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U.S. Dairy Forage Research Center 2000-2001 Research Report

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Appreciation is expressed to Peggy Carroll for her dedication to the task of typing and assembling this research summary.

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Preface

It is a pleasure to bring you these summaries of research conducted over the past two years at the U.S. Dairy Forage Research Center. The Center's mission is to build a knowledge and technology base for the dairy industry to fully exploit the use of forages in the production of milk. The Center was established in 1980 on the University of Wisconsin-Madison campus in Madison, WI, but is a federal unit of the Agricultural Research Service, U.S. Department of Agriculture (USDA). We employ agricultural engineers, plant and soil scientists, microbiologists, ruminant nutritionists, and a chemist who all work together to increase the efficiency of forage production and utilization by dairy farmers. At present, we have fifteen scientists: twelve at Madison, two cluster scientists at the University of Minnesota in St. Paul, MN, and one cluster scientist at Cornell University in Ithaca, NY. These scientists hold faculty appointments in university departments and provide supervision for approximately 6-8 graduate students and 4 postdoctoral fellows. We function in close cooperation with the agricultural experiment stations of several states.

The Center's 63-acre research farm is located in Prairie du Sac, WI and has facilities for housing and feeding 320 milking cows and 350 replacement heifers and dry cows. An additional 1,555 acres of adjacent land is utilized by the Center in agreement with the U.S. Department of the Army. In 1999, the U.S. Defense Department declared that the former Badger Army Ammunition Plant (BAAP), adjacent to our research farm, is excess property. The USDA has requested a no-cost transfer of custody of 1,718 acres of this excess federal land so that we can continue our research efforts. We are working with the Ho Chunk Nation, Wisconsin Department of Natural Resources, Sauk County, Sumpter and Merrimac Townships in Sauk County, the GSA, and the Army to develop a unified management strategy for the entire property to facilitate transfer of the land. We are encouraged by the cooperation of all parties to bring about a solution.

Regarding staff updates, we hired J. Mark Powell as a Soil Scientist/Agroecologist in December 2001. His expertise strengthens our integrated farming systems research effort. Mark earned degrees from the Clemson University, Cornell University, and Texas A&M University. He brings research experience with several international research organizations in which he developed an agroecology approach to ruminant livestock use of land bases to create economic alternatives with minimal ecologic impacts on land and soil resources. Check out the Enhanced Integrated Nutrient Management site on the DFRC web page, http://dfrc.ars.usda.gov/powell/ to see the immediate impact Mark brings to our research effort.

I am pleased to announce that Michael D. Casler, University of Wisconsin, has accepted our offer to fill the Research Geneticist position at USDFRC. Michael is no stranger to forage supporters, bringing 21 years of experience in forage breeding and genetics to the USDFRC effort. His contributions to improving forage grasses are recognized internationally. His efforts to improve cell wall digestibility of smooth bromegrass and perennial ryegrass, discover new varieties and improve management practices of perennial grasses for rotational grazers, as well as improvements in switch grass for biomass production, bring a unique expertise to dairy forage. He has the best skills to offer USDFRC using genetics and molecular genetics to develop new perennial grass and legume germplasm for dairy utilization, conservation uses, and value-added traits.

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Highlights of USDFRC 20th Anniversary Celebration – June 29 and 30, 2001
Neal Martin and Clive Holland

National recognition of research efforts of scientists, staff, and collaborators by citizens is the best accomplishment a group can receive. The U.S. Dairy Forage Research Center (USDFRC) Stakeholder Committee, chaired by Clive Holland, Pioneer Hi-Bred International, along with volunteers, private companies and staff, teamed together to tell citizens how our current research to increase utilization of forage crops by dairy cattle benefits communities. The theme "Cows, Crops and Communities" captured the importance of dairy forage research to a national audience.

The anniversary celebration occurred at 3 different venues: a recognition seminar for national media at the Fluno Center for Executive Education, University of Wisconsin-Madison; an open house at the USDFRC laboratory in Madison; and an open house at the research farm in Prairie du Sac.

Recognition Seminar

Over 14 national agricultural media representatives covered the seminar. The audience viewed a video tape from U.S. Senator Herb Kohl on the importance of dairy forage research, and heard a congratulatory letter from USDA Agriculture Secretary Anne Veneman, presented by USDA-ARS Administrator Floyd Horn. Dr. Horn and James Harsdorf, Secretary of Wisconsin Department Agriculture, Trade and Consumer Protection, also gave presentations. USDFRC scientists, along with industry representatives and farmers, completed the seminar with talks on the impacts of forage research. The video, presentations, and posters can be viewed in detail on the USDFRC web site: http://www.dfrc.ars.usda.gov/cd/StartHere.htm

Topics and speakers were: *U.S. Dairy Forage Research Achievements: Past, Present and Future*, Neal P. Martin, Center Director, USDFRC; Safe *Nutritious Milk from Improved Forage Crops,* Jim Hahn, Director, Membership & Procurement, Land O' Lakes, Arden Hills, MN; *Microbes Benefit Cows, Crops, and Communities, James* Russell, USDFRC Cluster Scientist (New York); *Improving Forage Crops is Good for Cows, Good for Communities*, Glen Broderick and Dave Mertens, Dairy Scientists, USDFRC; *Balancing Cows and Crops to Recycle Nutrients,* Michael Russelle, USDFRC Cluster Scientist (Minnesota); *New Crop Genetics Benefit Cows and Communities,* Mark McCaslin, Forage Genetics International, Savage, MN; *Dairy Farming in The West Needs Science of Cows and Crops to Improve Communities,* Steve Faber, Dairy Research Center, University of Arizona, Tucson, AZ; and *Importance of the USDFRC to the Upper Midwest Dairy Industry,* Dean Doornink - Jon-Dee Farm, Inc., Baldwin, WI.

Laboratory Open House

- The open house provided an opportunity for the public to better understand forages, dairy cattle, and the products generated from dairy cattle. Participants viewed displays and posters, a chef melted new cheese products to sample, and the Wisconsin Milk Marketing Board was on hand with a Volkswagon Beetle to promote Wisconsin dairy products.

Poster and display topics included:

- What is fiber, what is it made of, and how do dairy cows use it?
- Impact of increasing cell wall digestibility

- Cross-linking fiber limits digestion
- What is lignin?
- How forage samples are analyzed for structural studies
- Evolution of a new forage variety

- Forages for dairy production
- Breeding alfalfa with more digestible stems
- The rumen symbiosis: A partnership between the cow and its microbial population
- Molecular assessment of microbial populations in the rumen
- Inoculants-improving silage fermentation
- Bunker silo management
- Bag silos – Densities and losses
- Rapid forage analysis using NIRS
- Measuring fiber in feeds
- Responses of cows to differences in maturity and processing of corn silage
- Estimating nutritive value using a feed information expert system

- Reducing nonprotein nitrogen in alfalfa silage improves protein utilization
- Red clover silage as a replacement for alfalfa silage in dairy cow diets
- Rumen in vitro methods for testing the nutritional value of feeds
- Omasal sampling techniques for studying digestion in the rumen
- Milk urea concentration can be used to prevent overfeeding of protein
- Bacteriocins as an alternative to antibiotics
- New products from alfalfa
- Phosphorus requirements for lactating cows
- Roasted soybeans are an economical supplement for rumen undegraded (by-pass) protein
- Brown midrib corn silage for transition cows
- Grazing research at the U.S. Dairy Forage Research Center
- Integrated cropping systems and nutrient management on dairy farms
- Enhanced integrated nitrogen management on dairy farms
- Whole-farm phosphorous management on dairy farms
- Tannin-containing forage crops: A way to improve nitrogen use and profitability on dairy farms?
- Well-managed grazing helps protect ground water quality

Research Farm Open House

Various displays were set up (see below), and USDFRC scientists were available to answer questions. James Harsdorf, Wisconsin Secretary of Agriculture, Trade and Consumer Protection, spoke briefly on the value of the USDFRC to dairy farmers. After Secretary Harsdorf's presentation, Sheri Hicken, Wisconsin's 2001 Alice in Dairyland, addressed the audience.

Station 1: **Demonstration of Sampling the Ruminant Digestive Tract**
Reducing Nonprotein Nitrogen in Alfalfa Silage Improves Protein Utilization
Red Clover Silage as a Replacement for Alfalfa Silage in Dairy Cow Diets
Rumen In Vitro 'in Glass' Methods for Testing the Nutritional Value of Feeds

Station 2: **Integrated Cropping Systems and Nutrient Management of Dairy Farms**
Whole-Farm Phosphorus Management on Dairy Farms
Well-Managed Grazing Helps Protect Ground Water Quality
Tannin-Containing Forage Crops: A Way to Improve Nitrogen Use and Profitability of Dairy Farms
Enhanced Integrated Nitrogen Management on Dairy Farms
Russelle's Believe It or Not — Roots on Display

Station 3: **Learn How Improving Digestion of Cell Walls Enhances Animal Health and Milk Production**

Station 4: **New Uses for Alfalfa**
Breeding Alfalfa with More Digestible Stems

Station 5: **How New Forage Varieties are Developed**

Station 6: **Improving Silage Making and Storage**
Inoculants – Improving Silage Fermentation
Bunker Silo Management
Bag Silos – Densities and Losses

Station 7: **Feeding of Dairy Cattle**
Roasted Soybeans as an Economical Supplement for Rumen Undegraded (Bypass) Protein
Reducing Dietary Phosphorus to Lower Costs and to Improve the Environment
Responses on Performance and In Vivo Digestibility of High- and Low-Producing Cows To Maturity and Processing of Corn Silage.

History of the U.S. Dairy Forage Research Center

Congress and the United States Department of Agriculture/Agricultural Research Service (USDA-ARS) established the U.S. Dairy Forage Research Center (USDFRC) in 1978 with the mission to develop and disseminate knowledge and tools needed for enhancing sustainable and competitive dairy forage systems that are in harmony with the environment, promote animal health, and ensure a safe and healthy food supply.

USDFRC consists of a federally owned laboratory located on the University of Wisconsin-Madison campus and a federally owned support research farm (65 acres with buildings owned by USDA and 1400 acres leased from the Department of Army) located near Prairie du Sac, WI that includes a 320-cow research dairy herd.

Although USDFRC was first envisioned as a regional laboratory, the program quickly and clearly developed a national scope. About half of the Center's resources are used to support basic or fundamental research that has no geographical boundaries. The other half of the resources are used to support more applied research, and over half of this amount can be considered applicable to all of North America and to many locations around the world.

A crucial feature in the development of USDFRC has been the concept of central facilities at the University of Wisconsin, and cluster scientists located at several other universities. This concept has provided for a stronger scientific program and greater cooperation with other researchers, as well as widespread support from the industry and enhancement of the overall forage research program. USDFRC cluster scientists are now located at the University of Minnesota, St. Paul, MN, and at Cornell University, Ithaca, NY.

Research Overview

Research by the Center's thirteen scientists in Wisconsin plus the three cluster scientists focuses on important national questions and problems associated with forage and its relationship with other feedstuffs for the dairy industry and nutritional requirements of the ruminant, primarily the dairy animal.

ARS uniquely designs the scientific efforts at the Center such that research conducted at the laboratory is conducted with a multi-discipline approach. Disciplines represented at the Center laboratory are:
plant genetics, plant physiology, chemistry, rumen microbiology, soil science, agricultural engineering, agronomy, ruminant nutrition, and dairy science.

Research Accomplishments

Between 1980 to 1998, milk production in the United States increased 22%, while the number of cows declined by 15% and the total number of dairy farm operations dropped by 65%. Annual milk production per cow increased 45%, from 11,875 to 17,189 lbs. Dairy farm enterprises across the country generated almost $21 billion in cash farm receipts from milk sales. Factors that have contributed to this incredible increase are improvements in forage and feed consumption. Dairy cattle consume 100 million tons of forage each year valued at about $8 billion.

Research conducted at the U.S. Dairy Forage Research Center (USDFRC) on forage production, harvest, storage, and feeding forage quality has contributed to the dynamic changes in the dairy industry. The work of USDFRC has also advanced our understanding of the important role of forage crop quality to improve cow performance.

IMPROVEMENTS IN FORAGE-BASED RATIONS

The rumen sub model was developed for the Cornell Net Carbohydrate and Protein System, a method of feed formulation used by some dairy producers and nutritionists, and adopted at Level 2 by the National Research Council Beef Committee. Use of this model can result in feed savings as much as 17%.

- The NDF-Energy Intake System was developed as a way of directly using neutral detergent fiber (NDF) to formulate dairy rations that maximize forage use while promoting maximum milk production. The model is used to predict the intake portion of the Relative Feed Value Index, a quality index used to market cool-season legumes, grasses, and legume-grass mixtures nationally and internationally since 1990.

- The concept of physically effective NDF was created, combining the chemical and physical properties of fiber into a measurement used to meet the minimum fiber requirement of cows. The concept has been incorporated into several ration formulation software programs.
- Optimum conditions for roasting of soybeans to enhance bypass protein value were identified. This work has greatly contributed to widespread adoption of roasted soybeans in dairy cow diets and the generation of $20 to $40 million annually in added value.
- Improved digestibility of the vegetative part of the corn plant was demonstrated for brown midrib varieties or by cutting silage corn higher (20-28" vs. 8-10"), thus leaving some of the poorly digested stalk in the field. These improvements can increase milk production by 2-4 lbs per cow/day, and this increase more than pays for the loss of yield (or higher costs) associated with growing brown midrib varieties, or cutting silage corn at higher levels.
- The relative value was determined for various feed proteins used as supplements for lactating dairy cows fed alfalfa silages as their principal forage.
- A previously unrecognized group of ammonia-producing rumen bacteria were isolated and identified and shown to be sensitive to the ionophore, monensin. This group of bacteria wastes approximately $1 billion per year in protein.
- A sterilized, natural preparation of ruminal microorganisms was developed that can decrease calf diarrhea fivefold and nearly double the calf's rate of weight gain early in growth.

FORAGE DIGESTIBILITY

- The most comprehensive characterization of lignin structure and phenolic-carbohydrate cross-linking was completed. This information has dramatically altered scientists' views of cell wall structure and has identified routes to genetic improvement of forages for enhanced digestibility that are being pursued by several agricultural biotechnology companies. Some of these companies have established Cooperative Research and Development Agreements with USDFRC to further carry out this work.
- Pectin in alfalfa displayed rates of ruminal digestion equivalent to those of cereal starches. It produces a favorable fermentation product mix that maintains the level of butterfat in the milk. This work has stimulated breeding efforts by several companies, aimed at increasing pectin content of alfalfa.
- The anatomical features of forage corn that have greatest influence on its digestibility were identified. This information has provided breeders with a selection tool to develop varieties that have improved digestibility.
- Equations were developed that relate fiber particle surface area and ruminal pH to the rates of digestion of cellulose (the major component of forages). These equations will be useful in refining models of ruminal fiber digestion.

FORAGE AND FEED ANALYSIS

- The method used to analyze neutral detergent fiber (NDF), an important component for balancing dairy cattle rations, was improved. The modified NDF method has been adopted by the National Forage Testing Association (NFTA) to test the proficiency of at least 150 forage testing laboratories in the U.S. that analyze millions of forage samples each year for farmers, nutritional consultants, and feed industry representatives.
- Methods to quantify and characterize lignin in forages were developed and are now being used by researchers worldwide.

- Methods to measure how rapidly feed components are digested, based on production of fermentation gases, were improved and extended to soluble sugars in forages. This is the most difficult component for which to obtain accurate digestion rate data. The method has been used to identify new germplasm that have enhanced rates of carbohydrate digestion.
- A laboratory method was developed to rapidly determine rumen undegradable protein. This procedure is useful as a tool to optimize pretreatments to enhance the amount of bypass protein in a feed.
- Procedures have been developed to detect and eliminate non-random biases in forage composition estimated by Near Infrared Reflectance Spectroscopy (NIRS), a rapid analytical method widely used by forage testing labs. The methods have been used to improve protocols for selecting and evaluating calibration equations that underlie the use of NIRS technology.

FORAGE PRODUCTION AND MANAGEMENT

- Two varieties of red clover, Arlington and Marathon, were developed and released. The varieties have increased persistence, longevity, yields, and disease resistance, and annually save $140/acre/year on at least 250,000 acres in the Midwest.
- The first tetraploid and triploid red clover germplasm lines were developed. These varieties will be useful as genetic tools for development of new red clover varieties.
- Improved varieties of birdsfoot trefoil and kura clover were developed and released.
- A computerized DAiry FOrage SYstem Model (DAFOSYM) was developed that simulates growth, harvest, storage, feeding, and use of alfalfa and corn in dairy operations. The model has been distributed to end-users for use as a decision aid.
- The most comprehensive model to date was developed to predict and control aerobic deterioration of silages at the silo face during unloading.
- The first predictive tool was developed that allows farmers to assess the most effective means of increasing silage density to resist dry matter losses during ensiling.
- Drying rates, losses, and other performance measures of various types of forage harvesting methods were quantified. Application of this research provides the best management practice for harvesting hay, potentially saving 15% in field losses. If applied to 40% of the U.S. hay crop, the potential savings could top $4.4 million per year.
- Technology for farm-scale chemical conditioning of alfalfa was developed and transferred to end-users, returning $2 per dollar invested. If the technology is applied to just 25% of the U.S. alfalfa hay crop, it could yield a return of $1.6 million.

ADDING VALUE TO FORAGE OPERATIONS

- A novel field macerator machine was designed, built, and demonstrated. Manufacturing a field macerator to replace current mower-conditioners can potentially reduce drying time of alfalfa hay by two days, with increased dry matter digestibility and improved protein utilization.
- Conjugated linoleic acid (CLA), a natural anti-cancer agent produced in ruminants, was shown to be three- to fivefold higher in the milk of grazing cows than in cows fed conserved forages. In addition, the CLA content of milk from cows fed conserved forages increased to the levels found in the milk from grazing cows by feeding unsaturated vegetable oils, such as soybean oil.
- A simple method was developed to identify conditions under which use of silage inoculants yields an economic benefit. This information was distributed to farmers and extension agents.

- Technology was developed for wet-fractionation of alfalfa to produce a high-protein food for use in developing countries. A fiber residue suitable for fermentation to a variety of products was also developed. Simultaneous saccharification and fermentation (SSF) of alfalfa fiber produced during wet fractionation has been shown to make lactic acid at yields of up to 60% of fiber dry matter.
- The feasibility of using alfalfa stems as a biofuel was demonstrated. This cooperative research with the University of Minnesota paved the way for formation of the Minnesota Valley Alfalfa Producers, a farmer cooperative for production and dry fractionation of alfalfa to produce alfalfa stem fuel and alfalfa leaf protein meal.
- Collaborative work with the University of Wisconsin resulted in the development of transgenic alfalfa that produces Phytase, an enzyme lacking in the digestive tracts of swine and chickens. Feeding trials have revealed that this alfalfa serves as an effective feed, and the capacity of the enzyme to degrade phytic acids in feeds eliminates the need for phosphorus supplementation in the diet.
- Collaborative work with the U.S. Forest Products Laboratory (Forest Service-USDA) demonstrated the utility of woven mats of alfalfa fiber to remove heavy metals from wastewater and storm water runoff.
- Collaborative work with the U.S. Forest Products Laboratory also demonstrated the potential use of rumen bacteria for fermentation of forage fiber to biological adhesives. These adhesives may be used to partially replace environmentally unfriendly phenol-formaldehyde resins currently used in the forest products industry.

FORAGES AND THE ENVIRONMENT

- Reduction of phosphorus in mixed forage/concentrate diets by 20% from current NRC recommendations resulted in a 25-30% reduction in phosphorus excretion by the cow, and a potential savings to dairy producers of ~$100 million annually.
- Inexpensive addition of sodium carbonate to cow manure effectively reduced populations of *E. coli* in the manure.
- Practical use of deep-rooting varieties of alfalfa to remove nitrate from soils was demonstrated in soil contaminated by nitrogen from a railroad tank car derailment.

INFORMATION TRANSFER

- Hosted International Symposium on Forage Cell Wall Structure and Digestibility, October 7-10, 1991. One hundred sixty-five people representing 15 countries attended. ASA, CSA, and SSSA Monograph: "Forage Cell Wall Structure and Digestibility," Eds: H.G. Jung, D.R. Buxton, R.D. Hatfield, and J. Ralph, 794 pages, 1993. Now in second printing.
- Hosted Research Industry Conference in 1996. Fifteen scientists addressed the state of dairy forage research in 118-page proceedings.
- Hosted 15th *Trifolium* Conference, June 12-13, 1998.
- Published more than 1,000 publications (1000th publication published in November 1999).
- Distributed 16 annual research summaries to 700 extension and industry product specialists, farm advisors, and farmers that outlined the impact of research in 785 different short topics totaling 1,668 pages.
- Hosted many state and region farm field days

INTERNATIONAL COLLABORATION

Work at USNDFRC has created interest far beyond the borders of the United States. USDFRC scientists have collaborated with these foreign research institutes and universities:

AgResearch, New Zealand
Agriculture Canada, Quebec
Agriculture Canada, Nova Scotia
Danish Institute of Animal Science, Tjele
IMAG, Wageningen, The Netherlands
INRA, Reims, France
INRA, Paris, France
Institute of Wood Research, Japan
Kangweon University, Korea
Teagasc, Grange Research Centre, Ireland
Universite Paul Sabatier
University of Groningen, The Netherlands
University of Stuttgart Hohenheim, Germany
Volcani Center, Bet Dagan, Israel
Agricultural Research Institute, Norway
Agriculture Canada, PEI
CSIRO, Australia
ID-DLO, Lelystad, The Netherlands
INIA, Uruguay
INRA, Clermont-Ferrand, France
Institute of Food Research, Norwich, UK
INTA, Argentina
Swedish University of Agricultural Sciences
The Rowett Institute, Aberdeen, Scotland
University of Australia
University of Brisbane, Australia
University of Kiel, Germany
Wageningen Agricultural Univ., The Netherlands

Forage Genetics and Production

Polyphenol-Containing Forages: A Way to Improve the Profitability and Nitrogen-Use Efficiency of Dairy Farms?

J.H. Grabber, G.A. Broderick, R.D. Hatfield, J. M. Powell, M.P. Russelle and R.E. Muck.

Poor protein utilization is a problem on dairy farms

Forages like alfalfa are potentially a superb source of protein for livestock. The per acre protein yield of alfalfa is 50% greater than soybeans and its crude protein content should be high enough to meet the requirements of most dairy cattle. Unfortunately, most of the protein in alfalfa is degraded in the silo and rumen, impairing protein utilization by the cow. This leads to excessive nitrogen excretion in urine, increasing the amount of nitrogen that must be either recycled through crops or lost to the environment. Protein utilization by dairy cattle can be improved by several means, but each has shortcomings. Excessive protein breakdown in the rumen and nitrogen excretion by livestock is reduced if annual row crops like corn and soybeans are used to meet much of the nutritional requirements of lactating dairy cattle. Unfortunately, production of row crops in place of perennial forages dramatically increases the risk of soil erosion and nutrient loss from cropland to the environment. Alternatively, purchased protein supplements increase the cost of milk production and can lead to excess nitrogen and phosphorus accumulation in soils and greater loss of these nutrients from farmland. Post-harvest treatment of forage with formic acid or other additives can reduce protein degradation in ensiled forages and improve protein use by dairy cattle. However, concerns about cost, equipment corrosion, or safety currently preclude the widespread use of these treatments.

