chapter 17.

Bioavailability: Digestibility Trials

Nutrient bioavailability can be determined through digestibility studies, which measure the percentage of nutrients absorbed and used by fish, or, in the case of energy, the metabolizability (NRC 1993). Alternatively, bioavailability can be measured in growth assays. Digestibility describes the quantity of nutrients or energy from an ingested dietary component that is excreted in the feces, whereas metabolizability also includes the energy that is not excreted in the urine and through the gills. Determining ME values with fish is difficult because of the need to force feed and restrain the fish in metabolism chambers with the aid of a collar for simultaneous collection of fecal, gill, and urinary excretion. Determining DE values is easier because the fish feed voluntarily (NRC 1993). Proper collection of feces without leaching is important in determining DE for fish (Hardy and Barrows 2002). Digestibility can be measured either as "apparent" (does not take into account the nutrient losses into the feces from endogenous origin) or "true" (takes into account the contribution of endogenous derived nutrients in the feces) digestibility. Because the correction for endogenous loss is small, measurement of apparent digestibility is adequate (as described, for example, in NRC 1993).

There are indirect and direct methods of measuring digestibility (Hardy and Barrows 2002). The indirect method involves using an indigestible marker that is included in the diet at a known concentration. The digestibility of the target nutrient can be determined by assessing the difference between feed and fecal concentration and the target nutrient. The digestion coefficient of a nutrient utilizing the indirect approach is described, for example, in NRC (1993). This method has the advantage of not having to collect all the feces, and test fish can eat voluntarily. Various methods of feces collection have been compared and their advantages and disadvantages discussed (Austreng 1978, Brown 1993, Storebakken et al. 1998a, Vandenberg and de la Noûe 2001, Hardy and Barrows 2002).

The following indigestible markers have been used in digestibility studies with fish: acid insoluble ash (Einen and Roem 1997, Van Weerd et al. 1999, Vandenberg and de la Noûe 2001, Sales et al. 2003); chromic oxide (Austreng 1978, Aksnes 1995, Gomes et al. 1995, Aksnes et al. 1996, Brunson et al. 1997, Olsen et al. 1998, Bransden and Carter 1999, Apines et al. 2001, Papatryphon and Soares 2001, Vandenberg and de la Noûe 2001, Davis et al. 2002, Glencross et al. 2003a, Satpathy et al. 2003); yttrium oxide (Nordrum et al. 2000, Overturf et al. 2003, Sajjadi and Carter 2004, Ward et al. 2005); cholestane (Ishikawa et al. 1996, Glencross and Smith 2001), and titanium oxide (Ishikawa et al. 1996, Vandenberg and de la Noûe 2001). For more details, chapters have been written on this subject (NRC 1993, Lovell 1998c, Bureau et al. 2002).

Examples of published digestibility studies include: using phytase enzyme in feeds fed to flounder (Bransden and Carter 1999), striped bass (Papatryphon and Soares 2001), and salmon (Sajjadi and Carter 2004); evaluating

mineral digestibility in trout (Riche and Brown 1996, Overturf et al. 2003), abalone (Sales et al. 2003) and salmon (Ward et al. 2005); evaluating digestibility of protein/amino acid sources in trout (Aksnes et al. 1996, Apines et al. 2001), seabream (Glencross et al. 2003a), salmon (Nordrum et al. 2000), and African catfish (Van Weerd et al. 1999); evaluating digestibility of fat sources in seabream (Glencross et al. 2003b), prawns (Glencross and Smith 2001), salmon (Nordrum et al. 2003); and evaluating digestibility of general feed ingredients in shrimp (Brunson et al. 1997, Davis et al. 2002), trout (Gomes et al. 1995), and salmon (Hillestad and Johnsen 1994). Typically, three replicates were used per treatment in these studies.

The direct method of measuring digestibility includes measuring all feed consumed by the fish and all of the resulting excreta. Fish are force-fed a specified amount and all feces collected and nutrients analyzed. Special aquatic metabolism chambers have been developed allowing for the collection of gill, urine, and fecal excretions of rainbow trout (Smith 1971, 1976). Open circuit balance respirometers have been used with fish and eels to measure energy and nitrogen balance as well (Heinsbroek et al. 1993). These methods allow for nitrogen and carbon balances, and for DE and ME determinations (NRC 1993, Rumsey et al. 1993).

Because very few diets are comprised of a single ingredient, they may not be acceptable to fish as the sole component or have the proper physical characteristics (Bureau et al. 2002). Researchers (Cho et al. 1982, Wilson and Poe 1985) have determined digestible energy and digestible protein coefficients by comparing the digestibility of a reference diet with that of the test diet containing a mixture of the reference diet and test ingredient. This indirect method was used to determine the digestibility coefficients for the reference and test diet. The digestibility of the test ingredient was calculated as described, for example, in NRC (1993). The advantage of this method is that it takes into account synergistic effects of other ingredients mixed in the diet and the test diet may be more acceptable to fish than the test ingredient itself.

Bioavailability: Growth Studies

Growth studies provide a more absolute evaluation of the bioavailability of a nutrient in a feed ingredient than digestibility trials do. A digestibility trial may show two ingredients having equal digestibilities, but a growth trial may show a variation in growth because of differences in how the absorbed nutrient was metabolized. In a growth trial, test animals are fed various levels of the test nutrient or a standard reference nutrient in a basal diet that is deficient in the test nutrient but otherwise nutritionally adequate (Lovell 1998a, 1998e). Doses should be selected ranging from below to above the optimum dietary level.

Similar concentrations of the test nutrient from a standard source that is traditionally used and is of high bioavailability should be used in the reference diet. The two series of diets are fed to apparent satiation to young fish in aquaria for a period time and/or weight gain. A minimum of three replications per treatment group should be used. The nutritive-sensitive endpoints such as feed intake:body weight gain, body weight gain, or length from the dose-response data are obtained and analyzed using regression analysis, where the dietary nutrient concentration is the independent variable. The slope of the regression curve's linear portion for the test diet is compared with that for the reference diet (Littell et al. 1995). Examples of studies using the slope-ratio method include determining the bioavailability of zinc in channel catfish (Paripatananont and Lovell 1995) and of iron in salmon (Andersen et al. 1997).

Many factors can affect the reliability of the determination of the digestibility value whether determined in a growth or digestibility study (NRC 1993, Bureau et al. 2002). Meal size and processing of the diet such as extrusion has been shown to affect digestibility. Feeding frequency does not seem to have an effect. Water temperature effects have been variable with some sources saying it has no influence on digestibility (NRC 1993) and others reporting that it does (Bureau et al. 2002).

Performance Studies

Growth is usually the most important criterion for measuring fish response to experimental diets (Lovell 1998e). However, one should not assume that weight gain and growth are synonymous, especially if consumption of the diet may result in a difference in the composition of the gain (fat and protein content). Therefore, whole body and muscle composition should be measured for protein, fat, and moisture unless there is sufficient justification for not measuring it. If there is a difference in the composition of gain, then protein gain should be reported as well. Endpoints for growth studies include: body weight, length (Goddard 1996), average daily gain, thermal growth unit, daily instantaneous growth rate or specific growth rate, feed efficiency ratio, corrected feed efficiency ratio, condition factor, and mortality.

Other Measurements

Depending on the nutrient of interest, other endpoints may need to be incorporated into the experimental design. For example, a deficiency of the

fatty acid 22:6n-3 (docosahexaenoic acid) has been associated with reduction in the number of rods in the photocell receptor population and thus the visual performance of herring, such that they can no longer feed at low light intensities (Bell et al. 1995).

Product Assessment

Levels of polyunsaturated fatty acids and antioxidants in the diet may affect the level of oxidation in the tissue. Benzie (1996) has reviewed the causes, consequences, and laboratory measurements of lipid peroxidation. Color score may be included for evaluating pigments and bone mineral composition for minerals. In evaluating protein and amino acid quality, the following measurements should be considered:

Protein Efficiency Ratio (PER) is a measure of weight gain per unit of protein consumed and is useful to compare protein sources in a single experiment (Hardy and Barrows 2002).

- PER = (weight gain, g)/(protein consumed, g on dry matter basis)

Net Protein Utilization (NPU) is the measure of protein gained by fish during an experimental period per unit of protein absorbed by the fish (Hardy and Barrows 2002).

- NPU = [Fish protein content (end of study) – Fish protein content (start of the study)]/[protein consumed (g dry matter) × protein digestibility coefficient]

Clinical signs that may be indicators of nutritional effects may include lesions, hemorrhage, pigmentation abnormalities, gill hyperplasia, cataracts, and skeletal and cartilage deformities (Lovell 1998e).