Polyphenols can improve protein utilization

Protein utilization on dairy farms is enhanced if forages containing polyphenols are grown and fed to cattle. Modest levels of polyphenols (perhaps 2-4% of dry matter) bind to proteins, reducing proteolysis during ensiling and rumen fermentation by up to 50%. These levels, however, will permit extensive protein digestion in the abomasum and subsequent uptake of amino acids by the small intestine without adversely affecting carbohydrate digestibility or reducing feed intake. Some forage and grain crops (e.g. red clover, some varieties of birdsfoot trefoil, and grain sorghum) contain adequate levels of polyphenols for enhancing protein utilization by cattle. However, most feeds used on U.S. dairy farms (e.g. alfalfa, corn silage, corn grain, grasses, and soybean) contain very low levels of polyphenols (< 0.2% of dry matter), insufficient for improving protein use.

Feeding trials at the Dairy Forage Center have demonstrated the value of polyphenols in red clover for reducing wasteful protein breakdown during ensiling and rumen digestion and for improving protein use-efficiency of dairy cattle. This research is described elsewhere in our 2000/2001 Research Summaries. These trials also revealed that fiber digestibility of red clover based rations is 22% greater than alfalfa based rations, reducing excretion of manure by 19%. Although these traits increase the value of red clover, further adoption of red clover in dairy feeding systems will be limited unless improved cropping systems for this forage are developed.

The benefits of condensed tannins (another group of polyphenols) for improving protein utilization and ruminant performance are well documented in New Zealand for sheep and cattle fed pasture or green-chopped forages. Feeding trials with sheep demonstrated that increasing tannin concentrations from trace amounts to 4% of dry matter increased the flow of feed protein into the small intestine by 30%. Absorption of essential amino acids by the small intestine was increased by up to 60% in sheep diets containing as little as 2% tannin on a dry matter basis. In a recent study, milk production of non-supplemented Holstein cows was increased by 2.7 kg per day by tannins in birdsfoot trefoil. The potential for tannins to improve protein utilization and milk production of dairy cattle have not, however, been evaluated in forage-concentrate rations typically fed on U.S. dairy farms.

Tannins (and probably polyphenols in red clover) also shift nitrogen excretion from urine to feces and from soluble to insoluble nitrogen forms in feces. Moreover, a greater proportion of fecal nitrogen is in undigested plant residues—a form which mineralizes more slowly than microbial and endogenous nitrogen. These shifts in nitrogen forms could reduce ammonia and nitrate losses from dairy cow facilities, manure storage tanks, and manure-amended fields.

Tannins may also effect the overall cycling of nitrogen and carbon in forage-based cropping systems for dairy farms. In one study, three-year stands of alfalfa and birdsfoot trefoil, managed as hay, supplied about the same amount of nitrogen to three succeeding years of corn. Compared to alfalfa, birdsfoot trefoil provided 20% less nitrogen in the first year (without a reduction in yield), and about 50% more nitrogen in the second and third year of corn production. The more uniform mineralization of nitrogen from birdsfoot trefoil residues might be due to tannins; studies with green manure crops and tree litter indicate that polyphenols like tannins slow the mineralization of nitrogen and carbon in soil. A more gradual mineralization of nitrogen from crop residues and manure could reduce nitrate losses from cropland, especially when forage legume fields are manured and plowed prior to the first year of corn production (a common, albeit not recommended practice on dairy farms). Although not documented, similar shifts in nutrient partitioning and cycling on U.S. dairy farms (which often have excess nitrogen) could reduce nitrogen losses from manure during excretion, storage, and field application. These shifts may also improve crop uptake of nitrogen and the sequestering of carbon in soils. Because perturbation of nutrient cycles can have surprising outcomes, it is imperative that we measure the actual impact of polyphenols on dairy-forage systems before pursuing adoption of a new practice or technology.

Polyphenol Research at the Dairy Forage Center

Efforts are underway to understand the biochemical mechanisms behind the more efficient utilization of protein in red clover. We will continue feeding trials with polyphenol containing crops (birdsfoot trefoil and red clover) to identify optimal polyphenol concentrations for improving protein utilization and milk production of dairy cattle. Other studies will evaluate how polyphenols influence nitrogen loss from manure during excretion, storage, and land application and the cycling of nitrogen in crop rotations. We are also examining the role of forage polyphenols for improving the sequestration of carbon in soil. Short rotations of red clover with cereal crops and being designed and tested to take greater advantage of the aggressive establishment, slower maturation, and high initial productivity of this polyphenol-containing forage species. Integrated nutrient cycling, crop, and dairy nutrition models (e.g. DAFOSYM) are being used to plan studies and to assess the farm-scale and national impact of incorporating polyphenol-containing crops on to dairy farms. A preliminary DAFOSYM study evaluating polyphenol impacts on dairy farms is included in the current Research Summaries.

These efforts will identify optimal polyphenol concentrations and management practices for improving protein and nitrogen use on dairy farms. Our efforts will also stimulate and support work in the public and private sectors to develop forages with optimal polyphenol levels for enhancing protein and nitrogen utilization by dairy farms.

Potential Impact of Tannin-Containing Alfalfa on the Profitability and Nitrogen-Use Efficiency of a Wisconsin Dairy Farm

J. H. Grabber, C.A. Rotz, D.R. Mertens, and R.E. Muck

Introduction

Tannins bind to forage proteins, potentially altering protein and N availability during ensiling, ruminal digestion, and decay of residues in soil. Most feeds used on U.S. dairy farms (e.g. alfalfa, corn silage, corn grain, grasses, and soybeans) contain inadequate levels of tannins (< 0.2% of dry matter) for affecting N cycling. Plant breeding and biotechnology efforts are underway in the U.S. and abroad to develop alfalfa and other forages with modest amounts of condensed tannins. We used a dairy-farm simulation model (DAFOSYM) to predict the impact of growing and feeding an alfalfa with 2% tannin on a dairy farm in southern Wisconsin.

Methods

Based on limited published data, we assumed that tannin would reduce rumen-degradable protein of alfalfa by 20%, increase acid-detergent insoluble N of alfalfa by 30%, and reduce the N mineralization rate of alfalfa residues in soil by 30%. The simulated farm had 100 cows, 85 heifers, and 250 acres of medium silt-loam soil. In rotation with corn grown for silage and grain. Alfalfa for hay or silage was cut with a conventional mower-conditioner and grown. Alfalfa for hay was also cut with an improved mower-macerator developed by the Dairy Forage Center. High-forage rations were fed using homegrown feeds and purchased corn grain, roasted soybeans, soybean meal, fat, and minerals. Cows were injected with BST and milked twice daily. Manure and bedding were stored in a lagoon and shallow-injected into corn ground in the spring and autumn to minimize ammonia losses. Simulations were run using 25 years of weather data from Madison, Wisconsin.

Results and Discussion

In general, use of normal or tannin-containing alfalfa did not affect yields of forage or grain (Table 1). Due to lower manure N excretion by cattle and lower residue N availability with tannin-containing alfalfa, corn-based system (6) required small amounts of additional N fertilizer. Milk yields were greatest with rations based on tannin-containing alfalfa or corn silage. Use of tannin-containing alfalfa in place of normal alfalfa reduced protein purchases by 27 to 57 tons, reduced nitrogen losses by 6 to 30 lb per acre, and increased net return per cow by $62 to $118 per year. Tannins increased the value of alfalfa silage by $24 to $32 and alfalfa hay by $12 per ton of dry matter. Feeding tannin-containing alfalfa shifted grain purchases from lower yielding soybeans to higher yielding corn, reducing the need for off-farm production of potentially erosive and nitrate leaky row crops by 8 to 23 acres. Benefits of tannin were greatest for alfalfa silage based systems. Conventional hay systems were not competitive (data not shown), yielding annual net returns of

about $50 less per cow than alfalfa silage systems and $110 less per cow less than the macerator-hay system. We are conducting cropping and feeding studies with birdsfoot trefoil (containing 1 to 4% tannin) and alfalfa to identify optimal forage tannin concentrations and management practices for improving protein and N use on dairy farms.

Table 1. Feed production, feed use, profitability, and environmental impact of a 100-cow dairy farm growing and feeding normal verses tannin-containing alfalfa silage or hay. Alfalfa for hay was mown with a macerator to speed drying rate and to improve forage quality. Alfalfa and corn silage were produced and fed in ratios of 3:2 (systems 1–4) or 3:7 (systems 5 and 6).

	System					
	1	2	3	4	5	6
Alfalfa conservation	Silage	Silage	Hay	Hay	Silage	Silage
Crops (ha)						
Alfalfa (A)	150	150	150	150	75	75
Corn (C)	100	100	100	100	175	175
Rotation (crop-years)						
	A-3	A-3	A-3	A-3	A-3	A-3
	C-2	C-2	C-2	C-2	C-7	C-7
Tannins in alfalfa	No	Yes	No	Yes	No	Yes
Crop yields (t/a)						
Alfalfa silage	4.3	4.3	4.2	4.1	4.3	4.3
Corn silage	6.3	6.3	6.4	6.4	6.1	6.1
Corn grain	2.9	2.9	2.9	2.9	2.8	2.8
Feed (t DM)						
Alfalfa fed	502	502	517	515	236	237
Corn silage fed	329	329	351	351	565	565
Homegrown dry grain fed	116	116	108	108	124	124
Forage sold	14	9	15	12	16	11
Corn grain purchased	158	219	141	175	121	160
Soy meal 48% purchased	47	36	36	50	69	65
Roasted Soy purchased	64	18	55	14	71	36
Animal/vegetable oil	6	6	6	7	6	6
Milk production (lbs/cow)	27,370	27,770	27,090	27,430	27,900	28,100
Net return ($/cow/year)	1,975	2,093	2,033	2,095	1,976	2,052
SD of net return ($/year)	78	89	89	103	86	98
Manure on corn land (%)	100	100	100	100	100	100
Fertilizer N (lb/a)	0	0	0	0	50	75
Nitrogen losses (lb/a)						
N volatilized	82	66	65	59	68	64
N leaching	17	11	13	10	12	11
Denitrification	22	14	16	11	15	14
N in leachate (ppm)	11.8	8.0	9.1	6.9	8.4	7.9

Phytofiltration to Remediate High-Nitrate Ground Water: Initial Tests of the Concept.
M.P. Russelle, D.W. Kelley, M.D. Trojan, E.P. Eid, J.F.S. Lamb, and J.A. Wright.

Introduction

Nitrate moves readily through soil with percolating water, and is a common problem in shallow aquifers of the USA. Compliance with the public drinking water standard of 10 mg NO_3^--N/L often involves construction and maintenance of a water treatment facility. In the case of one public rural water supplier, Lincoln-Pipestone Rural Water in southwestern Minnesota, it involved building a $2 million facility that requires several hundred thousand dollars in operating expenses annually.
An alternative approach to remediate water from shallow aquifers in humid and subhumid areas may be phytofiltration. The concept is that NO_3^--laden water from the aquifer is irrigated onto a growing crop, using water rates that promote leaching of water back into the aquifer. In theory, the actively growing crop absorbs the NO_3^- to produce plant proteins, while a portion of the irrigation water returns as clean water to the aquifer. Perennial, cool-season forages offer several important advantages over annual crops in phytofiltration, including their long growing period, high yield, high nutrient need, high quality (and value), reduced runoff and soil erosion, improved soil quality, and deep root system.

Methods

We conducted this research at two sites in Minnesota, Pipestone in the southwest and Becker in central Minnesota. Alfalfa (*Medicago sativa* L.), orchardgrass (*Dactylis glomerata* L.), and bromegrass (*Bromus inermis* L.) were seeded in duplicated plots (23 X 23 m) on a silty clay loam soil at Pipestone in spring 2000, and these forages plus soybean [*Glycine max* (L.) Merr.] were seeded in replicated plots (2 X 6 m) on a sandy loam soil at Becker in spring 1999. About 2.5 cm of water was applied twice weekly during the growing season with a solid set sprinkler system at Pipestone and a surface drip system at Becker. Irrigation water concentrations ranged from approx. 15 to 50 mg N/L. At Becker, we added either ^{15}N or Br as a tracer for NO_3^- uptake by the crops. Forage harvests followed typical practices in the area, with three or four cuttings annually in established stands. Soybean was harvested at physiological maturity. Plant samples were analyzed for total N and for the tracers, where applicable. Ground water samples were obtained in spring 2000 at Pipestone to evaluate NO_3^- concentration at upgradient and downgradient locations under these large plots.

Results and Discussion

Estimated recovery of NO_3^- in irrigation water in 2000 at Becker was 55% in orchardgrass and alfalfa, but only 25% in soybean and bromegrass (Fig. 1). Highest yield and N harvest were obtained with alfalfa, lowest with smooth bromegrass. Soil solution nitrate concentrations were generally very low under the perennial forages and considerably higher under soybean (Fig. 2). The disparity between moderate removal of N (as measured in shoots) and the low soil solution concentrations indicates that denitrification likely was occurring.
No differences were observed in NO_3^- concentration in ground water at Pipestone. This may indicate that the method is not effective, or may have been due to insufficient water recharge (irrigation began only in mid-summer 2000), to rapid movement of the groundwater (causing us to miss the low-NO_3^- plume), or to N loss by denitrification.

Theoretically, we should be able to improve water quality, if only dilution, using this approach. For example, using a drinking water supply requirement of 3 ML/d, which is the need at the Pipestone location, 20 mg NO_3^--N/L in the aquifer, and leached N of 5 mg NO_3^--N/L, a total of 15.4 ML/week of clean water needs to be returned to the aquifer to reach a target NO_3^- concentration of 9 mg NO_3^--N/L. A standard quarter-section irrigation pivot applies 13.0 ML in a 5.0-cm depth, which implies that two such sections in the neighborhood of the drinking water supply wells should be sufficient during the cropping system.

Conclusion

Removal of nitrate appears to involve both N uptake and denitrification. This remediation approach has potential in areas where ground water can be readily influenced by leaching. More generally, it appears that perennial forages could be used to remove nitrate from sources such as wastewater or aerobic lagoon water applied through irrigation systems to prevent ground water contamination. Phytofiltration will be much less effective during periods when crop growth slows (immediately after harvest, in late autumn as plants enter dormancy, or when temperatures are too cold or hot for rapid growth). Even if water treatment can be avoided for 5 or 6 months of the year, the operating costs for the drinking water facility will decline.

Partial funding was provided by the Legislative Commission on Minnesota Resources through the Minnesota Future Resources Fund.

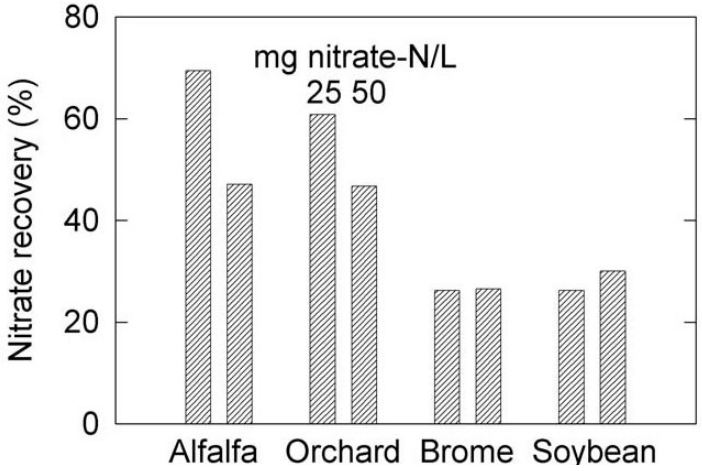


Fig. 1. Apparent NO_3^--N recovery by soybean and three perennial forage crops grown at Becker, MN, 2000, from irrigation water containing either 25 or 50 mg NO_3^--N/L.

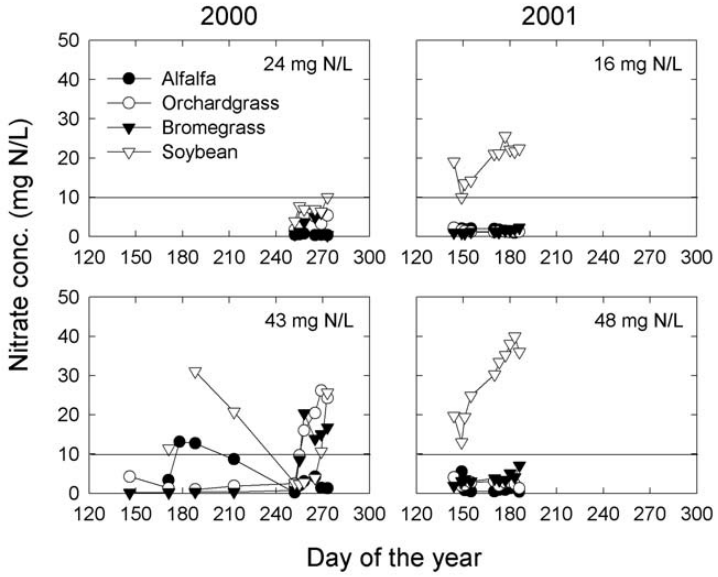


Fig. 2. Soil solution NO_3^--N concentration at the bottom of the root zone of four species during two growing seasons at Becker, MN.

Predicting Impacts of Crop Management on Nitrate Leaching in a Wellhead Management Area.

M.P. Russelle and D.W. Kelley

Introduction

Rural water supply quality is frequently compromised by high nitrate concentration. Although there are many sources of ground water nitrate, nonpoint sources such as fertilizer and manure N can be quite important in agricultural areas. We are working with the Lincoln-Pipestone Rural Water Supply District (LPRWSD) in southwestern Minnesota to devise ways to limit nitrate contamination of their aquifers. These shallow aquifers are easily affected by nitrate that leaches below the root zone, but leaching is highly dependent on the soil texture and depth in a field, what crop is grown, how much N is applied, whether supplemental irrigation is provided, and, of course, weather. Perennial crops can help reduce nitrate leaching by reducing both soil nitrate levels and water flow in spring, when leaching losses are usually highest in the North Central Region. Spring growth of alfalfa and other cool season perennial forages results in higher water use through evapotranspiration than with corn (evaporation only at this time), and reduces the amount of water loss by gravity through the soil. Nitrate leaching on fine-textured soils is uncommon during late summer, when crop water use is high, so neither corn nor alfalfa are likely to lose nitrate via leaching during this time. The majority of land in the LPRWSD wellhead management areas (WMA) is cropped to corn soybean. Our purpose in this research was to evaluate the likely impact of crop management on nitrate leaching in one of their WMAs.

Methods

We used a computer simulation model called GLEAMS (Groundwater Loading Effects of Agricultural Management Systems) with soils information from the Holland wellfield area (near Pipestone, MN) and ten years (1989-1998) of local historical weather data. We first calibrated and validated GLEAMS using detailed data from experiments conducted by others in the region. We simulated the effects of growing alfalfa, continuous corn at three N rates (115, 145, and 180 kg N/ha), and corn-soybean rotations at one N rate (100 kg N/ha on corn) on all major soils in the WMA (9000 ha). For the corn-soybean rotation, we ran the simulation with corn in even-numbered years, repeated the simulation with corn in odd-numbered years, and then averaged the results by year over the two crops. We assumed maximum yields were 8800 kg/ha (140 bu/acre) for corn, 4400 kg/ha (65 bu/acre) for soybean, and 10,000 kg dry matter/ha (4 tons/acre) for alfalfa, based on typical 'good' yields in the area.

In the model, fertilizer N was applied as urea and immediately incorporated in late April, one week before planting corn. Simulations were conducted twice, once using precipitation only and once with supplemental irrigation. The modeled irrigation regime was conservative; water was not applied until the soil dried to 25% of the available soil water holding capacity, and water was added only to 90% of the water holding capacity, so irrigation *per se* did not exacerbate leaching. Applied water was assumed to contain 5 ppm nitrate-N. No attempt was made to delay irrigation if precipitation would occur within a day or two, and thus, the model reflected the reality farmers face in needing to irrigate when precipitation is not a certainty.

Results

GLEAMS predictions supported our hypothesis that nitrate leaching under alfalfa is lower than under annual crops, like corn and soybean. The model predicted only rare leaching events under alfalfa, but it predicted high nitrate concentrations in the soil solution. This latter result does not agree with data in many experiments, which show that soil solution nitrate-N concentrations under alfalfa are typically much lower than 10 ppm. If water escapes the root zone of perennial forages during spring, it may help improve ground water quality as long as the nitrate concentration of this percolating water is low.

Average predicted corn grain yield increased on some soils with 130 compared to 100 lb N/acre, but little further gain was achieved with 160 lb N/acre. This result also occurred in simulations using a higher yield potential, lending credence to University of Minnesota fertilizer recommendations. The amount of water percolating below the corn root zone did not change with fertilizer N rate, but nitrate concentrations in that water increased rapidly when excessive fertilizer N was applied, leading to very high N losses on some soils.

Irrigation increased leaching losses, mainly due to increased water percolation during May through August, because of decreased soil water storage capacity when heavy rainfall occurred. In addition, late season irrigation reduces the capacity of soil to store snowmelt and rainfall in spring. Even with the conservative irrigation regime in this simulation, the amount of water percolating below the root zone increased by an average of 30 to 35% on most soils, and nitrate concentration increased to a variable degree.

We estimated the total average annual N loss via leaching by combining the per-acre loss and the area of each modeled soil in the Holland WMA. Even when per-acre leaching losses were small, total losses were predicted to be over 13,000 kg N if the entire WMA were growing continuous corn under nonirrigated conditions with 112 kg N/ha spring fertilizer applications. Under nonirrigated conditions, total nitrate-N losses under continuous corn tripled as fertilizer N rate increased from 112 to 145 kg N/ha, and doubled again when rate increased to 179 kg N/ha. Nitrate losses were similar for a corn/soybean rotation and for continuous corn with modest N rates under dryland conditions, but 40% more nitrate was lost under the corn/soybean rotation than under continuous corn under irrigation. We think this is due to lower water use and lower nitrate uptake by the soybean than by corn, even though more than twice as much fertilizer N is applied in the continuous corn system. We produced maps of predicted nitrate losses under different cropping scenarios. These color maps cannot be reproduced here, but provide an excellent means for the LPRWSD managers to visualize which fields may be contributing to ground water nitrate.

It is clear that nonpoint nitrate losses below the root zone of annual crops in the WMA may be contributing to the increasing nitrate concentrations measured in the water table. It is possible that less diffuse sources (e.g., barnyards with excessive manure deposition, leaky septic systems, surface water affected by tile drainage, etc.) are sources of nitrate, as well. This analysis does not include all possible management scenarios, and although results cannot be considered exact, they should be useful for designing cropping systems to improve and protect future ground water quality in the Holland WMA. Furthermore, this approach should be quite effective for water managers in other WMAs overlying shallow aquifers. Diversifying corn-soybean rotations with perennial forages, like alfalfa, can help protect water quality, particularly if 'leaky' soils are identified and targeted for perennial plantings.