Duration of the experimental period varies depending on the study's objective. Based on a survey of the literature, many of the studies have an experimental duration of eight to 16 weeks (R. Hardy, University of Idaho Aquaculture Research Institute, personal communication, 2007).

Fish should be acclimated to aquarium and environment for one to two weeks before beginning the experiment, during which time they can be given chemical baths for external pathogens (Lovell 1998e). If diet acceptability is in question, experimental diets should be fed to a group of similar non-study fish before the start of the experiment to confirm the acceptability of the diet. Fish should be fasted for 24 h (some researchers have even fasted fish for 48 h [Lygren et al. 1999, Ruyter et al. 2000, Hamre et al. 2004]) and anaesthetized before handling for length measurement and individual handling to minimize stress. Handling can be very stressful to fish and may result in temporary loss of appetite and abnormal feeding behavior (Goddard 1996).

General Guidelines

Regardless of the species, fish, shrimp, or prawns must be growing normally for valid conclusions to be drawn. A large enough pool of the target species should be available to select a uniform population for the study. Coefficients of variation should be < 10% between replicates. The desired difference among treatment means for target endpoints must be determined a priori so the correct number of replicates can be used. It was suggested the following standards for shrimp research: growth rate should be 85% of a commercially acceptable standard; vannamei weighing greater than 3 g should be growing 0.13 g/day), monodon weighing 3–10, 10–20, or 20–35 g should be growing at 0.13 g/d, 0.17 g/d, or 0.20 g/d, respectively; survival rate should be ≥ 85%; and feed conversion (feed consumed/body weight gain) should be a commercially acceptable value and definitely < 2:1 (D.M. Akiyama, Charoen Pokphand, Indonesia, personal communication, 2005). If feed conversions are higher than expected, possible causes include animals not consuming the diets, nutrients leaching before consumption, nutritionally imbalanced diets, and poor animal health.

Examples of published growth studies in which different nutritional traits in aquaculture were evaluated include: protein/amino acids–shrimp (Davis et al. 2002), fish (Gomes et al. 1995, Einen and Roem 1997, Nordrum et al. 2000); carbohydrates–fish (Aksnes 1995, Hemre et al. 1995, Martino et al. 2005); lipids–fish (Lie et al. 1993, Hemre and Sandnes 1999, Ruyter et al. 2000, Glencross and Smith 2001, Glencross et al. 2003b, Nordrum et al. 2003, Hamre et al. 2004, Solberg 2004, Martino et al. 2005); minerals-fish (Gatlin and Phillips 1989, Apines et al. 2001, Hamre et al. 2004); phytase–fish (Sajjadi and Carter 2004); and vitamins and antioxidants–fish (Lygren et al. 1999, Brønstad et al. 2002, Hamre et al. 2004). Similar designs should be considered for evaluating enhanced nutritional traits derived through biotechnology.

Processing Considerations, Carcass Characteristics, and Meat Quality

Overall response criteria for slaughter quality include: (1) consistency of fillet (scale 1 as soft and 4 as firm); (2) entrails (weight %); (3) pH in fillet; (4) pH in fillet after 24 h storage; (5) astaxanthin (mg/kg); (6) dry matter in the fillet; (7) protein in the fillet (%); (8) fat in the fillet (%); and (9) degree of maturation (%) (Aksnes 1995). Specific measurements will need to be included in the experimental design based on expected effects of the target traits. For example, changing dietary fatty acid consumption could affect the fillet oil content, the

fatty acid composition, and the peroxidation state.

Retail Product Assessment

For traits such as dietary oils and fatty acids, antioxidants, and pigments or others that may result in differences in appearance and shelf-life in the retail product, an assessment of the retail products derived from those treatments should be considered.

Sensory (Organoleptic) Evaluation

If a dietary trait that is fed in aquaculture has the potential to affect the organoleptic quality of the retail product offered to the consumer, a sensory evaluation should be done. It is not within the scope of this paper to delve into the specifics of sensory studies. Specific studies utilizing sensory analysis in evaluating fish fillets include: (1) a 10-member trained panel evaluating nine flavor descriptors in fillets derived from fish fed diets containing fishmeal or alternative ingredients (Adelizi et al. 1998); (2) a 16-person sensory panel evaluating color, texture, flavor, oiliness, and overall acceptability in fish fillets derived from fish fed canola oil and soybean oil as compared to fish oil (Glencross et al. 2003b); (3) a 13-member panel evaluating flavor, intensity of aromatics, tastes and feeling factors, and descriptive analysis spectrum prepared catfish fillets derived from fish fed diets containing various ingredients (Johnsen and Dupree 1991); and (4) a 7-member trained flavor profile panel evaluating the presence or absence of off-flavors in catfish fillets from fish fed diets containing 0, 1.5, or 3.0% fish oil (Morris et al. 1995).

Specific details on conducting sensory studies have been published (Johnsen and Kelly 1990, Meilgaard et al. 1999, Stone and Sidel 2004). Meilgaard et al. (1999) discussed in detail sensory attributes and human perception; controls for the test room, product, and panel; physiological and psychological factors affecting sensory verdicts; measuring responses; Triangle Test; Duo-Trio Test; Two-out-of-Five Test; Simple Difference test; "A" – not "A" Test; Difference-from-Control Test; Sequential Tests; Paired Comparison Designs; Directional Difference Test; Pairwise Ranking Test; numerous Multi-sample Difference Tests; determining thresholds; selecting and training panel members; descriptive analysis techniques; the Spectrum™ Descriptive Analysis Method; Affective Tests, including consumer tests and in-house panel acceptance tests; statistical methods; guidelines for choice of technique; and guidelines for reporting results. Stone and Sidel (2004) provide additional information on the organizing and operating a sensory evaluation program, measurements, test strategy and experimental design, discrimination testing, descriptive analysis, affective testing, and dealing with special problems.

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Chapter 17

Statistical Analysis and Interpretation of Results

This chapter is an updated version of Chapter 9 published in the ILSI document, *Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits* (ILSI 2003).

Good science is only as good as the process of conducting properly designed experiments, collecting data accurately, 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, data inaccuracies, unsound analysis, or incorrect data interpretation. 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, the researcher should address several important questions:

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 the experimental unit be—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 the experimental design be? 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 sufficient 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 GM crops are addressed. For additional information, readers are referred to other publications such as those by Cochran and Cox (1957), Gill (1978a, 1978b), Snedecor and Cochran (1980), Steel and Torrie (1980), Damon and Harvey (1987), Lentner and Bishop (1993), Hinkelmann and Kempthorne (1994), Morris (1999), Aaron and Hays (2001), Montgomery (2001), and Tempelman (2004).

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 assess a cereal grain with an improved amino acid profile for pigs. The treatments could be the GM grain in a low-protein diet, a conventional grain in a low-protein diet, and a positive control treatment consisting of the conventional grain in an adequate-protein diet. If possible, it would be best if the control grain is genetically similar, or near isogenic, to the GM grain except for the specific GM trait. In addition, the control grain should have been produced under environmental and agronomic conditions that are as similar as possible to the GM grain.

Treatment Selection

The selection of treatment diets for inclusion in experiments to evaluate the potential benefits of output traits is critical for drawing meaningful conclusions from test results. The first step in selecting experimental treatments should be to consider the expected commercial setting in which the GM product will be used. This will help determine the best conventional diet for use as a reference treatment. Next, the composition of the diet containing the GM crop or coproduct derived from a GM crop (the test diet) should be considered. Depending on the trait and the form in which it is added to diets, the test diet may be constructed in different ways. For example, a purified or semipurified plant-produced enzyme supplement may be included in a diet as a replacement for an enzyme supplement produced by a microbe, while a nutritionally enhanced GM grain may replace both conventional grain and one or more supplements in the diet. Another possibility is that a grain engineered to contain lower levels of an antinutrient may be substituted for its conventional counterpart at or above a commercial level without altering conventional dietary supplements. In the latter situation, greater amounts of a diet component may be possible if an antinutrient is typically the factor limiting its quantity in commercial feed.