Alfalfa Root System Architecture and Phosphorus Uptake
M.P. Russelle and J.F.S. Lamb

Introduction

Plant root systems have a genetically determined architecture that is typical of a species, but that architecture is also influenced by growing conditions. Plant root system architecture can have a large influence on the ability of a plant to obtain nutrients and water from the soil and to penetrate dense soils, and also affects the use of photosynthate, the food produced by the plant in sunshine.

Alfalfa is known as a deeply rooted perennial forage that can remove nitrate-nitrogen and other nutrients and water from deep in the soil. Plant breeding techniques have been used to develop alfalfas with different root system architectures – one being strongly tap-rooted with few fine lateral roots, the other being strongly branch-rooted with many fine lateral roots. The questions we asked with the research were: 1) Does alfalfa root architecture affect phosphorus uptake? 2) Does P availability in the soil affect the expression of root system architecture?

Methods

Two experiments, representing 5 site-year combinations, were conducted in Minnesota on either a loam or sandy loam soil. We grew a parent population (UMN2987, a composite of the best modern

varieties available in 1992) and selections for tap rootedness or branch rootedness (either first and second or only second cycles of selection). Plants were seeded in spring 1997, spaced 7.5 cm apart in a grid within each plot to allow individual plant sampling and equal rooting volume for each plant. Fertilizer P was injected after the first harvest in the 0 to 40-cm depth on a 3.8-cm grid spacing.

Forage was harvested twice in the year of establishment and 4 or 5 times annually thereafter. Roots were sampled by undercutting the plots (experiment 1) or taking 7.5-cm diam. cores directly over the plants (experiment 2) after one, two, or three cropping seasons. Forage yields, root system architecture (root mass, root length, fine vs. thick roots), and forage P uptake were measured.

Results

Bray-1 extractable soil test P levels ranged from 7.1 to 16.5 mg P/kg soil in unamended plots and were higher (16.3 to 25.8 mg P/kg) in P-amended plots, but P fertilization did not affect any measured plant parameter, except herbage P concentration and P uptake, in either experiment. There were no interactions of P rate and alfalfa entry.

Selections for root system architecture also involved plant size, as only the largest plants were kept in the selected populations. As a result, forage yields increased in both tap- and branch-rooted selections (mean 13,400 kg dry matter/ha in experiment 1, for example), compared to the parent population (mean 11,100 kg/ha in experiment 1). Fewer lateral roots generally were present in the tap-rooted selection and more generally were present in the branch-rooted selection, as compared to the parent population.

A greater proportion of the root system was present as secondarily thickened roots in the tap-rooted selection below 40 cm, but the tap-rooted selection also produced more fine-diameter roots than the branch-rooted selection. Fine root length densities ranged from about 10 cm/cm^3 in the upper 15 cm of soil to about 1 cm/cm^3 below 30 cm in 1997 in experiment 2 and from nearly 5 cm/cm^3 in the top 15 cm to 1.5 cm/cm^3 in the 45 to 60-cm depth in 1998.

Conclusions

It is revealing that simple selection for plants with large biomass within a composite of modern commercial cultivars increased herbage yield by 21%. This required selection for root and crown mass, rather than simply herbage mass, as is done in most commercial plant breeding programs, but it suggests that such a selection procedure may have substantial benefits to farmers.

Selection of alfalfa for these root system architecture characteristics was successful and was maintained in different environments. However, the two different root system architectures did not affect P uptake by the plant. In hindsight, this result is not unexpected, given the observation that most of the difference in root system architecture occurred below the 40-cm depth, and the treatments had been applied in the upper 40 cm.

Further research should be conducted on the effect of these two root system architectures on nutrient and water extraction below 40 cm. In addition, it is important to evaluate the effect of root system architecture on other poorly mobile nutrients, such as K, which can be absorbed in luxury quantities by alfalfa and other forages, to the detriment of dry cows that consume this feed.

Forage Handling, Preservation and Storage

Separation and Concentration of Soluble Protein from Plant Juice
R.G. Koegel, B.P. Lamsal, M.E. Boettcher, R.J. Straub

Introduction

Wet fractionation is the separation of fresh green herbage into a juice fraction and a high fiber fraction. In the case of alfalfa, the juice contains approximately 25% of the initial herbage dry matter and 35-40% of the herbage protein. This protein is in two forms: (1) particulate, green chloroplastic protein and (2) soluble, cream-colored cytoplasmic protein. The latter makes up approximately 40% of the juice protein or approximately 15-16% of the initial green herbage protein.This soluble protein is potentially food-grade with desirable functional properties (e.g. solubility, emulsification, foaming, etc.) In order to realize the potential high value of this protein it must be separated from the particulate fraction and concentrated without damaging functional properties.

Methods

The process of juice clarification by removal of the green particulates requires heating to 60C (142F) to aggregate particles, followed by centrifugation. This was carried out in a 15 cm (6 inch) diameter decanter centrifuge which provided 3000xG force. Since proteolysis takes place relatively rapidly, due to endogenous enzymes, it is desirable to quickly reduce juice temperature to near freezing as soon as possible and to hold it there for the remainder of processing. Protein concentration and removal of dissolved solids from the clarified juice was accomplished by ultrafiltration. A polysulfone membrane with 10,000 molecular weight cutoff was used to retain protein molecules while allowing smaller molecules and liquids to pass through the membrane. A dynamic filtration apparatus was designed and built. This apparatus has a 140 mm (5.5 inch) diameter rotor which can rotate up to 3000 rpm to create fluid shear forces for reducing retentate concentration on the membrane surface. The apparatus can be operated in two modes: (1) with the membrane attached to the surface of the rotor or (2) the membrane can be attached to the stationary surface separated by a small gap from the rotor surface. Typically the apparatus was operated with the outlets adjusted to give a permeate:retentate ratio of 4:1 giving a theoretical protein concentration of 5:1. A two level, three factorial experiment was conducted to determine the relative importance on flux (flow rate) of (1) rotor speed, (2) transmembrane pressure, and (3) gap between stationary and rotating surfaces. The two levels of the three variables used were: speed 1500,3000 rpm; pressure 25,50 psi, and gap 2,5mm.

Results

For the levels of variables chosen, the most important variable affecting flux was rotational speed, followed closely by transmembrane pressure. Contrary to expectations, gap between stationary and rotating surfaces had insignificant effect for the two levels of variable chosen. Since both rotational speed and gap contribute to shear rate which help to reduce buildup of retained materials on the

membrane, its lack of effect is surprising. Flux tends to decrease slowly with time. Typical flux values for clarified alfalfa juice generally ranged from 35-50 liters/sq. m/hr after two hours while initial flux with distilled water, by contrast, was 1100-1200 liters/sq. m/hr for relatively new membranes.

Conclusions

The flux observed for clarified alfalfa juice was consistent with that reported by other workers and is also consistent with that of similar proteinacious materials such as whey. The importance of the gap variable needs to be evaluated for values outside the 2-5mm range. At a flux of 40 liters/sq. m/hr, it would require about 32 membranes of 1m diameter in parallel to achieve a flow through the membranes of one cubic meter or 1000 liters per hour. At an initial protein concentration of 1.5-2% in the clarified juice, this would result in 15-20 kg (33-44lb) of protein being concentrated to about 7.5-10%.

Production of Hydrolytic Enzymes by Solid Substrate Cultivation

R. G. Koegel, H.K. Sreenath, M.E. Boettcher, and R.J. Straub

Introduction

The fermentation of plant fiber (ligno-cellulosics) to ethanol, lactic acid, or other chemicals is a two step process. The first step is enzymatic depolymerization of the fiber to fermentable sugars which can then be fermented, as the second step, by an appropriate microorganism to the selected product(s). The two steps may be carried out concurrently in what is referred to as simultaneous saccharification and fermentation (SSF). While inexpensive raw materials, such as crop residues, can be converted to relatively valuable products in this way, the current high cost of commercially available enzymes makes the economics of these conversions dubious. For example, the US Department of Energy has estimated that the cost of enzymes needed for producing one gallon of ethanol from lignocellulosics to be around $0.60. Enzymes are typically produced commercially by growing organisms in liquid culture in large aerated vats. The resulting enzymes must then be extracted from the dilute nutrient broth, concentrated, dried, and packaged for shipment to the point of use. It has been proposed that enzymes could be produced much more cheaply by cultivation of the microorganisms on solid substrate. One proposed scheme is to inoculate a small fraction of the fermentation substrate with aerobic fungi, known to be good enzyme producers under appropriate conditions. After the fungus had adequate time to multiply and to produce abundant enzymes, this fraction of the substrate would be mixed with the main portion as a source of the needed enzymes. No separation, concentration, drying, packaging, or shipping of the enzymes would take place. The total substrate would then be inoculated with the desired fermentation organism and conditions maintained to maximize fermentation while terminating growth of the enzyme-producing fungus. An analagous system has traditionally been used in Asia in the production of fermented rice products. Biopulping of wood to reduce the energy required for comminution is a related process. Difficulties which have been identified in scaleup of solid state cultivation of fungal enzymes to industrial scale include uniform aeration throughout the substrate mass and uniform removal of excess heat generated by the metabolism of the organisms. Pulsed pressure aeration has been advocated to overcome both problems.

Methods

A 20 liter pulsed aeration bioreactor was designed and built to address the problems of gas and heat transfer throughout the substrate. Pressure typically cycled between 0 and 15 psi gage with around 6 cycles per minute. Incoming air was bubbled through a heated water bath to achieve the desired temperature and humidity. Sterilized substrate was inoculated with the selected organism. The substrate was typically sampled and assayed at 24 hour intervals for enzyme activity. This was accomplished by washing the sample substrate and measuring enzyme activity in the filtered wash water. The two most frequently used organisms were *Aspergillus niger* and *Trichoderma reesei* RUT C-30, a mutant strain developed for cellulase production. The most frequently used substrate was alfalfa fiber resulting from wet fractionation. However, spent cellulose sausage casings were also used after shredding. In addition to the almost pure cellulose of the casing material, these also contained meat juices. Certain other trace nutrients were added to the substrates in various trials of fungal cultivation.

Results

Initially heat was added to the bioreactor by the inflow of air from the heated water bath. As the organisms multiplied, their metabolic activity added heat causing the substrate temperature to increase. Whenever either the substrate temperature or the water temperature exceeded the target temperature, the waterbath heater was automatically turned off until temperatures fell below the target. The substrate temperature was thus maintained between narrow limits while also maintaining moisture in the substrate. *Aspergillus niger* grew well on alfalfa fiber and other agricultural residues, appeared to out-compete any contaminants, and yielded high xylanase activity (in the range of 500-1000IU/g substrate dry matter). Results from *Trichoderma reesei* RUT C-30 were inconsistent. Adequate cellulase activity was sometimes achieved. However, on a number of occasions, either cellulase activity was low and/or the *Tricoderma* appeared to be inhibited by competing organisms. When growing well, however, it effectively hydrolyzed the cellulose sausage casings.

Conclusions

Solid substrate cultivation of organisms to produce enzymes for the saccharification of lignocellulosic feedstocks appears to have potential for reducing enzyme costs. The degree of success, to date, appears to depend on the robustness of the organism chosen. Therefore, additional work is required to identify the most appropriate organisms along with environmental and nutritional conditions which will allow the organisms to out-compete invading organisms while producing enzymes abundantly. Pulsed aeration showed potential for dealing with the dual problems of heat build-up and inadequate oxygenation as operations are scaled up.

Medium Density Fiberboard from Alfalfa Fiber

R.G. Koegel, R.J. Straub, and M.E. Boettcher

Introduction

A number of non-traditional products can be made from forage crops, like alfalfa, by means of wet fractionation. Wet fractionation consists of dividing herbage into juice and fiber fractions. Products from the juice fraction (about 25% of the initial dry matter) include feed-grade and food-grade protein concentrates, carotenoids, chlorophyll, and enzymes. Products from the fiber fraction (about 75% of the initial dry matter) include chemicals such as ethanol and lactic acid produced by fermentation, biofilters, fuels by means of gasification or direct combustion, and structural products such as fiberboard. Currently most fiberboard is made from wood fiber which is a byproduct of wood milling. It depends on synthetic, petroleum-based adhesives for its strength and integrity. This adhesive is also the major expense in fiberboard production. While it is doubtful that use of agro-based fibers could significantly reduce the cost of fiberboard, any reduction in the requirement of synthetic adhesive could make a contribution to decreased cost.

Methods

Fiberboard was made from alfalfa fiber obtained either from wet fractionation of alfalfa herbage or by washing feces from dairy cattle fed on a ration high in alfalfa (>90%). The adhesive used was alfalfa juice from wet fractionation. The mixture of fiber and juice was placed between 6 inch x 6 inch platens in a hydraulic press where pressures ranging from 200-400psi were applied while heating each platen with two 600 watt heaters from 350-450 deg.F. It was postulated that the proteins and carbohydrates in the juice in the presence of heat and moisture would undergo the Maillard reaction to form an insoluble complex which would act as an adhesive. The ratio of juice weight: fiber weight was in the range 0.5-1.0. Softening and flowing of the lignin in the fiber above 300 deg.F was thought to act as a second adhesive. The temperature was raised to the target value, held for approximately five minutes, heaters were turned off, and the temperatures were allowed to return to ambient. Samples were soaked in water for 24 hours and % increase in weight noted. Resulting materials were cut into rectangles and broken, in a testing machine, in beam bending to determine strength and rigidity.

Results

Initial results indicated that fiber washed from feces made board which was stronger and more stable than that made from fresh fiber. Removal of the more easily digested fiber components by the bovine digestive system appeared to reduce shrinking and swelling which lead to delamination. Therefore only manure-derived fiber was used in subsequent trials. Rifts or tears due to steam formation between the platens was a problem in materials with higher levels of juice. This was overcome by predrying the material to moisture contents below 20%. In materials with no juice added, strength was significantly lower and moisture absorbtion significantly higher, indicating that the “cementing” due to the thermal softening of lignin played a secondary role. Densities of the board generally exceeded 50 lb/cu ft which ranked it as “high density”. The most stable boards (400 psi,450 deg.F, fiber:juice=1.0:0.67) increased in weight by 8-13% during 24 hours of soaking. The strongest board had a modulus of rupture ranging from 21-26 Mpa (3100-3800psi) just sufficient to meet the requirements of “medium density” while the strength of most boards ranked as “low density” or

below. The modulus of elasticity of the most stable boards exceeded the requirement for “medium density” board (2400 Mpa) and frequently exceeded requirements for “high density” board. According to US Patent 5,371,194, the integrity of board with this type of adhesive can be improved by ammoniation to alkalinity prior to pressing. This effect could not be detected, however.

Conclusions

Alfalfa fiber, washed from bovine feces, and mixed with alfalfa juice at a ratio of around 1: 0.67, made medium density fiberboard of good integrity when pressed at 450 deg.F and 400 psi. The modulus of rupture of this board was marginal relative to the ANSI requirement, however. Modest addition of synthetic adhesives should be tried to ascertain if strength could be augmented to meet or exceed ANSI standards at nominal cost.

Ohmic Heater for Treatment of Plant Juice
R.G. Koegel, R.J. Straub, M.E. Boettcher

Introduction

Rapid, uniform heating of plant juice is an important step in the removal of particulate (chloroplastic) protein. Heating with conventional heat exchangers is problematic due to localized overheating and to fouling of heat exchanger surfaces. Direct steam injection into the juice has been used to overcome these problems. Localized, instantaneous overheating can still result and the steam concentrate dilutes the juice. Since the juice has high electric conductivity, it is possible to heat it by direct passage of electric current through the juice. It has been claimed by workers in the former Soviet Union that the passage of alternating current through the juice also ruptures any intact chloroplast membranes freeing soluble protein held within the membrane.

Methods

A batch type heater was designed and built which used three-phase, 208 volt or 480 volt alternating current (Fig. 1). The electricity was conducted to and from the juice via three graphite paddles equally spaced on a vertical shaft rotor. Slip rings provided a path from the electrical source to the rotor. The rotor was driven by a three-phase electric gear motor equipped with a variable frequency speed control to allow varying the rotational speed. It was typically run at 30-50 rpm. Rotation served dual purposes of stirring the juice to keep the temperature uniform throughout while avoiding any buildup on the paddles. The capacity of the juice container was 55 gallons (~200 liters) The three paddles each had an area of 36 sq. inches (~230 sq. cm) and were at a radius of 8 inches (20cm) from the rotor axis.

Results

As observed in earlier research, juice conductivity, and thus current flow, increase as temperature increases. Juice was generally heated from around 65F (18C) to 131F (55C). Batch size did not generally exceed 20 gallons (77 liters). Initially the heater was run on 480 volts which led to a current flow exceeding 50 amps for a calculated power of around 42 kw. This resulted in the juice

temperature being raised approximately 65F (36C) in about four minutes. Because the current, at 480 volts, tended to exceed the 50 amp rating of the circuit used, the heater was subsequently run on 208 volts. At this voltage the current was about 20 amps and the heating time around 15 minutes. No fouling of the graphite electrodes was observed over more than a month of use. At the higher current flow of 50 amps, current density on the electrodes was about 0.2 amps/ sq. cm. No attempt was made to verify whether soluble protein was augmented by rupture of chloroplast membranes.

Conclusions

The ohmic heating apparatus functioned as intended. Lack of fouling at the electrodes indicates absence of localized overheating. Reducing the time that juice is at elevated temperatures is desirable to minimize proteolysis caused by endogenous enzymes. Therefore, running the heater at 480 volts would be an advantage. This should reduce heating time to roughly 20% of that for 208 volts. Evaluation of whether the alternating current electrical treatment increases availability of soluble protein by rupturing intact chloroplast membranes should be carried out.

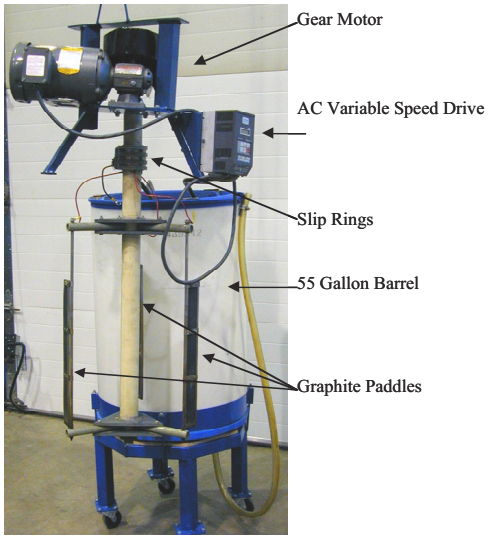


Fig. 1: Electrical Juice Heater

Inoculant Effects on Aerobic Stability of Corn Silage
R.E. Muck

Introduction

Inoculants are the most common additives used in making silage in the U.S. These products provide selected lactic acid bacteria to supplement the natural population of lactic acid bacteria on the crop and ensure a rapid and efficient silage fermentation. While these products have provided improvements in dry matter recovery and animal performance, aerobic stability (the time until the silage begins to heat during feed out) has sometimes been made worse by inoculants, particularly in corn and other whole-crop grain silages. Inoculant manufacturers are aware of this problem and have been working on developing inoculants that more consistently improve aerobic stability. The objective of this study was to test several new types of inoculants and compare their effects on aerobic stability with current products.

Methods

Whole-crop corn was harvested with a forage harvester in each of three years. The chopped corn was ensiled in 60 x 10 cm dia. PVC silos sealed with a rubber end cap on one end and with black plastic secured with duct tape on the other. The number of treatments varied from year to year depending on product availability, but most of the products were tested all three years. Treatments included an uninoculated control, three standard corn silage inoculants available in the market, a new product with improved homofermentative strains, several new products with the heterofermentative species *Lactobacillus buchneri*, and a prototype product (1 year only) with a standard inoculant plus a chemical spoilage inhibitor. All products were applied at recommended rates but were diluted with water such that each treatment was applied at 1 g/50 g crop. The control received 1 g water/50 g crop. The silos were opened after a minimum of 90 days ensiling. Silos were weighed prior to emptying. The spoiled silage on the top was removed and weighed. The rest of the silage was removed, mixed and analyzed for microbial groups, pH, fermentation products and moisture content. The remainder was placed in Styrofoam buckets, and silage temperatures were recorded hourly until heating occurred.

Results and Discussion

The pHs and aerobic stabilities of the silages in all three years are summarized in Table 1. The standard inoculants and the improved standard inoculant had no effect on silage pH relative to the control in any year; all had excellent pHs of 3.62 to 3.90. The *L. buchneri* inoculants raised pHs significantly from the control, typically 0.1 to 0.3 units. The cause of the higher pH was a shift in fermentation – increased acetic acid and ethanol concentrations, reduced lactic acid concentrations.

The *L. buchneri* inoculants produced the most consistent improvements in aerobic stability across the three years. Only in year 2 with *L. buchneri* 3 was the improvement in aerobic stability not statistically significant ($p < 0.05$). The standard inoculant plus chemical spoilage inhibitor was tested in only one year but provided a 3-day improvement in aerobic stability relative to the uninoculated control. The standard inoculants generally had trends toward reduced aerobic stability, as expected, although there was only one significant effect (a reduction in stability in year 3 by Standard 1). The improved standard inoculant was similar to the standard inoculants in the first two

years but provided significantly greater stability than the control in year 3. Aerobic stability across the treatments was negatively correlated with yeast counts as indicated in Fig. 1. No other factors were well correlated with aerobic stability. These results suggest that the primary means of improving aerobic stability in the effective products were by lowering yeast populations, the frequent initiators of heating in corn silage.

The standard inoculants and the improved standard inoculant generally had the lowest dry matter losses although trends were not statistically significant. The *L. buchneri* products typically had dry matter losses numerically between the standard inoculants and the control. Experience in these trials and earlier ones with *L. buchneri* suggest that dry matter recoveries are approximately one percentage point less than those with standard inoculants because of the shift to more heterofermentative products with *L. buchneri*.

Overall, the *L. buchneri* inoculants were more consistent than the improved standard inoculant in enhancing aerobic stability. This came with a small cost to dry matter recovery. While these trials addressed fermentation and aerobic stability, the principal return to the farmer from using inoculants has been in improved animal performance. Animal research trials with the *L. buchneri* products are beginning to be published. These have found similar improvements in aerobic stability but have yet to show improved animal performance compared to that from uninoculated silage.

Conclusions

Of the new corn silage inoculants available to farmers, the *L. buchneri* inoculants provided the most consistent improvement in aerobic stability. An improved standard inoculant was better than standard inoculants in one year of three relative to aerobic stability. At this stage of testing, selection of a corn silage inoculant appears to hinge on the most important goal(s) of the farmer. If poor aerobic stability in corn silage and its effect on animal performance are consistent problems that have not been solved by improved silo management, then the *L. buchneri* products show the most promise. However, if the primary goals are improved animal performance and dry matter recovery, then the improved standard and conventional homofermentative inoculants are more likely to achieve success.

Table 1. Characteristics of the silages.

		pH		Aerobic Stability Relative to Control, h		
Inoculant	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
None (Control)	3.82	3.87	3.64	0	0	0
Standard 1	3.85	3.89	3.71	16	-13	-40
Standard 2	3.84	3.90	3.65	-4	-20	-6
Standard 3	3.83	3.90	3.62	-25	-6	-10
Improved Standard	3.81	3.90	3.64	-24	-27	29
Standard + Inhibitor	3.83	—	—	76	—	—
L. buchneri 1	4.01	4.11	4.01	142	100	811
L. buchneri 2/3*	3.90	4.06	3.84	103	22	454

* *L. buchneri* 2 in year 1; *L. buchneri* 3 in subsequent years.

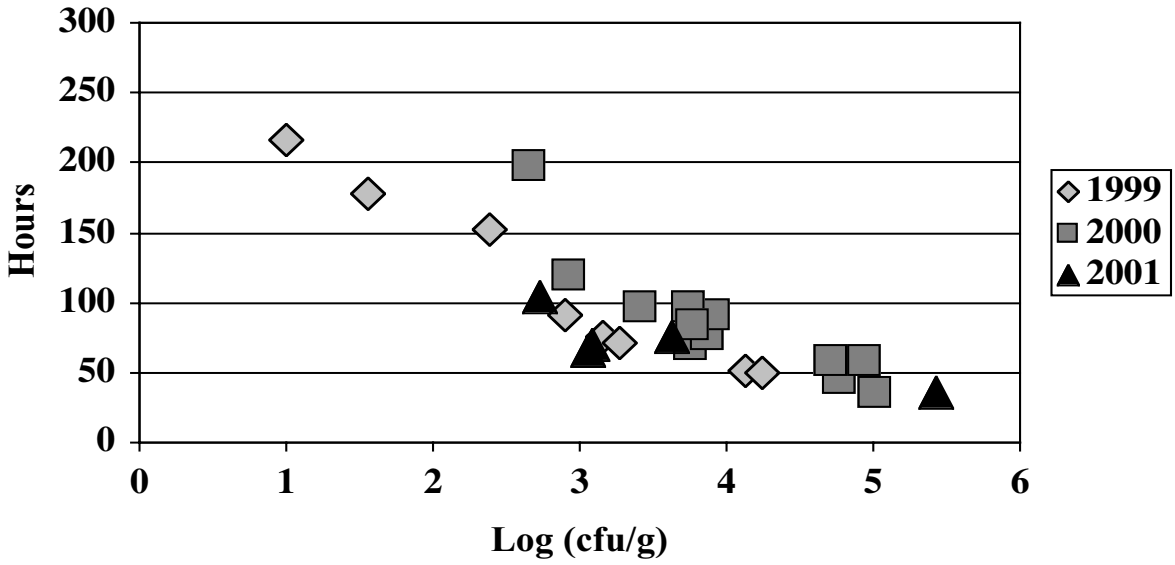


Fig. 1. Aerobic stability of the treated silages as correlated with yeast counts in the silages at opening. A yeast count of 1 log(colony-forming units/g crop) indicates that all four silages in the treatment were below detectable level (100 cfu/g crop).

Density and Losses in Pressed Bag Silos
R.E. Muck and B.J. Holmes

Introduction

The pressed bag silo is an increasingly popular method of making silage. It is relatively inexpensive. Storage size varies with the quantity of forage harvested. For farms that are expanding in herd size, silo capacity can be added with little capital cost. Small diameter bags allow small farms to consider making silage rather than hay. Finally, bag silos make it easy for farmers to inventory and manage silage, e.g., reserving high quality silage for the best animals.

Unfortunately there are limited data on the densities and losses from bag silos. This makes accurate economic assessment of bag silos difficult for farmers considering using them. It also hampers farmers with bag silos in assessing their silage inventory.

Methods

We monitored the filling and emptying of bags at our research farm at Prairie du Sac and two University of Wisconsin Agricultural Research Station farms (Arlington, West Madison) over the 2000 harvest season. Most of the silages were alfalfa or whole-plant corn. All loads of forage entering the bags were weighed and a sample taken for analysis. After each load was pressed into the bag, the side of the bag was marked to indicate the distance filled by the load. Each load sample was

analyzed for moisture content. The remainders of samples were composited by field and date and analyzed for particle size distribution, crude protein, neutral detergent fiber, moisture and ash.

At emptying, the weight of all silage removed from a bag was recorded. Any spoiled silage not fed was weighed and specifically identified as such on the emptying log. A grab sample from the face of each silo was taken periodically, one per filling load. Samples from emptying were analyzed for pH and fermentation products in addition to those performed on the load samples. However, the samples from emptying were not analyzed for particle size distribution. Densities for the bags were calculated based on the weight ensiled, length and nominal bag diameter.

Results and Discussion

Over the course of the 2000 harvest season, a total of 25 bag silos were made at the three farms. The average dry matter densities of all the bags are shown in Figures 1 and 2. Dry matter (DM) density increased the drier the crop at ensiling, 2.9 and 5.3 kg/m^3-% DM for alfalfa and corn silages respectively. Estimating densities at a constant DM content (40%), DM densities in alfalfa silages were approximately 200 kg/m^3. Densities in corn silage were 3 to 8% lower than those in alfalfa with one bagging machine whereas densities were 16 to 35% higher in the other bagging machine. One bagging machine was shared between two farms, and one farm consistently achieved a higher density (approximately 10%) than the other, indicating the operator affects density.

So far 15 bags have been completely emptied and results analyzed. Average DM losses were 8.4% gaseous/seepage loss (i.e., weight loss out *vs.* in) and 5.8% spoilage loss (i.e., silage removed from the bag but not fed) for a total of 14.2% loss. The average spoilage and total losses were inflated by three bags with substantial spoilage (26 to 38% total loss). One of those bags sustained major bird damage on the top that was not noticed immediately and repaired. In contrast, eight bags had no spoilage loss or very minor spoilage at the ends. Removing the three bad bags from the average reduced average total losses to 9.7%. Gaseous losses increased with low feed out rates (<30 cm/day) whereas spoilage losses were associated with drier (>40% DM), more porous silages. Overall, these results suggest that DM losses similar to those in tower silos are achievable with good silo bag management.

Conclusions

Densities in bag silos were affected by crop DM content, crop type, bagging machine and how the operator set up the bagging machine. At 40% DM, DM densities in alfalfa averaged 200 kg/m^3. Densities were higher in corn silage with one machine and lower in the other. DM losses in bag silos can be similar to those in tower silos (i.e., less than 10%), but large losses (>25%) can occur if management is less than ideal. Losses will be minimized by ensiling between 30 and 40% DM, routinely monitoring for and patching holes, and feeding out at least 30 cm/day from the face.

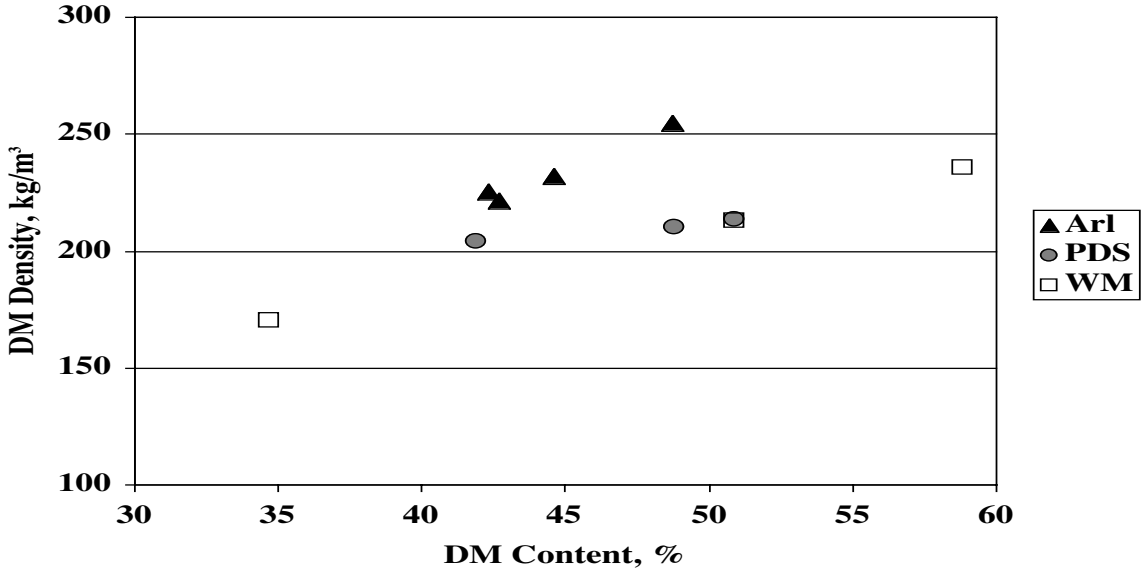


Fig. 1. Average dry matter densities of hay crop silages made in bag silos at different dry matter contents and farms (Arl – Arlington, PDS – Prairie du Sac, WM – West Madison).

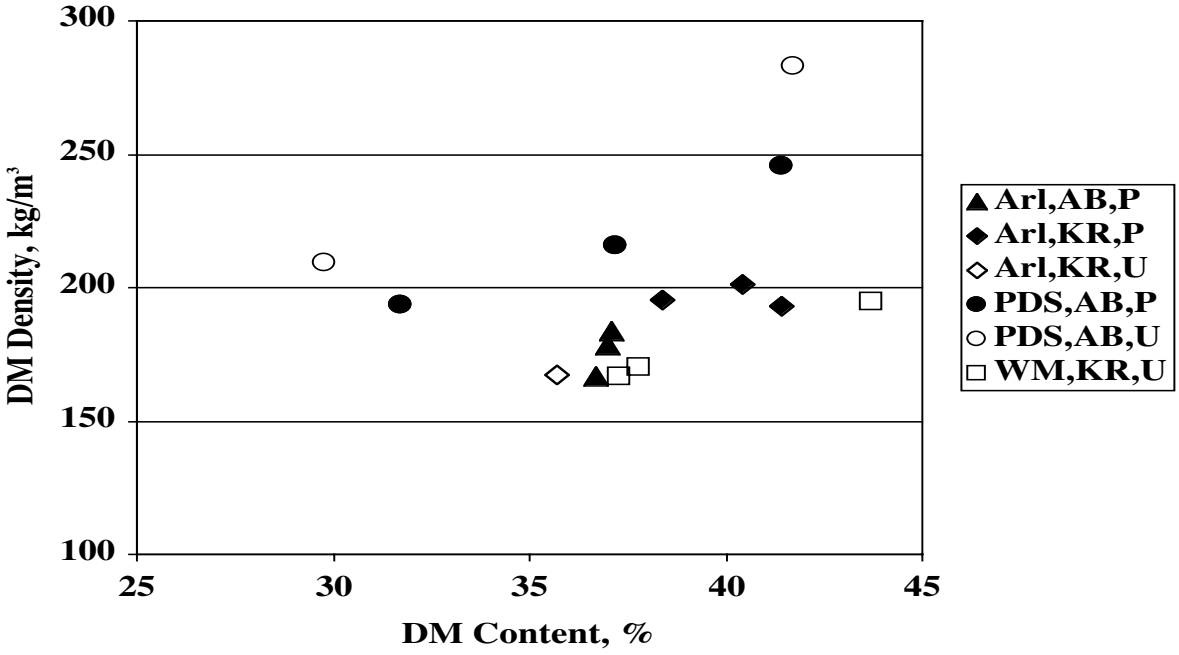


Fig. 2. Average dry matter densities of corn silages made in bag silos at different dry matter contents, farms (Arl – Arlington, PDS – Prairie du Sac, WM – West Madison), machines (AB – Ag Bag, KR – Kelly-Ryan) and kernel processing (P – processed, U – unprocessed).

Model Studies of Ferulate-Coniferyl Alcohol Cross Products Formed in Primary Maize Walls

J.H. Grabber, J. Ralph and R.D. Hatfield

Introduction

During cell-wall biosynthesis in grasses, feruloylated xylans become extensively cross-linked by coupling of ferulate into diferulates (Fig. 1) and by copolymerization ferulate and diferulate xylan esters with monolignols to form xylan-lignin complexes. As demonstrated in earlier studies, nonlignified primary walls isolated from maize cell suspensions are a valuable model system for studying the dimerization of ferulates and their subsequent incorporation into lignin. In the current study, this system was used to characterize the types of cross-products formed between ferulates and coniferyl alcohol (**3**). Coniferyl alcohol, with or without *p*-coumaryl and sinapyl alcohols, is the most abundant and consistently observed monolignol secreted into cell walls at the onset of lignification.

Methods

Cell walls from maize cell suspensions were treated with dilute hydrogen peroxide to dimerize ferulate via wall bound peroxidase. These walls, containing 52 mmol g^{-1} of ferulate monomers and 33 mmol g^{-1} of diferulates, were then partially lignified with 110 mmol g^{-1} of coniferyl alcohol for subsequent identification of ferulate- and diferulate-lignin cross-products in alkaline hydrolysates by GC-MS and NMR.

Results and Discussion

Alkaline hydrolysis and GC-FID analysis revealed that about 50% of the total ferulate in walls (11.8 mg g^{-1}, 47 mmol) copolymerized with about 19 mg g^{-1} (105 mmol) of coniferyl alcohol. As noted previously, ferulate and 5–5-coupled diferulate had the greatest propensity to copolymerize with coniferyl alcohol, accounting for 82% of the cross-coupled structures formed. GC-MS analysis revealed that ferulate monomers were coupled to coniferyl alcohol by 8-β', 5-β', 4-*O*-β' linkages (Fig. 2 and 3). Coupling of ferulate to the β'-position of coniferyl alcohol suggests that ferulate can act as a nucleation site where lignification begins. The observed 8-β' product **18** was probably present in the walls as **15** with structure **18** being formed during saponification and acidification. Based on GC analysis, 4-*O*-β', 8-β', and 5-β' structures comprised about 52, 35, and 13% of the dimeric cross-products recovered from cell walls. If these proportions are representative of grass cell walls, then substantial amounts ferulate-mediated cross-links between xylans and lignin could be lost via de-esterification if **18** was the major product of 8-β' coupling. As a result, oxidative coupling of ferulate into 8–8-, 8–5-, and 8–*O*–4-coupled diferulates takes on added importance; these dimers not only mediate cross-linking among xylans and between xylans and lignin, their lack of 8-β'-coupling with monolignols precludes the loss of cross-links via this pathway. In addition to 4-*O*-β', 8-β', and

5-β' cross-products, minor correlations of 8–*O*–4'- and 8–5'-coupled cross-products were detected by HMBC-NMR (Fig. 4), indicating that some ferulate or 5–5-coupled diferulate coupled with dimers or oligomers of coniferyl alcohol. Coupling of ferulate with lignin oligomers suggests that ferulates do not act solely as nucleation sites for lignin formation.

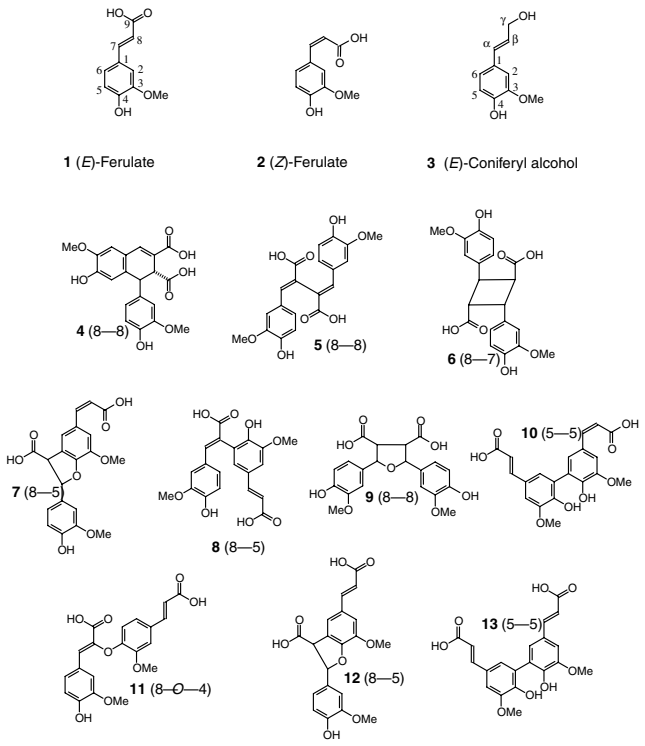


Fig. 1. Ferulates (**1**, **2**) and diferulates (**4**-**13**) released from primary walls of maize by alkaline hydrolysis. Ferulate xylan esters and coniferyl alcohol (**3**) may undergo oxidative coupling at the 8- or β-, 5- and 4–*O*-positions. Diferulate esters may undergo oxidative coupling with monolignols at their 5- and 4–*O*-positions; only 5–5-coupled diferulate has the potential to couple with monolignols at its 8-position.

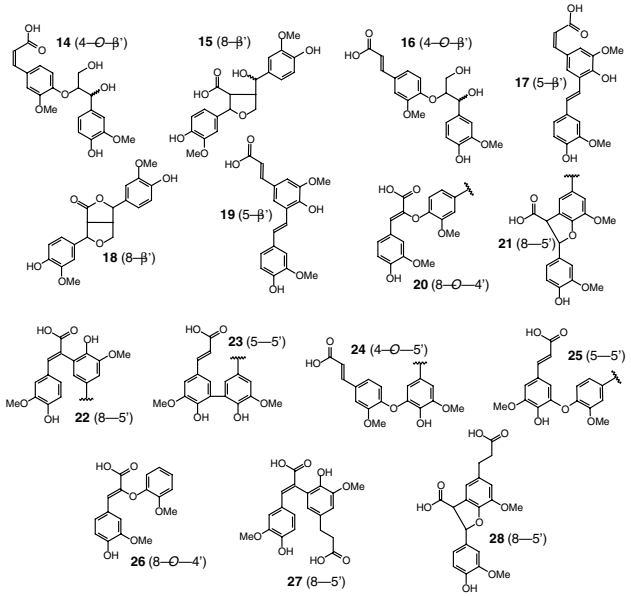


Fig. 2. Potential cross-products between ferulate and coniferyl alcohol released by saponification of cell walls (**14**-**25**). Diferulates can form similar types of cross-products with coniferyl alcohol. Several cross-product models (**26**-**28**) were prepared for NMR studies.

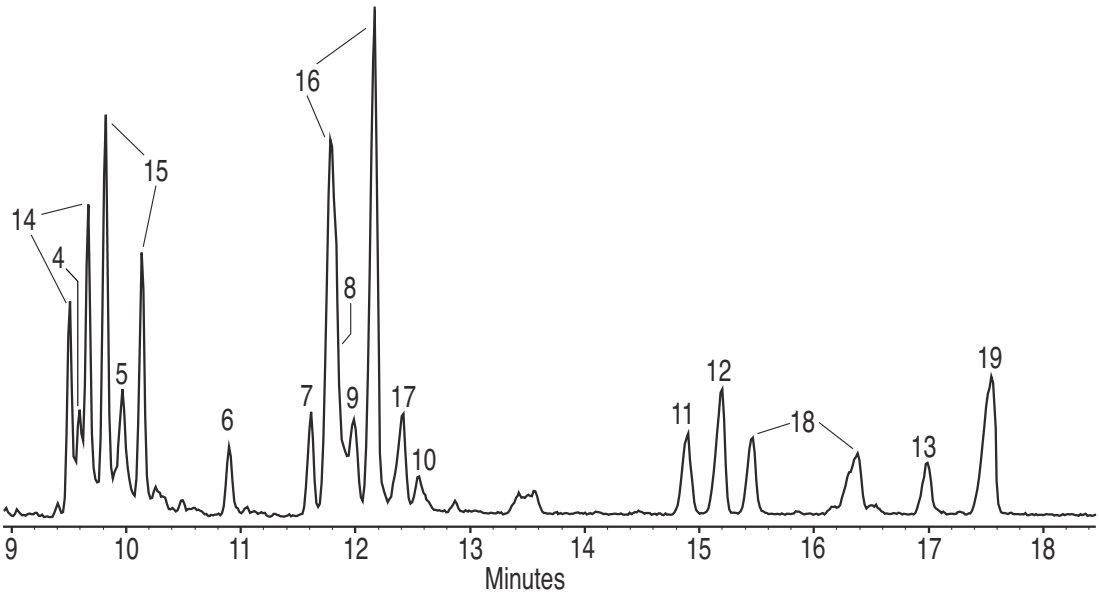


Fig. 3. GC-MS total ion chromatogram of diferulates and ferulate-coniferyl alcohol dimers recovered following room-temperature alkaline hydrolysis of partially-lignified primary walls from maize suspension cultures.

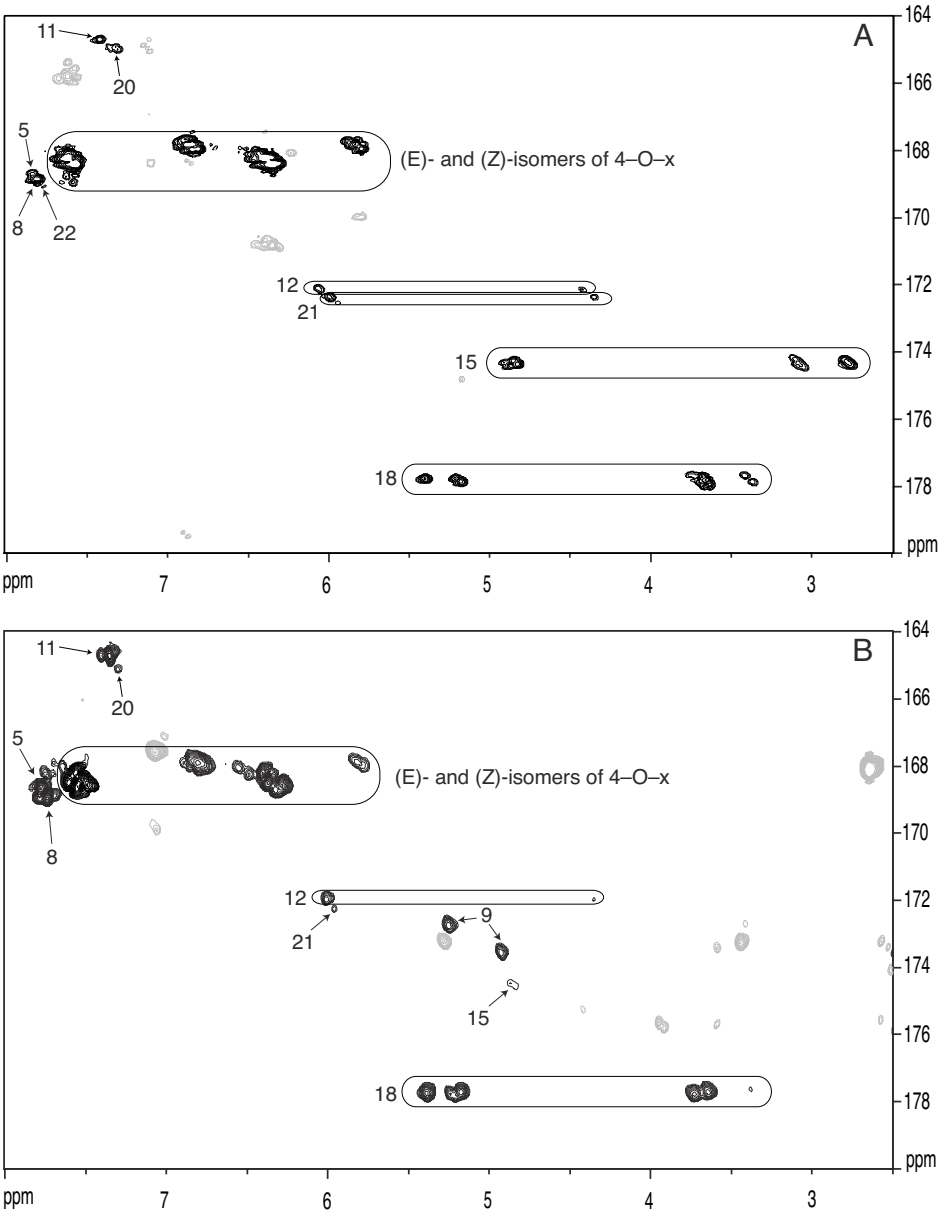


Fig. 4. Long-range C-H correlation (HMBC) spectra of ferulate, diferulates, and their cross-products recovered following room-temperature alkaline hydrolysis of partially-lignified primary walls from maize suspension cultures. (A) Fraction 1 recovered by EtOAc extraction (B) Fraction 2 recovered from the $MgSO_4$ used to dehydrate the EtOAc extract.