In addition to the reference and test diets, one or more control diets may be desirable depending on the nature of the test diet. If the test diet is designed to replace a dietary supplement, then it is desirable to include a deficient diet treatment that excludes that particular supplement. This will enable a comparison between the conventional diet and the deficient diet to determine if the experiment had sufficient power to detect the benefit of the supplement, and, by extension, the enhancement in the test diet. Another useful control may be a diet fortified with a nontransgenic supplement matched to the level provided by the test diet. This fortified control will be useful to evaluate whether the results observed with the test diet can be entirely attributed to the nutritional enhancement provided by the GM component of the test diet. When the test diet component is being tested at a concentration that is above typical commercial levels (e.g. when an antinutrient has been reduced), a variation of a fortified control might be a diet containing an excess of the same conventional component compared to the commercial control. This latter control will be useful in evaluating if the experiment had sufficient power to detect the benefit of the test diet.

It is important to realize that fortified and/or deficient diet treatments may not be feasible for certain types of test diets. For example, if an antinutrient is reduced in a GM feedstuff, a fortified control is not applicable and a deficient diet may not be obtainable. Likewise, it may not be possible to mimic transgenic modifications that alter the concentrations of multiple constituents (e.g. amino acids) with either deficient or fortified controls.

Thus, a commercial reference diet should be included in experiments designed to evaluate the potential benefits of output traits. A GM test diet treatment will also be included, but its form will depend on the transgenic modification. Likewise, deficient and fortified controls will often be included in experiments, but the composition of these controls will depend on the transgenic modification and the intended commercial setting in which it will be used.

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, animals are not uniform in most cases 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 to carry over into another period. Modifications of a Latin square design, such as a crossover or switchback design (a 2×2 Latin square), are typically 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 more likely 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.

Number 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 may be estimated using procedures described by Cochran and Cox (1957) or Berndtson (1991). Table 17-1 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.

Table 17-1. Estimated Number of Replications (Blocks) Needed to Detect a Treatment Difference at P < 0.05)[a]

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

[a]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).

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 experiment's outcome. An example of preplanned nonorthogonal contrasts is comparing 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 conduct. 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 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. However, the LSD test should only be used for the comparisons planned before the data have been examined (Steel and Torrie 1980). Carmer and Walker (1985) presented 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).

Unexpected Effects

Animal feeding trials are designed to evaluate the potential benefits of diets containing a GM feedstuff and/or to demonstrate their nutritional equivalence with conventional feedstuffs. However, such experiments are also commonly used to test for unexpected effects. Because there is a clear rationale and hypotheses associated with evaluations of expected benefits, the statistical tools employed for testing these hypotheses should be different from those used for seeking unexpected effects. Typically, a small number of variables are investigated to determine the potential benefits of a GM feedstuff. Thus, the number of principal response variables is normally small. When multiple treatments are being compared, the family-wise error rate is typically controlled for each response variable being considered to maintain the desired Type I

error rate (chance of falsely declaring a difference when none exists) across all the comparisons that are made within each response-variable (e.g. Tukey's, Dunnett's, Bonferroni adjustments).

However, if multiple response variables are collected, no control of the Type I error is provided across the different response variables using these corrections. This is inconsequential when relatively few primary variables are analyzed, such as for benefits testing, but can be quite significant for testing unexpected effects across many response variables. For example, at the 95% confidence level, the chance of falsely declaring at least one significant difference when three different response variables are evaluated is ~14% (1 - 0.953), while the chance of falsely declaring at least one significant difference with thirty different response variables is ~79% (1 - 0.9530). Thus, there is a need to control the false discovery rate across the experiment when looking for unexpected effects across many response variables.

The false discover rate (FDR) is a technique specifically formulated for controlling the false discovery rate across multiple response variable analyses (Benjamini and Hochberg, 1995). This method controls the rate of declared significant differences at the specified significance level independent of the number of comparisons and response variables in the analysis. Therefore, if a 95% confidence level is specified for declaring a significant difference, each significant difference that is declared has a 95% chance of being true.

Thus, different types of statistical analyses are typically appropriate when testing for expected benefits or unexpected effects because in the former situation, a small number specific hypothesis is being tested, whereas in the latter case, a large number of unexpected effects are being screened. While testing for the benefits and/or nutritional equivalency of GM feedstuff is the main objective of animal feeding experiments, unexpected effects may also be evaluated. The false discovery of significant differences is likely when many response variables are evaluated, unless the false discovery rate is controlled. The use of FDR or similar techniques allows this control and improves the probability of discriminating real differences from those generated by random chance.

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' pre-experimental 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 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, UK; http://www.vsn-intl.com/genstat/).

Interpretation of Experimental Results

Researchers should have background and training that will enable them to interpret 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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