Cross-Products Between Ferulate and Coniferyl Alcohol Act as Nucleation Sites for Lignin Formation in Primary Maize Walls

J.H. Grabber, J. Ralph and R.D. Hatfield

Introduction

Ferulate-xylan esters may act as initiation or nucleation sites for lignin formation in grasses—the site at which lignification begins in cell walls. Our group obtained the first evidence for this in HMBC-NMR studies of ^{13}C-labeled ryegrass lignin; initial coupling reactions exclusively involved ferulate coupled to the β-position of monolignols. The milled "wood" lignin isolated from ryegrass was likely derived from secondary cell walls, whereas lignification in grasses starts in the middle lamella and primary cell wall. Consequently, we know little about the role of ferulates as nucleation sites where lignification begins in cell walls.

Methods

A dilute H_2O_2 solution was added to primary walls isolated from maize cell suspension to stimulate oxidative coupling of ferulate into diferulates by wall bound peroxidase. Walls were then slowly lignified *in situ* by adding solutions of coniferyl alcohol and H_2O_2. Samples were analyzed for Klason lignin and for alkali-labile ferulates and diferulates by GC-FID.

Results and Discussion

Primary maize walls were lignified with varying levels of coniferyl alcohol to study the incorporation of ferulate and diferulate isomers into lignin and the growth of ferulate/diferulate-lignin complexes. For our purposes, the ferulate and diferulate isomers were combined into two functional groups—ferulate/5–5-coupled diferulate and 8-coupled diferulates—based on their kinetics of incorporation into lignin. Coniferyl alcohol was efficiently polymerized into cell walls, forming cross-coupled structures with up to 95% of the ferulates and diferulates in cell walls (Fig. 1a). Ferulate and diferulates incorporated as at least two pools with the larger pool incorporating earlier and more rapidly than the smaller pool. Incorporation of the larger pool was 3.5-fold faster for ferulate/5–5-coupled diferulate than for 8-coupled diferulates, confirming previous observations that initial cross-coupling reactions with monolignols overwhelmingly involve ferulate and 5–5-coupled diferulate. During lignification, the size of complexes increased linearly even as the incorporation of total ferulates per unit coniferyl alcohol declined dramatically (Fig1b). As illustrated in Figure 2, this suggests that cross-products between coniferyl alcohol and ferulate/5–5-coupled diferulate, formed at the onset of lignification, act as preferred sites for continued lignin polymerization. Preferential growth of lignin at these sites occurred although more numerous non-incorporated ferulates and diferulates were available for cross-coupling with coniferyl alcohol. Our group is currently conducting additional model studies with maize walls and investigating means of isolating additional lignin from the ^{13}C-labelled ryegrass, in order to more definitively establish whether ferulates act as nucleation sites for lignin formation in grasses.

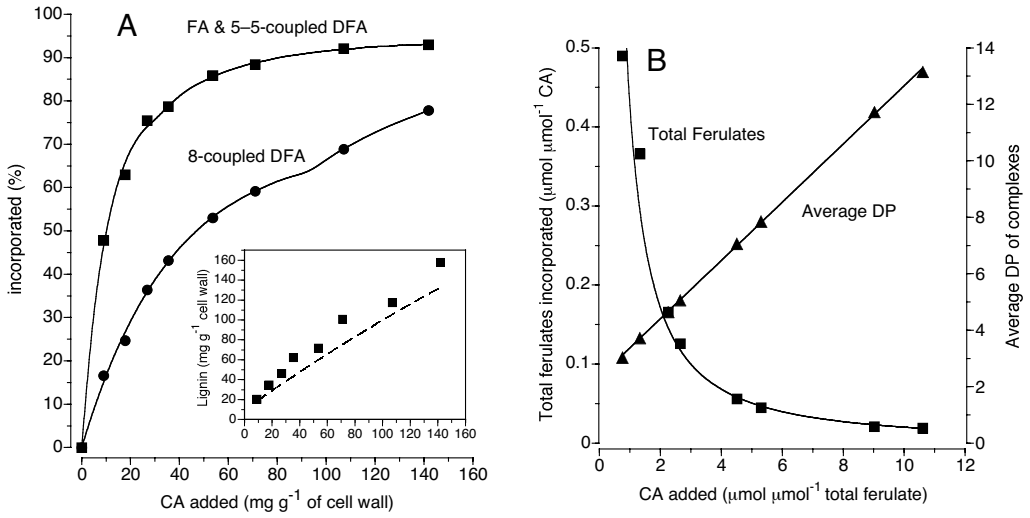


Fig. 1. (A) Incorporation of Ferulate (FA) and 5–5-coupled diferulate (DFA) and of 8-coupled DFA during lignification of primary maize walls with coniferyl alcohol (CA). The insert shows the Klason lignin content of walls compared to the predicted lignin content of walls with complete incorporation of CA (dashed line). (B) Comparison of the incorporation of total ferulates (ferulate monomers plus dimers) with the apparent degree of polymerization (DP) of complexes formed between total ferulates and CA.

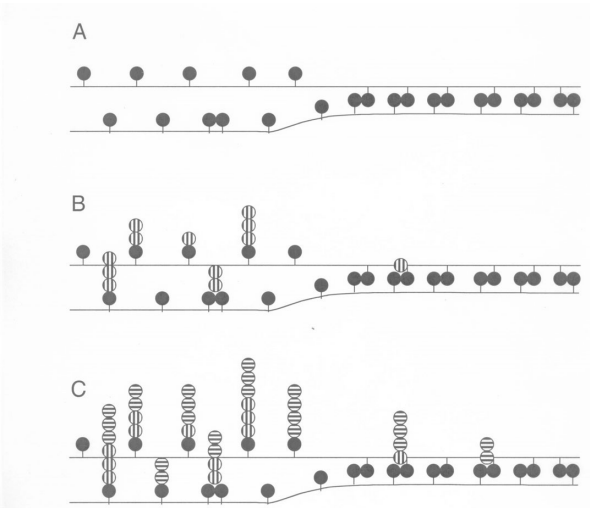


Figure 2. A schematic drawing illustrating the role of cross-products between ferulate/5-5-coupled diferulate and coniferyl alcohol as nucleation sites for continued lignin formation. (A) Non lignified walls with xylans substituted with ferulate and cross-linked with diferulates (solid circles). (B) At the onset of lignification, coniferyl alcohol (circles with vertical stripes) couples mainly with ferulate monomers, 5-5-coupled diferulate, and their cross-products. (C) As lignification continues, additional coniferyl alcohol (circles with horizontal stripes) preferentially couples with existing cross-products, even though unincorporated ferulate monomers, 5-5-coupled diferulate, and particularly 8-coupled diferulates predominate as potential coupling sites.

Relationship of Growth Cessation with the Formation of Diferulate Cross-Links and *p*-Coumaroylated Lignins in Tall Fescue Leaf Blades

J.W. MacAdam and J.H. Grabber

Introduction

The discovery of 5–5-coupled diferulic acid linkages between adjacent feruloylated xylan chains led to the hypothesis that such bonds control cell wall extensibility and tissue growth in grasses. Numerous studies have demonstrated a correlation between an increase in the content of 5–5-coupled diferulic acid in cell walls of coleoptiles and a decrease in growth and cell wall extensibility. The 5–5-coupled dimer, however, represents only a small portion of the total diferulates in grass walls. In addition, 5–5-coupled diferulate (unlike 8-coupled diferulates) is probably formed by intramolecular dimerization of ferulate polysaccharide esters. Therefore, most if not all cross-linking mediated by ferulic acid involves 8–*O*–4, 8–8-, and 8–5-coupled diferulates, yet relationships between 8-coupled diferulates and cell wall extension have been ignored, even in recent studies with cereal coleoptiles. Lignification of primary walls has also been associated with reduced extensibility and growth of coleoptiles. Although very small quantities of *p*-coumarate are esterified to arabinoxylans, most *p*-coumarate deposition occurs in tandem with lignification, making *p*-coumarate accumulation a convenient indicator of lignification. In previous work, we observed that the elongation rate of tall fescue leaf blades increased exponentially and then stopped abruptly, implying a rapid change in cell wall mechanical properties. Therefore, the main objective of this study was to investigate relationships between the cell-wall deposition of ferulate monomers, all major diferulates, and *p*-coumarate with changes in the segmental elongation rate of tall fescue leaf blades.

Materials and Methods

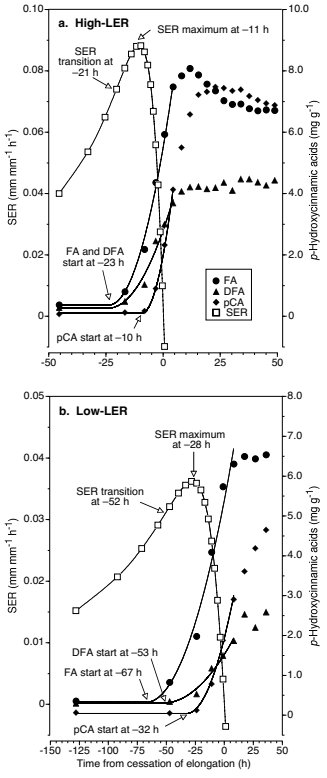
Vegetatively propagated tillers of tall fescue with high and low leaf elongation rates (LER) were grown in a controlled environment chamber. Prior to sampling, mean daily elongation rates were calculated by measuring the length of nine leaves over a 5-d period following leaf emergence. At the time of destructive sampling, epidermal cell lengths were measured from Formvar replicas prepared from the abaxial surface of leaf blades. Segmental elongation rates (SER) were calculated from leaf elongation rates and cell length profiles. The time of maximal SER was estimated by solving a quadratic equation fit through SER data points. The time at which SER transitioned from an increasing rate of increase to a declining rate of increase was estimated by solving a quadratic equation fit through the first derivative of SER points. The first derivative of each point of the SER curve was calculated by fitting a quadratic equation through that point and its nearest neighbors. Cell walls isolated from 5-mm segments cut from leaf blades were analyzed for alkali-labile *p*-hydroxycinnamates by GC-FID. A segmented quadratic-plateau regression model was used to estimate the time at which the deposition of *p*-hydroxycinnamates began during leaf development.

Results and Discussion

Leaf elongation rate averaged 1.35 mm h^{-1} for the high-LER genotype and 0.54 mm h^{-1} for the low-LER genotype. The length of the elongation zone, determined from cell length profiles, was about 22 mm for both genotypes. Residence time of epidermal cells in the meristem and elongation zones averaged 140 h for the high-LER genotype and 400 h for the low-LER genotype. SER of both genotypes initially increased at an increasing rate and then transitioned to a declining rate of increase

at about -21 h (time to cessation of elongation) in the high-LER genotype and at about -52 h in the low-LER genotype (Fig. 1). SER reached a maximum of 0.089 mm/mm/h at about -11 h in the high-LER genotype and 0.036 mm/mm/h at about -28 h in the low-LER genotype. After reaching a maximum, SER declined rapidly as epidermal cells stopped elongating.

Accretion of ferulate and diferulates in cell walls of high-LER leaf blades began about 23 h before elongation stopped (Fig. 1a). In the low-LER genotype, ferulate accretion began about 67 h before elongation ceased (Fig. 1b). The accumulation of diferulic acids in the low-LER genotype was delayed, beginning about 14 h later; the expanded time frame of low-LER elongation allowed a better separation of the data for deposition of these two cell wall components. In both genotypes, deposition of diferulates into cell walls began within 1 or 2 h of the time when SER transitioned from an increasing to a declining rate of increase. During growth of the high LER genotype, concentrations of 8–5- and 8–*O*–4-coupled isomers increased 10-fold from -20 to +20 h from cessation of elongation (Fig. 2a), Concentrations of 8–8- and 5–5-coupled isomers increased only 5-fold over the same 40 h period. Similar changes in diferulate concentrations were apparent from -50 to +50 h for the low-LER genotype (Fig 2b). The 5–5-coupled dimer comprised only a small and variable proportion (12-17%) of the total diferulates in cell walls, making it a poor indicator of diferulate cross-linking in developing leaf blades.



The deposition of *p*-coumarate occurred later in leaf development, beginning about 10 and 32 h before elongation ceased in the high- and low-LER genotypes, respectively (Fig. 1). Accretion of *p*-coumarate, and therefore of *p*-coumaroylated lignins, began just before or at the time when SER reached a maximum and then rapidly declined to zero. These results, using genotypes with differing growth characteristics, suggest that leaf elongation is slowed somewhat at the onset of diferulate cross-linking and ended by the deposition of *p*-coumaroylated lignin in cell walls. The role of ferulate cross-linking in halting growth may, however, be greater than indicated by this analysis since incorporation of ferulate and diferulate xylan esters into lignin dramatically increases ferulate mediated cross-linking of cell walls. Indeed this type of cross-linking may represent a primary mechanism by which lignin reduces cell wall extensibility. The temporal association of ferulate cross-linking and *p*-coumaroylated lignin deposition with growth cessation does not, however, demonstrate a cause and effect relationship. Further work is underway to clearly demonstrate whether ferulate cross-linking and lignification are mechanisms by which growth is stopped in grasses.

Fig. 1 Segmental elongation rate (SER) and cell-wall concentrations of ferulate (FA), diferulates (DFA), and *p*-coumarate (pCA) in leaf blades from the (a) high-LER and (b) low-LER genotypes of tall fescue. The segmented curves used to predict the start times of *p*-hydroxycinnamate accretion are indicated on the figure.

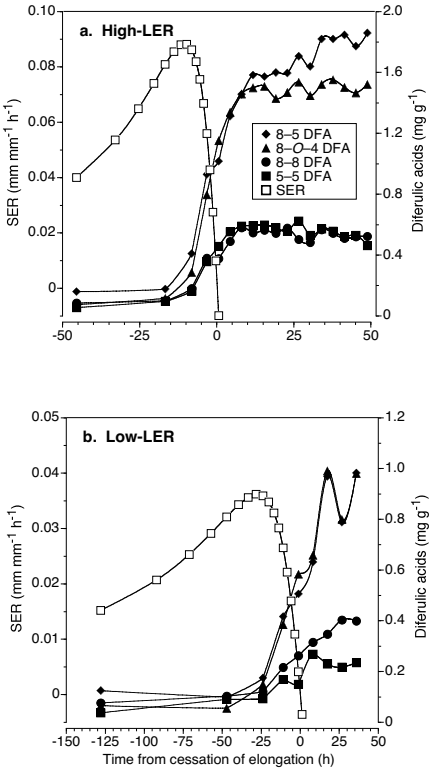


Fig. 2 Segmental elongation rates (SER) and cell-wall concentrations of diferulate (DFA) isomers in leaf blades of the (a) high-LER and (b) low-LER genotype of tall fescue

Deposition of *p*-Hydroxycinnamates Into Fescue Leaf Blades During Primary and Secondary Cell Wall Development

J.H. Grabber and J.W. MacAdam

Introduction

Ferulates are esterified to α-L-arabinose sidechains on xylans in grasses. During wall biosynthesis and lignification, xylans are cross-linked by oxidative coupling of ferulate monomers into dehydrodimers. During lignification, ferulate and diferulate esters copolymerize with monolignols, thereby cross-linking xylans to lignin. These cross-links probably contribute to wall stiffening and growth cessation in grasses and to poor degradation of grass walls by hydrolytic enzymes. The fate of ferulate and diferulates in walls are difficult to track because ferulate deposition, dimerization, and copolymerization into lignin are overlapping processes during cell wall formation in grasses. This difficulty is further compounded by our inability to fully recover or characterize, by solvolytic or spectroscopic methods, ferulates in lignified walls. In previous studies, cell-wall concentrations of ferulate were reported to decline during secondary wall formation in grass tissues. This reduction in *measurable* ferulate during secondary wall formation has been used to support the contention that most ferulates are deposited in primary cell walls. The current study with tall fescue leaves provides evidence that most ferulate and diferulate accretion occurs during secondary cell wall formation. The accretion of *p*-coumarate was also examined in relation to cell wall deposition.

Materials and Methods

Vegetatively propagated tillers of high and low leaf elongation rate (LER) genotypes of tall fescue were grown in a controlled environment chamber. Prior to sampling, mean daily elongation rates were calculated by measuring the length of nine leaves over a 5-d period following leaf emergence. At the time of destructive sampling, epidermal cell lengths were measured from Formvar replicas prepared from the abaxial surface of leaf blades. Segmental elongation rates (SER) were calculated from leaf elongation rates and cell length profiles. The time of maximal SER and minimal cell wall mass were estimated by solving a quadratic equation fit through data points. Cell walls isolated from 5-mm segments cut from leaf blades were analyzed for alkali-labile *p*-hydroxycinnamates by GC-FID. A segmented quadratic-plateau regression model was used to estimate the time at which the deposition of cell walls and *p*-hydroxycinnamates ended during leaf development.

Results and Discussion

Cell wall mass initially decreased toward the distal end of the elongation zone with water uptake and cell expansion (Fig. 1), reaching a minimum within a few hours after cell elongation stopped in leaves. Cell wall mass then increased, due to secondary cell wall deposition, ending about 46 h after elongation ceased.

When plotted on a mg mm^{-1} basis, it is clear that the deposition of ferulate and particularly diferulates continued into the later stages of secondary cell wall formation (Fig. 2). Based on our data, at least 70% of *measurable* alkali-labile total ferulates (monomers and dimers) were deposited during secondary wall formation. The concentration of *p*-coumarate appeared to plateau about 33 h after elongation ceased, about 13 h before secondary cell wall deposition ended. Although the dataset for the low-LER genotype is less complete, similar patterns of ferulate, diferulates, and *p*-coumarate deposition during cell wall formation were apparent (data not shown).

Our analyses, however, underestimate the concentrations of ferulate and diferulates in more mature tissues because ferulate and diferulates readily copolymerize into lignin. Once incorporated into lignin, ferulate and diferulates are not released from lignin by room temperature alkaline hydrolysis. Indeed, most ferulate- and diferulates-lignin cross-links are not cleaved even by high-temperature alkaline hydrolysis or by other solvolytic methods currently used to estimate such cross-linking in cell walls. Therefore, researchers should exercise caution when relating ferulate or diferulate concentrations to cell-wall deposition, extensibility, or growth when lignification of tissues has commenced.

Since *p*-coumarate is primarily a component of lignin, these observations may indicate that lignification stops before secondary wall formation is completed. An alternative interpretation is that acylation of monolignols by *p*-coumarate slows or stops at the latter stages of lignification. In contrast to ferulate, *p*-coumarate esters on lignin units form few, if any, cross-linked structures mediated by radical coupling reactions. *p*-Coumarate (and ferulate) can, however, undergo a photocatalyzed cyclodimerization during tissue development to form truxillic and truxinic acids but none were detected by GC-FID. Therefore, room-temperature alkaline hydrolysis, as used in this study, provides a good estimate of the total quantity of *p*-coumarate in walls.

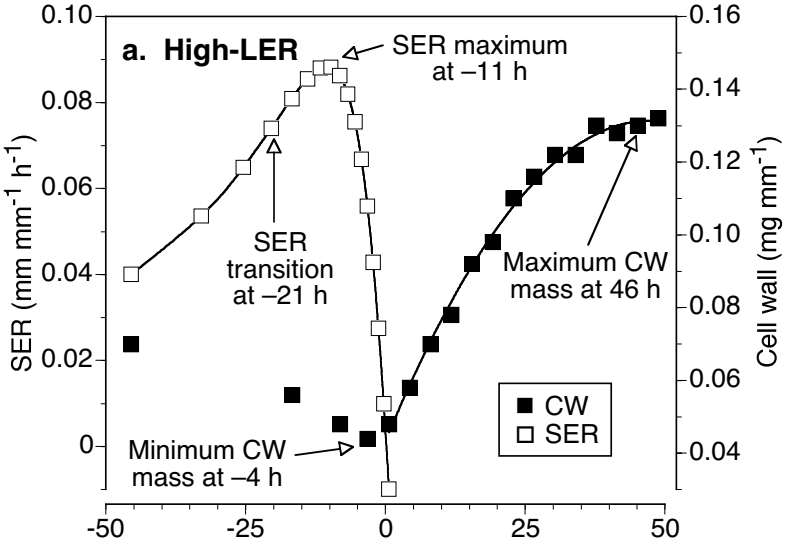


Fig. 1. Segmental elongation rates (SER) and mass of cell walls (CW) of leaf blades from the high-LER genotype of tall fescue. Data are plotted at the centers of 5-mm-long leaf blade segments. The segmented curves used to predict the time of maximum CW accretion are indicated on the figure.

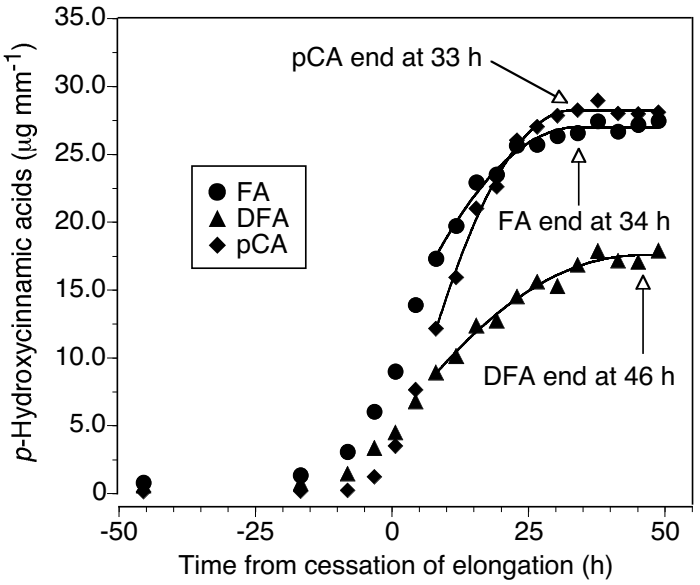


Fig. 2. Per-unit-length contents of ferulate (FA), diferulates (DFA), and *p*-coumarate (pCA) in leaf blades from the high-LER genotype of tall fescue. The segmented curves used to predict the end of *p*-hydroxycinnamate accretion are indicated on the figure.

Diferulates Analysis: Diferulates and Disinapates in Insoluble Cereal Fiber

M. Bunzel, John Ralph, J.M. Marita, F. Lu, R.D. Hatfield, H. Kim, J.H. Grabber, S.A. Ralph, G. Jiminez-Monteon, and H. Steinhart.

Introduction

Grasses have substantial amounts of hydroxycinnamic acids intimately associated with the cell wall. Ferulate, in particular, has a significant role in cross-linking. Polysaccharide-polysaccharide cross-linking is achieved by ferulate dehydrodimerization by either photochemical or, more importantly, radical coupling reactions of ferulate-polysaccharide esters. The whole range of ferulate dimers **1**-**9**, Fig. 1, are now routinely being found in a variety of samples. Radical cross-coupling of (polysaccharide-linked) ferulates with lignin monomers results in lignin-polysaccharide cross-linking. Even ferulate dimers cross-couple with lignin monomers/oligomers to incorporate into the lignin polymer resulting in extensive polysaccharide-polysaccharide-lignin cross-linking.

Methods of Analysis

Although HPLC methods have been used for qualitative and quantitative analysis of diferulates, HPLC really cannot afford the dispersion and resolution of GC. In addition, alkaline hydrolysates of plant materials produce a wide range of products that cannot be easily pre-fractionated. For example in grass stem samples, in addition to the diferulates from radical coupling of ferulates are the photochemical dimers and numerous ferulate-monolignol crossed dimers. There are many components still to be identified. As indicated below, two new diferulates have recently been found, along with dimers of another hydroxycinnamate, sinapate. Only cursory analyses are currently possible using HPLC; GC-FID and/or GC-MS should be used if component detail is required.

Mass spectral data for the diferulates have been requested by many groups. Spectra from both a quadrapole EI instrument and an Ion Trap are given in Fig. 1. We have been impressed with the ion trap instrument for providing enhanced sensitivity in the high-mass region. The ion-trap spectra of all of the diferulates have a molecular ion peak that is quite abundant; quadrapole instruments tend to favor the uninformative trimethylsilyl peak at 73, and typically yield weak molecular ions.

A Better GC Standard for Diferulates Analyses

The standard used for the original quantification of diferulates and most subsequent studies has been *o*-coumaric acid. Unfortunately this standard has neither ideal elution nor satisfactory response factors for the diferulates. A standard having better structural similarity was sought. Rather extensive surveys failed to unearth a satisfactory commercially available standard. We decided that the 5–5-coupled dimer which had been monomethylated might be a good candidate, and this has been used in our labs. However, it was discovered somewhat late in the analysis process that the "standard""**IS** was contaminated with the di-methylated compound **IS*** (see the chromatograms in Fig. 2), from which separation was extremely difficult. We are now examining the use of the fully methylated dimer as well as seeking cleaner and simpler methods to prepare the monomethylated dimer. Our lab will eventually provide the chosen reference compound to other labs interested in using it.

Discovery of the Full Range of Ferulate Dimers (Diferulates)

The sole ferulic acid dehydrodimer reported from plant cell walls before 1994 was 5–5-diferulic acid **7**. The more recent determination (and authentication) of a range of diferulates from grasses stemmed from a recognition that radical coupling of ferulates, necessary to produce the 5–5-coupled dehydrodimer **7**, could produce other dehydrodimers by anticipated 8–5-, 8–8-, 8–O–4- and 4–O–5-coupling reactions, analogous to those observed for coniferyl alcohol during lignification. Unless the radical coupling was directly controlled by an enzyme or, as been more recently revealed in lignan biosynthesis, a dirigent protein other dimers would be expected to be more prevalent than the 5–5-dimer. This has been found to be the case in every plant material subsequently examined.

The only dehydrodimer not found until recently was 4–O–5-DFA–**9**, Fig. 1. This diferulic acid has also now been found (in rather small amounts) in several insoluble cereal fibers, as described in the following report ("Identification of the Last Elusive Diferulate Linkage"). The finding completes the spectrum of ferulate dehydrodimers to be found in plants, and supports the concept of free-radical coupling of cell wall components independently of enzymes or proteins which might otherwise confer a strict regiochemical course, i.e. produce only a single diferulate.

Another 8–8-coupled Diferulate?

Most alkaline hydrolysates of grass cell walls also contain another previously unidentified peak. MS analysis suggests that it is the tetrahyrdrofuran dimer **4**, Fig. 1. As can be seen in the chromatogram in Fig. 2, it is a substantial component that should also be quantified as resulting from 8–8-dimerization. Work is currently underway to isolate sufficient amounts of the compound for structural elucidation by NMR and to synthesize it.

Levels of Diferulates in Cereal Grain Insoluble Fiber

Grain fiber is known to be beneficial for human nutrition. The levels of diferulates in a range of cereal grain fibers was recently surveye: maize 12.6, wheat 2.4, spelt 2.6, rice 4.0, wild rice 2.8, barley 3.7, rye 4.0, oat 3.6 and millet 5.7 mg/g of insoluble fiber. Very low levels were found in the soluble fiber fraction as might be anticipated. The high levels in maize make this an ideal secondary standard to check column performance and variations in the analyses over longer periods of time.

Disinapates Cross-linking Cell Wall Polysaccharides??

Although sinapic acid has been identified in plant extracts and can be released in small quantities from grass cell walls by low-temperature base, it has not been determined if it acylates polysaccharides or other components. Nor has sinapate been shown to be involved in radical coupling reactions to produce dehydrodimers. Preliminary identification of such radical dehydrodimerization products in the insoluble fiber fraction from wild rice samples is presented here.

GC-MS total ion chromatograms of saponified extracts from wild rice (*Zizania palustris* L.) insoluble fiber showed additional peaks in the dimer region; two peaks were especially predominant. The mass spectra were analogous to those of ferulate dimers, with masses of various peaks offset by 30 or 60 mass units (corresponding to one or two additional methoxyls). The products could also be obtained by saponification of dimers prepared by oxidative coupling of ethyl sinapate. We assume

that they are the two 8–8-coupled disinapate analogs of the 8–8-diferulates **1** and **2**, one ring form and one open-chain. We are currently independently synthesizing both products to identify them conclusively.

Although it is logical that sinapoylated polysaccharides might be cross-linked by radical dimerization of the sinapates in an analogous way to the ferulates, we have not demonstrated that they are in fact esterified to polysaccharides. It is also curious that disinapates would form with little evidence (from MS data so far) of any cross-coupling products between ferulate and sinapate; however, we have not yet examined the propensity for such cross-coupling. If disinapates are found in cell wall fractions from stems or grains of plants other than wild rice, such issues should be clarified by further research.

Conclusions

Diferulates continue to emerge in varied roles as significant components of many plant fibers and have interesting implications for human and animal health as well as for their properties in limiting cell wall digestibility by ruminants. The 4–O–5-coupled dimer has now been found in small quantities in many cereal grains in which the insoluble fiber fraction contains high levels of total diferulates, up to 12.6 mg/g in maize. A new form of the 8–8-dimer (a tetrahydrofuran) has also been found. Many dimers of ferulate cross-coupled with monolignols (not detailed here) can also be found in grain and stem fibers. Sinapates in wild rice, like ferulates in all grasses, appear to dimerize via radical coupling reactions to produce at least two sinapate dehydrodimers, which can be detected by GC(-MS). We assume new roles for such hydroxycinnamate dehydrodimers will continue to be discovered and researchers will learn more about their impact on other physiological processes.

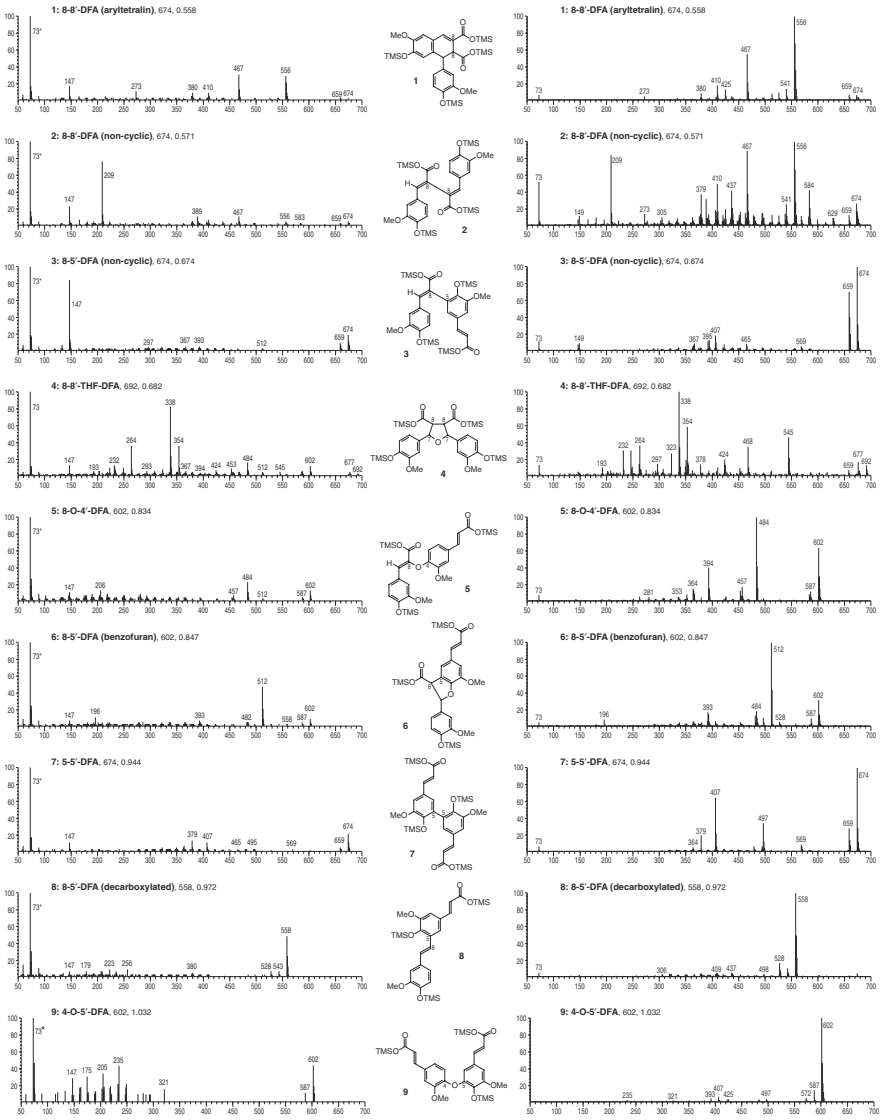


Fig. 1. Structures and mass spectra from the nine diferulate products resulting from saponification of cereal insoluble fiber. The left column of spectra are traditional EI spectra from an HP 5970 bench-top quadrapole instrument. Note that spectra in which the TMS peak is noted as 73* have had all peaks but the 73 peak doubled in intensity for easier viewing of the important high-mass peaks. The right column of spectra are from a Thermoquest Polaris GCQ ion-trap instrument, and generally show superior molecular ion peaks. Spectrum labels include, in addition to the compound number and name, the nominal mass and the retention time relative to **IS**, the monomethylated 5-5-coupled ferulate dimer.

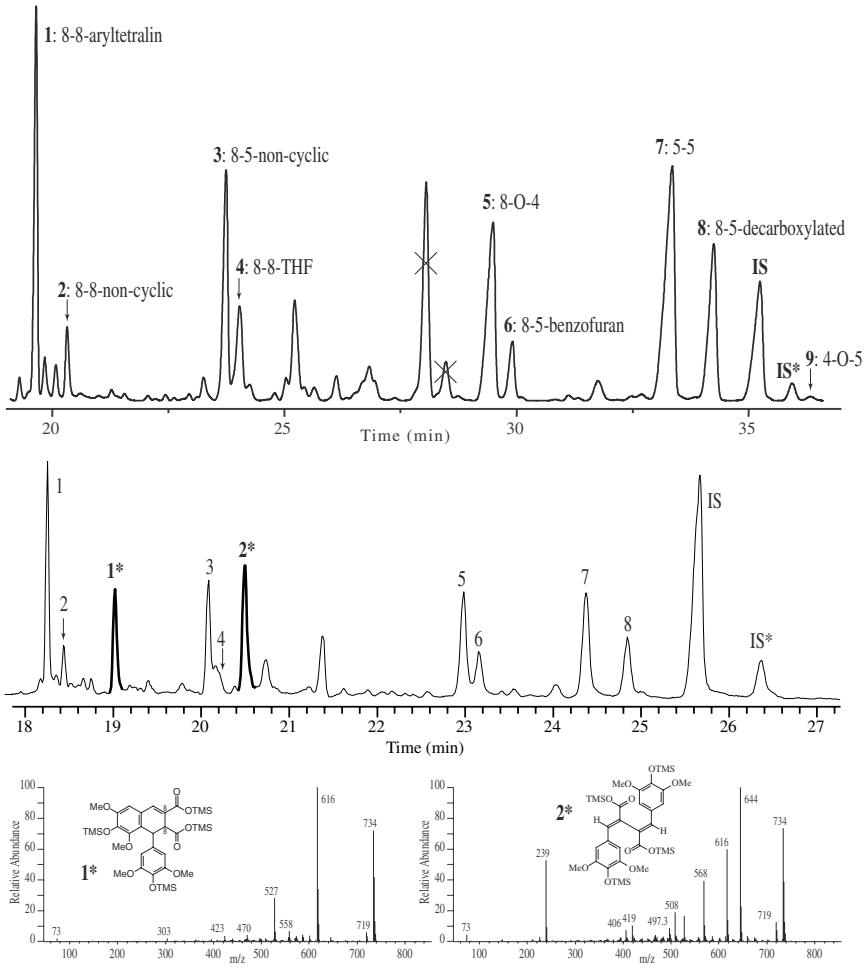


Fig. 2. *Top.* GC-MS total ion chromatogram of the dimers region from saponification of maize grain insoluble fiber showing diferulate products **1**-**9**. The crossed out peaks are apparently artifacts that are not present in more recent chromatograms from such samples. **IS** is the mono-methylated derivative of the 5–5-dimer **7**; the di-methylated derivative is also present, **IS***. *Middle.* Similar data (different machine and conditions!) for wild rice insoluble fiber hydrolysate showing diferulates and two new disinapates. *Bottom.* Ion-trap mass spectra of disinapates (structures to be confirmed).

Identification of the Last Elusive Diferulate Linkage

M. Bunzel, J. Ralph, J.M. Marita, G. Jimenez-Monteon, R.D. Hatfield and H. Steinhart

Introduction

Ferulates play an important role in modifying the mechanical properties of cell walls as well as in limiting polysaccharide degradation by exogenous enzymes by acting as cross-links between polysaccharides and between polysaccharides and lignin. Dimerization of ferulates is a mechanism for cross-linking cell wall polysaccharides.

The more recent determination of a range of diferulates from grasses stemmed from a recognition that radical coupling of ferulates, necessary to produce the 5-5 dehydrodimer, could produce other dehydrodimers by anticipated 8-5-, 8-8-, 8-O-4- and 4-O-5-coupling reactions. These other dimers have been found to be more prevalent than the 5-5-dimer in every plant material subsequently examined (see discussion section). Prior to this study, the only dehydrodimer *not* found was 4-O-5-DFA (Fig. 1). This paper reports its identification and semiquantitative determination in several insoluble cereal fibers.

Methods

Internal Standard (E,E)-4-hydroxy-4,5,5-trimethoxy-3,3-bicinnamic acid. Since the currently used internal standards (tetracosane, 2-hydroxycinnamic acid) have non-ideal retention times or response factors that are too large, an internal standard more like the dimers being analyzed was sought. In this study, we used monomethylated 5-5-DFA produced by methylation of diethyl 5-5-diferulate using dimethyl sulfate, followed by column purification on silica gel and saponification. Although it worked well for this study, the standard cannot be recommended at this time for the following reasons. It was not discovered until well into this study that the standard was contaminated by its dimethylated analog, peak IS* in Fig. 2. Subsequent attempts to purify the compound failed. Since response factors were derived for IS against the authentic diferulates, the quantitative aspects of this study are sound, but in future studies it will be necessary to find or prepare a pure internal standard (see previous report — "Diferulates Analysis: Diferulates and Disinapates in Insoluble Cereal Fiber").

Plant Material. Whole grains of corn, wheat, spelt and rice were obtained from a German supplier.

Preparation of insoluble fiber. Samples were milled to a particle size smaller than 0.5 mm. The sample material (10 g) was suspended in phosphate buffer (0.08 M, pH 6.0, 300 mL) and 750 μL of α-amylase were added. The beakers were placed in a boiling water bath for 20 min and shaken gently every 5 min. The pH was adjusted to 7.5, and samples were incubated with 300 μL protease at 60 °C for 30 min with continuous agitation. After adjusting the pH to 4.5, 350 μL amyloglucosidase were added and the mixture was incubated at 60 °C for 30 min with continuous agitation. The suspension was centrifuged, the residue was washed two times with hot water, 95% ethanol and acetone and finally dried at 60 °C overnight in a vacuum oven.

Saponification of insoluble fiber and extraction of ester linked phenolics. Insoluble fiber (40–90 mg) was weighed into a screw-cap tube, internal standard (5–50 μg) dissolved in dioxane was added and saponification with NaOH (2 M, 5 mL) was carried out under nitrogen and protected from light for 18 h at room temperature. Samples were acidified with 0.95 mL concentrated HCl (resulting pH < 2) and extracted into diethyl ether (4 mL, three times). Extracts were combined and evaporated under a stream of N_2. Finally, samples were dried under vacuum.

GC-FID and GC-MS analysis of dehydrodiferulic acids. Dried extracts were silylated by adding 10 μL pyridine and 40 μL BSTFA and heating for 30 min at 60 °C in sealed vials. Trimethylsilylated derivatives of phenolic acids were separated by GLC using a 0.2-mm × 25-m DB-1 capillary column (0.33 μm film thickness) (J&W Scientific) and identified by their electron impact mass data collected on a Hewlett-Packard 5970 mass-selective detector. He (0.54 mL/min) was used as carrier gas. GLC conditions were as follows: initial column temperature, 220 °C, held for 1 min, ramped at 4 °C/min to 248 °C, ramped at 30 °C/min to 300 °C, held 40 min; injector temperature 300 °C, split 1/50. Mass spectra in the electron impact mode were generated at 70 eV. Semiquantitative determination of 4-O-5-DFA was carried out by GLC using the same column and GLC conditions and a flame ionization detector (detector temperature 300 °C). He (0.4 mL/min) was used as carrier gas.

Results and Discussion

Dietary fiber is defined as that part of foodstuff which is not digested by secretions of the human gastrointestinal tract. Although there are other minor sources, plant cell walls constitute the major part of dietary fiber.

In all investigated insoluble cereal fibers the 8-5- and 8-8-coupled diferulic acids as well as the 8-O-4- and 5-5-coupled diferulic acids were identified after saponification by their relative retention times, Fig. 2, and their mass spectra. These diferulic acids have previously been identified in cocksfoot, switchgrass and suspension-cultured corn, sugarbeet, water chestnuts, corn bran, and carrots. Interestingly, the 8-O-4, 8-5- and 8-8-diferulic acids but not the 5-5-diferulic acid were identified in elongating pine hypocotyls. In none of these investigations could the theoretically possible 4-O-5-DFA be identified unambiguously.

In the extracts of saponified corn insoluble fiber 4-O-5-coupled diferulic acid was identified by comparison of its mass spectrum and its relative GLC retention time with that of the genuine compound, which was synthesized and authenticated by NMR. Fig. 1 of the previous report (“Diferulates Analysis: Diferulates and Disinapates in Insoluble Cereal Fiber”) shows the MS spectrum of silylated 4-O-5-DFA (compound **9**); with originally one phenolic and two acid groups, its nominal molecular mass is 602. The relative retention time of 4-O-5-DFA against the internal standard was 1.032. From the insoluble fibers of wheat, spelt and rice the 4-O-5-DFA was identified by its relative GLC retention time, and detection of the molecular peak m/z 602 in selected ion chromatograms.

Semiquantitative determination of 4-O-5-DFA was carried out by setting the response factor as 1.0. Determination of the accurate response factor was not possible because of the tiny amounts of synthesized 4-O-5-DFA available. The response factors of the other diferulic acids, which could be synthesized in larger amounts, against the internal standard were close to 1 (0.91-1.18, with the exception of the 8,5-cyclic-coupled DFA, which is 2.2). The amounts of 4-O-5-DFA in insoluble cereal fibers were 33±3, 13±1, 10±1, and 8±2 μg/g in corn, spelt, wheat, and rice, respectively. Consequently, the amounts of 4-O-5-DFA are approximately 70–100 times lower than the amounts of the sum of 8-5-coupled diferulic acids, which were identified as the major diferulic acids in the cereal fibers investigated. These results therefore provide evidence for the full range of possible ferulate radical coupling products in cereal and presumably in other plant cell walls containing ferulates and diferulates. They also confirm the prevalence for coupling at ferulate’s 8-position (to give the more predominant 8-5-, 8-8-, and 8-O-4 dimers), as also observed in ferulate cross-coupling into lignin .

Conclusions

Alkali-releasable 4-O-5-DFA has been detected in plant materials. The relatively high signal-to-noise chromatograms from the methods described here allow the product to be definitively identified. Its identification as an, albeit minor, diferulate supports its role in the important cell wall cross-linking reactions achieved by ferulate dehydrodimerization. The finding completes the spectrum of ferulate dehydrodimers to be found in plants, and supports the concept of free-radical coupling of cell wall components independently of enzymes or proteins which might otherwise confer a strict regiochemical course, i.e. produce only a single diferulate.

The complete manuscript is available from our website at:
http://www.dfrc.ars.usda.gov/DFRCWebPDFs/2000-Bunzel-JAFC-48-3166.pdf

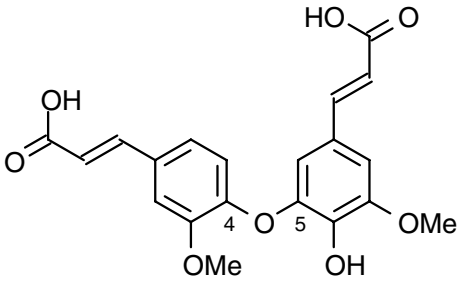


Fig. 1. Structure of 4-O-5-coupled diferulic acid.

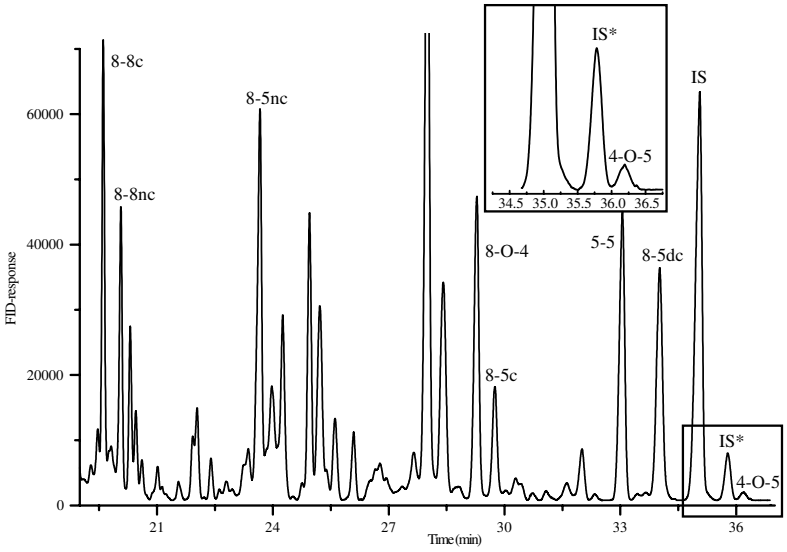


Fig. 2. GC-FID chromatogram of the extract of saponified spelt insoluble fiber.
8-8-coupled diferulic acids: c – cyclic, nc – non cyclic; 8-5-coupled diferulic acids: nc – non cyclic, c – cyclic, dc – decarboxylated. Many of the unlabelled peaks have been assigned as ferulate cross-products, as will be detailed elsewhere.

5-Hydroxyconiferyl Alcohol as a Monolignol in COMT-deficient Angiosperms

J.M. Marita, F. Lu, J. Ralph, R.D. Hatfield, C. Lapierre, S.A. Ralph, C. Chapple, W. Vermerris, W. Boerjan, and L. Jouanin.

Introduction

Recent advances in genetic engineering have allowed researchers to perturb the monolignol biosynthetic pathway producing often significantly altered lignins. This approach provides valuable insights into the control of lignification and into the apparent biochemical flexibility of the lignification system.

Here we present NMR and DFRC data from recent studies on mutants and transgenics deficient in COMT (caffeic acid *O*-methyl transferase), the favored substrate for which now appears to be 5-hydroxyconiferyl aldehyde. Downregulating these enzymes dramatically affects the composition of lignins and the structures contained in those lignins.

COMT-Deficient Poplar: Benzodioxanes from Incorporation of 5-Hydroxyconiferyl Alcohol.

COMT is one of two enzymes required to 5-methoxylate guaiacyl monomeric units to produce sinapyl alcohol and eventually produce syringyl units in angiosperm lignins. If COMT is downregulated, 5-hydroxyconiferyl aldehyde might be expected to be reduced to 5-hydroxyconiferyl alcohol if the next reductase enzyme is sufficiently non-specific. In fact, it appears that 5-hydroxyconiferyl alcohol is indeed formed, shipped out to the wall, and incorporated into lignin analogously to other lignin monomers (although it produces some novel structures in the final lignin), Fig. 1.

NMR provides beautiful evidence that benzodioxane structures are produced in lignins which incorporate 5-hydroxyconiferyl alcohol. Figure 2b shows the sidechain region of an HMQC spectrum from an (acetylated) isolated lignin from a COMT-deficient poplar (*Populus tremula* x *Populus alba)* described recently. The benzodioxanes **H** are readily apparent. Well separated contours at δ_C/δ_H of 76.8/4.98 (α), and 75.9/4.39 (β) are diagnostic for the benzodioxanes **H**; the γ-correlations overlap with those in other lignin units, but are elegantly revealed in 3D TOCSY-HSQC (see accompanying Report—"The first 3D NMR of un-enriched lignin. Proof of benzodioxanes in a COMT-deficient angiosperm") or 2D-HMQC-TOCSY spectra. The sidechain correlations are consistent with those in a model compound for the *trans*-benzodioxane, synthesized by biomimetic cross-coupling reactions between coniferyl alcohol and a 5-hydroxyguaiacyl unit.

A reasonable quantification of this unit can be achieved by measuring volume integrals in the 2D spectra, particularly if the similar Cα–Hα correlations are used. The ratios in the transgenic poplar (and in the wild-type control) are given in Table 1. The 5-hydroxyconiferyl alcohol-derived benzodioxane **H** units are the second most abundant (~18%) interunit type in the COMT-gene-silenced sample. Since the lignins analyzed by NMR represent 65% of the total lignin in this transgenic, it is logical that the benzodioxane structures would remain a significant component even if the lignins were drastically partitioned by the isolation process. The total β-ether frequency (normalβ-ether**A** plus dibenzodioxocins **D** plus benzodioxanes **H**) in the transgenic is around 78%, lower than in the control because of the higher guaiacyl content. However, there are a few units not covered by these percentages since they have no resonances in the aliphatic sidechain region of the NMR spectra (cinnamaldehyde endgroups, β–1-structures). Lignins from COMT antisense poplars

also contain benzodioxane units at a lower level. Although the data are limited at present, it appears that 5-hydroxyconiferyl alcohol may largely make up for the sinapyl alcohol deficiency.

Evidence for 5-hydroxyconiferyl alcohol incorporation and of benzodioxane units in the lignins also comes from degradative analyses (see the accompanying report — "Marker Compounds for Enzyme Deficiencies in the Lignin Biosynthetic Pathway. 2. COMT"). What the degradative and NMR data show is that 5-hydroxyconiferyl alcohol is behaving like a normal monolignol does in lignification. It reacts at its β-position with the phenol at the end of the growing lignin polymer, and new monolignols then react with the resulting new 5-hydroxyguaiacyl phenolic end. This endwise polymerization is characteristic of the major lignification pathway. It also indicates that 5-hydroxyconiferyl alcohol appears to be incorporating intimately into the polymer in the same way that the traditional monolignols do. We see no reason therefore why 5-hydroxconiferyl alcohol should not be considered an authentic monolignol in these systems.

Benzodioxanes in F5H-Upregulated Arabidopsis.

There is another interesting variant in the COMT-deficiency class. Arabidopsis transgenics with upregulated F5H have previously been shown to have only a minor guaiacyl component. Contours previously unidentified in the 2D NMR spectra of their lignins now obviously result from benzodioxane structures, Fig. 2c. The observations here imply that, whereas syringyl production was enormously up-regulated in these transgenics, the methylation could apparently not keep pace with the accelerated production of 5-hydroxy-units (e.g. 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol). The result is a significant incorporation of 5-hydroxyconiferyl alcohol into the monolignol pool for the most heavily F5H-upregulated transgenics, ~10% as measured from contour volumes in the HMQC spectra — see Table 1.

Benzodioxanes in *bm3* Maize Mutants

Maize has four brown-midrib (*bm*) mutants, all of which have reddish-brown vascular tissue in the leaves and stems. Two are known to have mutations in the monolignol biosynthetic pathway; *bm1*, CAD; *bm3*, COMT.

Lignins in the *bm3* mutant show the now characteristic signs of 5-hydroxyconiferyl alcohol incorporation. Early on, thioacidolysis suggested that 5-hydroxyconiferyl alcohol was a constitutive unit of those lignins. The dimeric benzodioxane products from thioacidolysis and DFRC are also readily identified in the maize mutant. Preliminary NMR of isolated lignins shows the substantial presence of these new **H** units in the mutant (Table 1) where it accounts for some 25% of the interunit linkages characterized (although we have not yet examined how this structure is partitioned between the soluble fraction used for NMR and the residues).

Implications for Pulping

How is offsetting syringyl units in lignins with 5-hydroxyguaiacyl units likely to affect pulping performance? The new benzodioxane units in the lignin are still ether structures (a,b-diethers), but will they cleave under pulping conditions? In preliminary studies with etherified benzodioxane model compounds, very little ether cleavage occurs under soda pulping conditions— the models are recovered intact in high yields. Kraft pulping conditions are unlikely to affect the outcome. With less

cleavable b-ethers in the lignin, pulping efficiency would therefore likely be reduced. Recent pulping trials with COMT-deficient poplars confirm a lower pulping efficiency (Jouanin et al., 2000). In part this may also be attributed to the slightly higher guaiacyl content in the transgenic lignins compared with the wild-type control, but it is suspected that much of the effect can be attributed to the alkaline stability of the benzodioxanes.

Implications for Ruminant Digestibility

No clear correlations between lignin structure and cell wall digestibility in ruminants are evident. However, various mutants and transgenics are of interest because of their potential to improve digestibility. At present, such studies remain empirical. Dixon's group at the Noble Foundation are examining digestibility implications of various alfalfa transgenics, including those deficient in COMT, and we are currently structurally analyzing their plants.

Conclusions

As further evidence accumulates from degradative and NMR methods that 5-hydroxyconiferyl alcohol monomers integrate into the polymerization process, it becomes evident that 5-hydroxyconiferyl alcohol can be used as a lignin monomer by plants, in part offsetting the deficiency in sinapyl alcohol monomers. A salient observation is that the process of lignification appears to be flexible enough to incorporate phenolic phenylpropanoids other than the traditional monolignols. The incorporation of 5-hydroxyconiferyl alcohol (as well as hydroxycinnamyl aldehyde monomers in CAD-deficient angiosperms) also implies that the plant is sending these products of incomplete monolignol biosynthesis out to the cell wall for incorporation. The resultant modified lignins apparently have properties sufficient to accommodate the water transport and mechanical strengthening roles of lignin and to allow the plant to be viable. Whether such plants will be able to confront the rigors of a natural environment replete with a variety of pathogens remains to be determined. However, the plants' approach toward lignification, i.e. polymerizing monolignol precursors and derivatives along with the traditional monolignols, is a testament to a flexible survival strategy; in a single generation, these plants have circumvented genetic obstacles to remain viable. The recognition that monolignol intermediates and other novel units can incorporate into lignin provides expanded opportunities for engineering the composition and consequent properties of lignin for improved utilization of valuable plant resources.

Fig. 1. Production of benzodioxanes **7** in lignins via incorporation of 5-hydroxyconiferyl alcohol **1** into a guaiacyl lignin. Only the pathways producing β-ether units are shown. Cross-coupling with syringyl units is less pronounced in lignins which have a low syringyl content due to COMT downregulation. Cross-coupling of 5-hydroxyconiferyl alcohol **1**, via its radical **1·**, with a guaiacyl lignin unit **3G**, via its radical **3G·**, produces a quinone methide intermediate **4** which re-aromatizes by water addition to give the β-ether structure **5** (possessing a 5-hydroxyguaiacyl end-unit). This unit is capable of further incorporation into the lignin polymer via radical coupling reactions of radical **5·**. Reaction with the monolignol coniferyl alcohol **2G**, via its radical **2G·**, produces a quinone methide intermediate **6**. This time, however, quinone methide **6** can be internally trapped by the 5-OH phenol, forming a new 5–O–α-bond, and creating the benzodioxane ring system in **7**. The presence of structures **7** in COMT-deficient transgenic plants is diagnostically revealed by NMR, units **H** in Fig. 2.

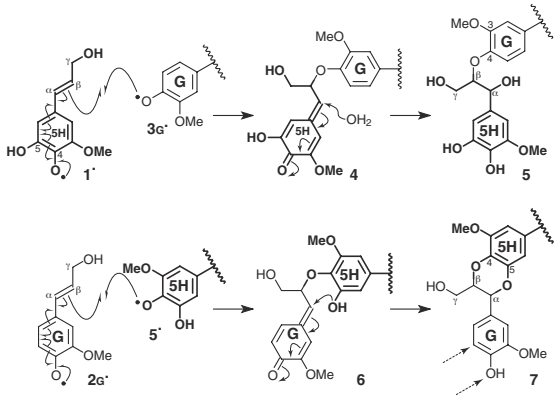


Fig. 2. Partial spectra from gradient HMQC NMR experiments highlighting new peaks for benzodioxane units **H**. Lignins were from a) a control poplar, b) a COMT-downregulated transgenic, c) an F5H-upregulated Arabidopsis, and d) similar correlations from a benzodioxane model.

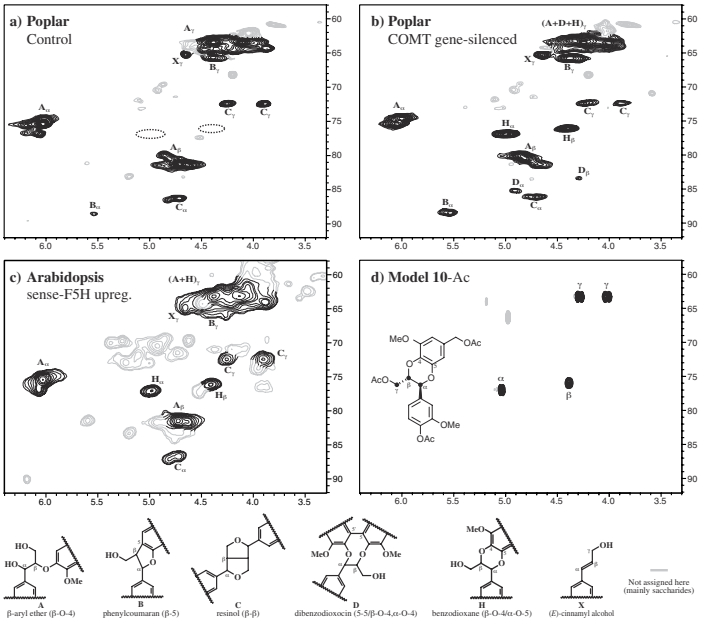


Table 1. Subunit ratios derived from volume integrals of contours in the sidechain region of ^{13}C–^{1}H correlation spectra of acetylated isolated lignins.

Lignin	A	B	C	D	H	X	β-O-4
Poplar							
WT	88	3	7	0	0	2	88
COMT-silenced	53	13	5	6	18	5	78
COMT-anti	65	12	5	3	10	4	78
Maize							
WT	86	7	0	0	0	6	86
bm3-mutant	60	7	0	0	25	8	85
Arabidopsis							
WT	67	13	6	6	0	7	73
F5H-sense	81	<1	6	0	10	4	91

A = β-ether (β-O-4), **B** = phenylcoumaran (β-5), **C** = resinol (β-β), **D** = dibenzodioxocin (β-O-4/α-O-4), **H** = benzodioxane (β-O-4/α-O-5); the last column is the sum of all β-O-4 components (**A**+**D**+**H**). Note: these values are simple area ratios of these 6 units, and should not be interpreted as percentages of interunit linkages in lignin, since 5–5, β–1, and other linkages are not included.

Marker Compounds for Enzyme Deficiencies in the Lignin Biosynthetic Pathway. 1. CAD

H. Kim, J. Ralph, F. Lu, I. Mila, B. Pollet, and C. Lapierre.

Introduction

Perturbations of the lignin biosynthetic pathway have the potential to enhance the utilization of plant cell walls in various natural and industrial processes. In forages fed to ruminant animals, lignins inhibit the rumen-degradability of polysaccharides. In chemical pulping for paper production, the aim is to selectively remove the lignin from the cellulose fibers. Recently it has become evident that there is potential beyond simply down-regulating lignification itself to produce low-lignin plants. Inducing structural and compositional changes in the polymer may also be beneficial for many processes.

The definitive way to determine a plant's response to up- or down-regulation of lignin-biosynthetic-pathway genes/enzymes and the impact on the lignification process itself is by lignin structural analysis. However, full structural analysis is a lengthy and difficult process inappropriate for screening. Marker compounds that allow the degree of change to be elucidated are vital particularly when various levels of down-regulation need to be assessed.

CAD (cinnamyl alcohol dehydrogenase) is the last enzyme on the pathway to the monolignols, primarily coniferyl and sinapyl alcohols, from which polymeric lignin is derived. CAD deficiency in various mutant and transgenic plants has been reported to induce the increased incorporation of hydroxycinnamyl aldehydes into the lignin polymer. Recent NMR studies revealed that, in a CAD-deficient tobacco, sinapyl aldehyde readily 8–O–4-coupled with both syringyl (S) and guaiacyl (G) lignin units, and that coniferyl aldehyde also 8–O–4-cross-coupled but only with S-units.

Radical coupling of an hydroxycinnamyl aldehyde at its 8-position results initially in a quinone methide. Unlike in the β–O–4-coupling of hydroxycinnamyl alcohols, where re-aromatization is by nucleophilic addition of water to the quinone methide, the hydroxycinnamyl aldehyde quinone methide 8–O–4-coupling product re-aromatizes by elimination of the acidic 8-proton to give the unsaturated 8–O–4-product **3**, Fig. 1; this elimination was observed in the dimerization reactions of coniferyl aldehyde and has recently been established for both coniferyl and sinapyl aldehydes in lignification as well.

Hydroxycinnamyl aldehydes either involved in 8–O–4-linkages or incorporated as 4-O-β-end-groups **6**, Fig. 1, will release diagnostic monomers following thioacidolysis, which therefore serve as marker compounds for hydroxycinnamyl aldehyde incorporation. Consequently, they also serve as maker compounds for CAD-deficiency. This paper details the identification of thioacidolysis CAD marker compounds, and provides evidence that 8–O–4-coupled hydroxycinnamyl aldehyde units in lignin are their source.

Results and Discussion

Two new isomeric monomers have been systematically detected following thioacidolysis of a range of CAD-deficient dicots in the INRA laboratories. The compound levels appeared to vary with the amount of CAD-downregulation (see Fig. 55 later). These isomers had a nominal MW of 312 and an apparent formula $C_{15}H_{20}O_3S_2$. Raney nickel desulfurization generated a single new monomer (nominal MW 194, $C_{11}H_{14}O_3$). The latter was found by GC-MS to be not the commercially available

syringyl prop-1-ene **7** (Fig. 2), nor the pro-2-en **8** that could be prepared by Rh(III)-assisted isomerization, both of which had different GC retention times. Syringyl cyclopropane **9** appeared to be a possibility particularly since DFRC-degradation of cinnamyl aldehydes produced cyclopropanes. However, synthesized **9** also had a GC retention time which differed from the thioacidolysis-Raney-Ni product. The only possibility left (while retaining the syringyl moiety) was the indane **10**. However, its authentication was required and the exact structures of the precursor thiol products also remained unresolved. Rather than synthesize suspected compounds independently, we sought to elucidate the source of the thioacidolysis products using model compounds, and to prepare sufficient products for isolation and full structural characterization, along with establishing their identity to the thioacidolysis products.

Since thioacidolysis rather specifically cleaves ether linkages, the only likely source of the compounds was β-ether units **6** or **3**, Fig 1. However, we already know that products R–CHSEt–CH_2–$CH(SEt)_2$ result from coniferyl and sinapyl aldehydes and from their ethers **6**. The likely source of the new compounds was therefore logically from 8-O-4-coupled hydroxycinnamyl aldehyde units **3**.

Model compounds for hydroxycinnamyl aldehyde 8–O–4-units **3** were synthesized. Thioacidolysis of the model compound for **3SG** did indeed cleanly give the same two isomeric compounds as thioacidolysis of CAD-deficient plants, in approximately equal amounts, Figure 3. With similar mass spectra, the structures could only be reliably elucidated by NMR. The isomers were separated by preparative TLC or HPLC.

Proton NMR showed that one of the isomers (the faster moving isomer) had two aromatic or double-bond protons and one aliphatic proton (presumably with a thioethyl group on the same carbon), whereas the slower isomer had only one aromatic and two aliphatic protons. Full structural elucidation by the usual complement of 1D and 2D NMR methods revealed them to be isomers **11S** and **12S** related by a 1,3-sigmatropic proton shift by which they presumably equilibrate under the thioacidolysis conditions. These structures are logical from the anticipated thioacidolysis mechanisms, Figure 4, at least in hindsight. Raney nickel desulfurization of either (or a mixture) of the two isomers **11S/12S** gave the indane **10S** reasonably cleanly. Only one guaiacyl analog of compound **12S** could be observed, together with the product G–CHSEt–CH_2–$CH(SEt)_2$ derived from coniferyl aldehyde end-groups. This result suggests that down-regulating CAD activity in poplar specifically affects the reduction of sinapyl aldehyde into sinapyl alcohol.

The importance of the new markers (**11S + 12S**), relative to the conventional syringyl monomers (S–CHSEt–CHSEt–CH_2SEt), was found to increase together with the level of CAD deficiency (Fig. 5). This signature was observed even before any wood phenotype (red coloration of the xylem) could be seen or before any other lignin structural alteration could be detected.

Conclusions

Marker compounds (released following the application of degradative methods) for CAD-deficiency have been identified and their sources in the novel lignins elucidated. The thioacidolysis CAD-markers come from hydroxycinnamyl aldehyde units linked 8–O–4 to generic lignin units. Such marker compounds become valuable in assessing the effects of various levels of down-regulation in CAD-deficient plants.

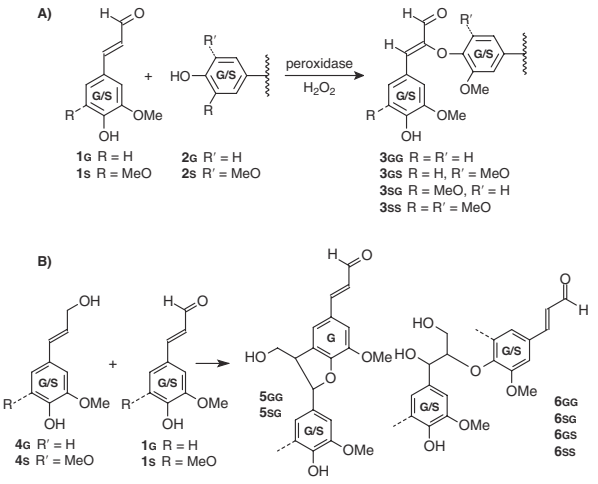


Fig. 1. Possible modes of incorporation of hydroxycinnamyl aldehydes **1** into the lignins of CAD-deficient plants. A) The four products possible from 8–O–4-cross-coupling with lignin S and G units **2**. **3GG** is not observed in vitro or in vivo (in tobacco). B) Possible products from coupling at the aromatic sites (4-O- or 5-), resulting in hydroxycinnamyl aldehyde end-units (**5** or **6**) in lignins.

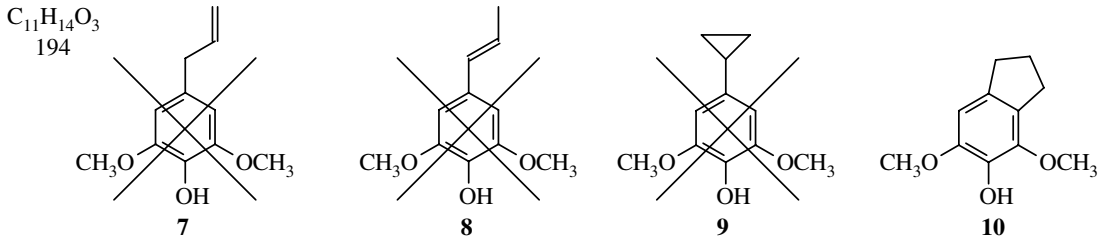


Fig. 2. Possible structures for the MW 194 marker compounds from thioacidolysis followed by Raney-Ni desulfurization.

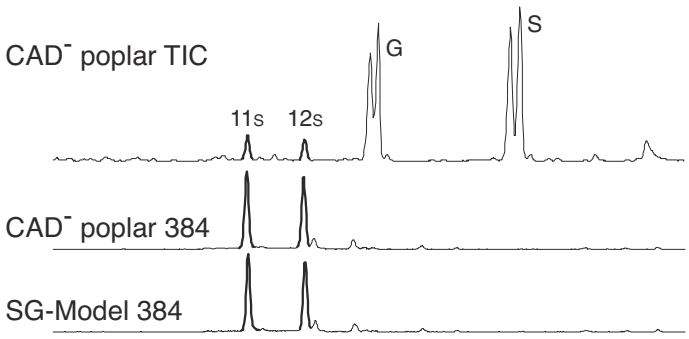


Fig. 3. GC-MS traces showing the two thioacidolysis CAD markers in the transgenic poplar (total ion chromatogram and selected-ion chromatogram (m/z 384, silylated) and the analogous selected-ion chromatogram from thioacidolysis of the 8–O–4-model compound for **3SG**.

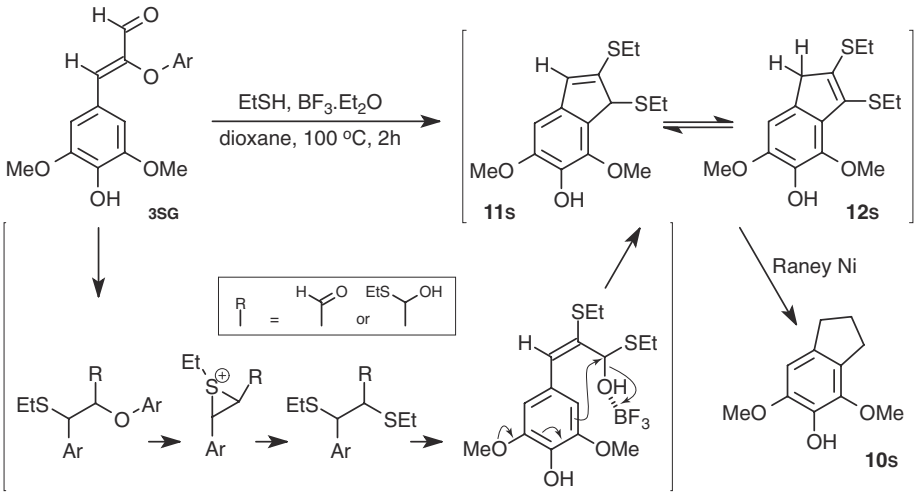


Fig. 4. Thioacidolysis of the model compound for **3SG** or of similar units in the lignin.

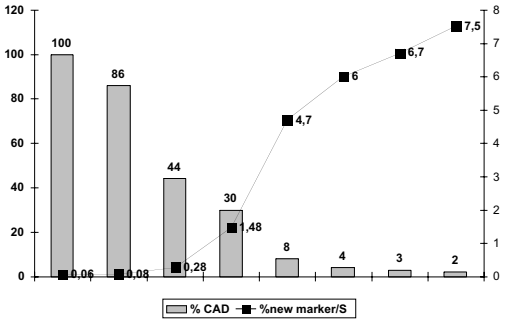


Fig. 5. Relative importance of the thioacidolysis markers of CAD deficiency in poplars, [%(**11S** + **12S**)/Conventional S], as a function of residual CAD activity level (100% = control level). Sample with 44% residual activity does not show any red xylem phenotype. Sample with 30% residual activity correspond to the ASCAD21 line displaying a red phenotype. Samples with activity <10% are OGY70ASCAD different lines. Data from Lapierre, INRA, France.

Marker Compounds for Enzyme Deficiencies in the Lignin Biosynthetic Pathway. 2. COMT

F. Lu, J.M. Marita, J. Ralph, I. Mila, B. Pollet, and C. Lapierre.

Introduction

As described more fully in the introduction to Part 1, the preceding article, marker compounds that are diagnostic for a given gene downregulation and allow the degree of change to be elucidated are vital particularly when various levels of down-regulation need to be assessed.

COMT is the final methylating enzyme in the production of sinapyl alcohol monolignols. Its down-regulation results in a build-up of 5-hydroxyconiferyl alcohol which is exported to the wall and intimately incorporated into lignins forming novel benzodioxane structures in the polymer, as shown in Fig. 1 of a preceding Report "5-Hydroxyconiferyl alcohol as a monolignol in COMT-deficient angiosperms". Thioacidolysis, and also our DFRC method, produce diagnostic marker compounds from these novel lignin units.

Results and Discussion

Thioacidolysis of COMT-deficient brown-midrib mutants had earlier suggested that 5-hydroxyconiferyl alcohol was a constitutive unit of those lignins. A monomeric compound **1** was released, Fig. 1. More recently, a dimeric benzodioxane compound **2** was also observed following Raney-Ni desulfurization of thioacidolysis products from COMT-deficient poplar transgenics. The DFRC degradative method also produced diagnostic benzodioxane dimers **3**, but no monomeric 5-hydroxyguaiacyl products. Model studies showed that the DFRC method leaves benzodioxanes completely intact, whereas thioacidolysis partially cleaves them (into monomeric products). The absence of monomeric 5-hydroxyguaiaicyl DFRC products strongly suggests that all such units in the lignins are present in benzodioxane structures. GC(-MS) data for the DFRC products are shown in Fig. 2.

These benzodioxane marker compounds are not released in great amounts, but are nevertheless diagnostic and their detection is sensitive enough that they are likely to become useful measures of COMT-downregulation.

Mechanistic Implications

The release of compounds **3G** and **3S** following DFRC degradation are more diagnostic than might at first be assumed. The following observations are relevant.

1. The double bond implies that the 5-hydroxyconiferyl alcohol unit had coupled β-O-4 to a syringyl or guaiacyl unit in lignin. It could not have been another 5-hydroxyguaiacyl unit or dimer **3** would not be released. In fact, it also is not likely to have been incorporated by reaction with another monolignol, since cross-coupling studies are showing that it is coniferyl or sinapyl alcohol that couples at the β-position and the 5-hydroxyconiferyl alcohol couples at the 4-O-position. This is borne out in the literature by the isolation of several lignans similar to **3** but none have been reported with reverse coupling modes (i.e. coupling at the β-position on the 5-hydroxyconiferyl alcohol unit).

2. Since we can readily identify both compounds **3S** and **3G**, we obviously know that monolignols, sinapyl or coniferyl alcohol, then react at their β-positions with the newly formed hydroxyguaiacyl unit (at its 4-O-position) on the growing polymer.
3. The NMR data already indicates that the phenol on the guaiacyl unit attached to the benzodioxane in the lignin is etherified. Since DFRC releases units **3**, there must also be further etherification presumably by another monolignol (at its β-position)– again, it could not be another 5-hydroxyconiferyl alcohol or it would not release dimer **3**. Further DFRC experiments will be able to establish what proportion of these released benzodioxanes were originally etherified.

Conclusions

Marker compounds (released following the application of degradative methods) for both COMT-deficiency have been identified and their sources in the novel lignins elucidated. Thioacidolysis monomeric and dimeric, and DFRC dimeric COMT markers originate from benzodioxane structures in the lignins which themselves result from the incorporation of 5-hydroxyconiferyl alcohol monomers. Most importantly they establish that the novel 5-hydroxyconiferyl alcohol monomer is coupling with normal lignin units, and that coniferyl alcohol will then couple with the new 5-hydroxyguaicyl end unit that is formed. As such, the novel monomer appears to be incorporating intimately into the lignin. The thioacidolysis and DFRC marker compounds become valuable in assessing the effects of various levels of down-regulation in COMT-deficient plants.

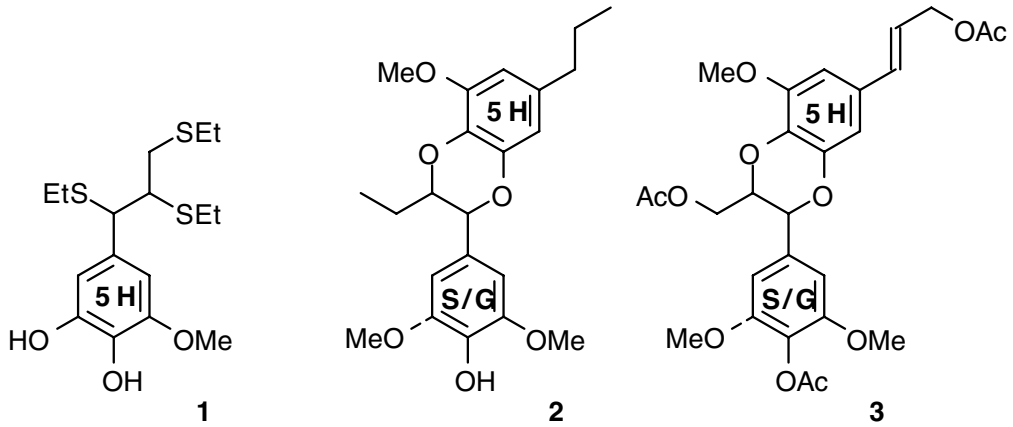


Fig. 1. Marker compounds for 5-hydroxyconiferyl alcohol incorporation into lignins (and COMT deficiency); thioacidolysis monomeric marker **1**, dimer **2** (following Raney-Ni desulfurization), and DFRC marker **3**.

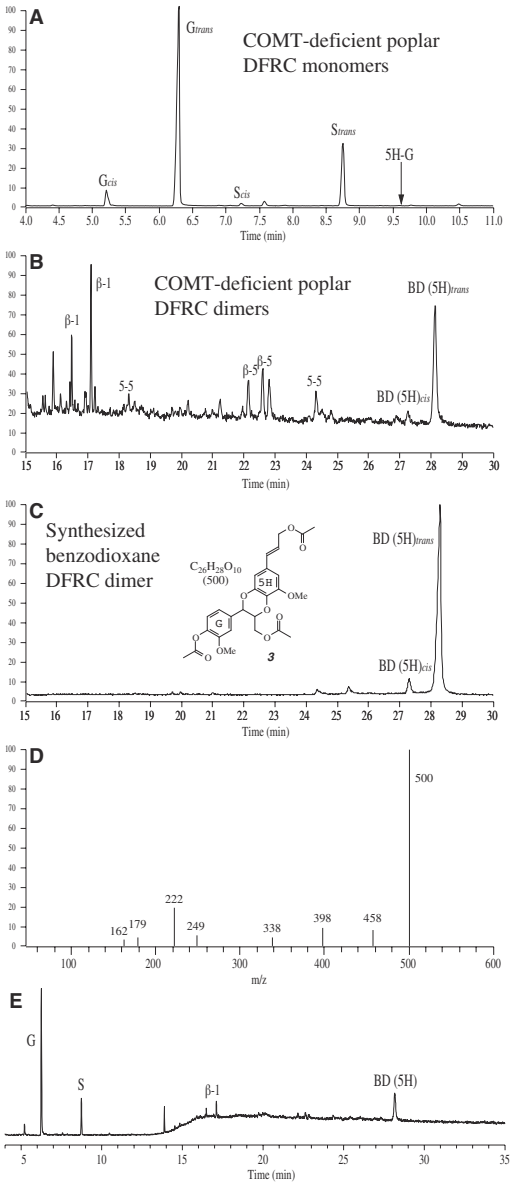


Fig. 2. DFRC Benzodioxane Markers for COMT-deficiency. A. DFRC monomer region of COMT deficient poplar showing no 5-hydroxyguaiacyl monomers — thioacidolysis monomers contain the diagnostic 1,2,3-trithioethylguaiacylpropane. B. The DFRC dimers region showing the benzodioxane components. C. The synthesized DFRC benzodioxane product for authentication. D. MS of the guaiacyl benzodioxane. E. Low-res GC-MS TIC of entire chromatogram (monomer and dimer regions) showing that the benzodioxane in this gene-silenced poplar lignin is released in easily detectable amounts.

The First 3D NMR of Un-enriched Lignin. Proof of Benzodioxanes in a COMT-deficient Angiosperm

J. Ralph and J.M. Marita

Introduction

NMR spectroscopy is an exceptionally powerful tool for determining organic structures, even in a polymer as complex and heterogeneous as lignin. We have recently reviewed the application of 2D NMR to lignin, providing a multitude of colored figures to aid interpretation - this article is also available from our web site: http://www.dfrc.ars.usda.gov/DFRCWebPDFs/1999-Ralph-TAPPI-55.pdf.

Three–dimensional (and/or higher-dimensional) NMR experiments are routinely applied to labeled proteins. Some success has come from applying the 3D HMQC-TOCSY (with one ^{13}C and two ^{1}H axes) to synthetic lignins, and to uniformly ^{13}C-enriched lignins, but the literature suggests that such experiments are difficult with real isolated unlabeled lignins. Here we report not only the first 3D NMR of an isolated lignin that has not been ^{13}C-enriched, but one which firmly establishes the presence of benzodioxane units in a COMT transgenic, as described further in an accompanying Report ("5-Hydroxyconiferyl alcohol as a monolignol in COMT-deficient angiosperms"). The "isolation" of the novel structural unit by 3D NMR is, in our humble opinion, spectacular.

Experimental

The 2D (two-dimensional) NMR spectra were taken on our Bruker DRX-360 instrument fitted with a 5-mm ^{1}H/broadband gradient probe with inverse geometry (proton coils closest to the sample). The conditions for all samples were ~100 mg of acetylated lignin in 0.4 ml of acetone-d_6, with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). The standard Bruker implementations of gradient-selected versions of inverse (^{1}H-detected) heteronuclear multiple quantum coherence (HMQC) experiments were used. The 3D (three-dimensional) NMR experiment was acquired on a Bruker DMX-750 instrument fitted with a 5-mm triple-resonance (^{1}H, ^{13}C, ^{15}N) gradient inverse probe. The 3D gradient-selected TOCSY-HSQC experiment was trivially modified to a two-channel version from "mlevietf3gs3d" (a 3 channel experiment). The TOCSY spin lock period was 70 ms in this case; 2D TOCSY experiments indicated that, whereas 125 ms was optimal, 70 ms also provided suitable TOCSY transfer especially for new benzodioxane structures. Carbon/proton designations are based on conventional lignin numbering. Lignin sub-structures are labeled by the convention established in a recent book chapter (Advances in Lignocellulosics Characterization).

Results and Discussion

Figure 1 shows the first 3D gradient-selected TOCSY-HSQC spectrum of a natural ^{13}C-abundance lignin. It is the acetylated lignin from the sense-suppressed COMT transgenic taken on a high magnetic field 750 MHz instrument. In less than 24 hours, the 3D experiment provided ample sensitivity to authenticate all of the major units in the natural ^{13}C-abundance sense-suppressed COMT transgenic lignin. Analogous experiments run on a 360 MHz instrument over 60 h were similarly successful.

In the 3D TOCSY-HSQC experiment, spectra are acquired with three orthogonal dimensions, labeled F_1, F_2, and F_3. The acquired dimension is F_3 (proton). F_2 is carbon and F_1 is proton. A slice in the F_2-F_3 plane is basically a 2D ^{13}C-^{1}H HSQC spectrum at a given proton chemical shift (defined by the position along the proton F_1 axis). 2D F_2-F_3 projections/slices for the prominent structures in the lignin are shown in Figs 1d-g. The slices show: a) the 3D contour map; b) a 2D HMQC for comparison with; c) the first 2D slice in the F_2-F_3 plane (which is essentially a 2D composite spectrum of all the units on all the other planes); d-g) F_2-F_3 slices at various proton frequencies (in F_1) showing almost perfect isolation of the major structural units in HSQC-type sub-spectra.

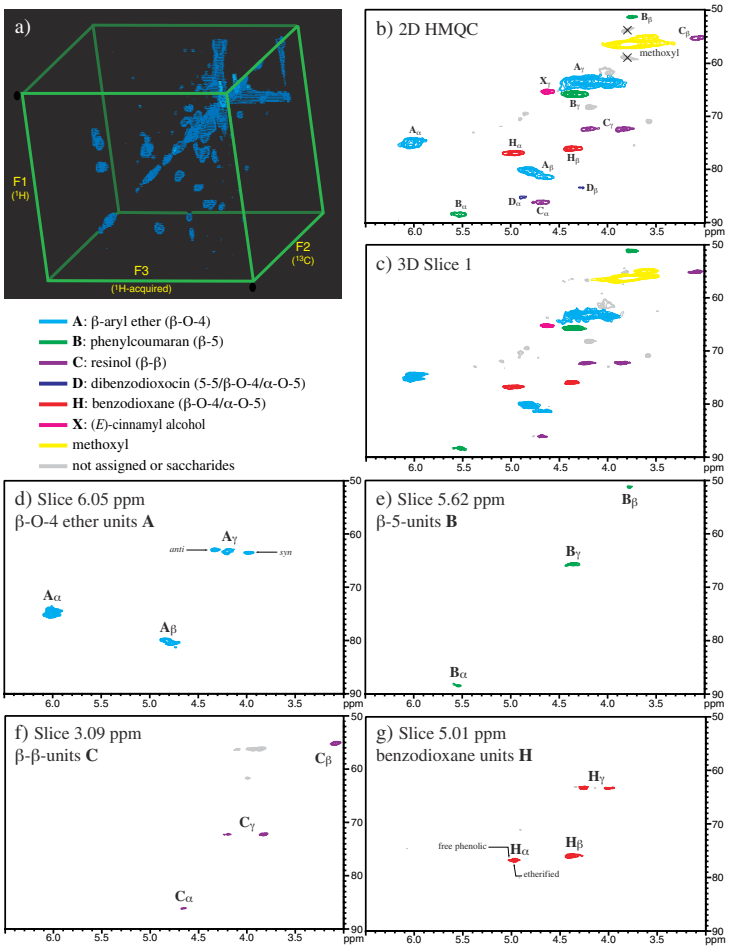
When a proton frequency is unique to a given structure, a "pure" F_2-F_3 slice and HSQC of only that structure can be obtained. F_2-F_3 slices for both the β-aryl ether **A** units and phenylcoumaran **B** units at their respective α-proton frequency along the proton F_1 axis show this phenomenon nicely, Figs 1d,e. Only ^{13}C-^{1}H correlations for the given unit are seen in each slice. Fig. 1d is a "pure" slice of β-aryl ether **A** units at the α-proton frequency of 6.05 ppm. This slice shows both *syn*- and *anti*-β-ether isomers. Slices either side of this slice (not shown) resolve *syn*- from *anti*-isomers. Fig. 1e is a "pure" slice of phenylcoumaran **B** units at the α-proton frequency of 5.62 ppm. However, a "pure" F_2-F_3 slice of the resinol **C** units is not obtainable at any of its proton frequencies. The best slice represents resinol **C** units at its β-proton frequency of 3.09 ppm, Fig. 1f. At this particular frequency some saccharides/methoxyl peaks are also detected but the slice uniquely isolates all of the resinol **C** units. The F_2-F_3 slice of the new benzodioxane structure is spectacular with its α-, β-, and γ-correlations fully resolved, Fig. 1g. In the 3D experiment, the γ-correlations of benzodioxane in the F_2-F_3 plane are nicely resolved and isolated from the plane at its α-proton frequency (5.01 ppm) as well as at the β-proton frequency (~4.47 ppm; not shown) along the F_1 axis. By obtaining a "pure" slice, there is no ambiguity between correlations of different structures. For example, the γ_1- and γ_2-correlations of the benzodioxane units that are unresolved from the γ_1- and γ_2-correlations of the β-aryl ether **A** units and the dibenzodioxocin **D** units in 2D spectra are unique to their respective 3D slices. This is the first reported identification of a new lignin component using 3D NMR experiments at natural abundance. The data in this slice agree with those from a benzodioxane model compound.

Another detail regarding benzodioxane units **H**, the degree of etherification, is revealed in the 3D spectra (and 2D — see Fig. 2 in the accompanying Report, "5-Hydroxyconiferyl alcohol as a monolignol in COMT-deficient angiosperms"). Unfortunately, the ball-milling step in the lignin isolation process produces extra phenolic groups, so isolated lignins have a higher phenolic content than natural lignins. In acetylated lignins, units that were free-phenolic in lignin become phenol-acetylated, whereas those that were originally etherified remain so. Phenol acetylation causes H_α in **H** units to move to a lower field (higher ppm). Thus, unetherified units have H_α at 5.04 ppm, whereas etherified units are at 4.96 ppm. The 2D slice for the 3D experiment shown in Fig. 1g has only a trace of correlations for the acetylated component. Other slices reveal slightly more. However, it appears that the benzodioxane units **H** are substantially etherified and therefore have been fully integrated into the polymer by further monolignol coupling reactions during lignification. Monomer substitution (5-hydroxyconiferyl alcohol for sinapyl alcohol) therefore appears to have been successfully accommodated in these transgenics. The substitution of L-fucose with L-galactose in fucose-deficient *mur1* mutants of Arabidopsis is a previously documented monomer substitution occurring in polysaccharides (Science 272: 1808), where the polymer biosynthesis is more highly structurally controlled than in lignification.

Conclusions

The presence of these novel benzodioxane units in an isolated lignin from a COMT-deficient angiosperm is diagnostically revealed by a 3D NMR experiment. The experiment is able to completely isolate the complete carbon-proton sidechain network onto its own 2D plane, authenticating the structure and establishing the elusive gamma-correlations. From the plant point of view, the finding indicates that the plant is capable of sending the intermediate monolignol 5-hydroxyconiferyl alcohol out into the cell wall for incorporation. The realization that novel units such as benzodioxanes can be tolerated in lignins should encourage further research into bioengineering plants with broad compositional changes in their lignins in order to achieve enhanced cell wall digestibility and/or reduction of negative environmental impacts of chemical pulping and bleaching related to papermakin

Fig. 1. 3D NMR (750 MHz) "isolation" of the major units in COMT-deficient transgenic poplar lignins. (a) A 3D gradient-selected TOCSY-HSQC spectrum (70 ms TOCSY mixing time) of a natural ^{13}C-abundance lignin (acetylated) from the sense-suppressed COMT transgenic; (b) 2D gradient-selected HMQC spectrum; (c) the first F_2-F_3 plane which is essentially a 2D ^{13}C-^{1}H HSQC spectrum; (d-g) 2D F_2-F_3 slices for the major structural units (**A**, **B**, **C**, and novel **H**). Note: this Figure is made to be viewed in color; please see the web version of these Research Summaries on our web site, or see the original paper containing these spectra: http://www.dfrc.ars.usda.gov/DFRCWebPDFs/2001-Marita-JCSPerkin-2939.pdf



Preliminary Evidence for Sinapyl Acetate as a Lignin Monomer in Kenaf

F. Lu and J. Ralph

Introduction

What does kenaf have to do with forages? Lignins of many agriculturally important crops and woody plants are acylated by various acids, although the biochemistry associated with such acylation remains unresolved and the genes are unknown. Nor is it known if lignin monomers (the hydroxycinnamyl alcohol monolignols) are first acylated to produce ester conjugates which are then incorporated by coupling and cross-coupling into lignin by the traditional free-radical coupling reactions, or whether acylation occurs *following* the monolignol radical coupling reactions or on the lignin polymer itself. These issues are becoming important to resolve as genes controlling the various processes and the functions of such acylation are sought in order to improve the utilization of plant resources, by ruminant animals for example. Acylated components have also been found to increase (on a lignin basis) when lignification is decreased by down-regulating enzymes in the monolignol biosynthetic pathway. Once we understand what the plant is trying to achieve with this lignin acylation, it might be possible to introduce additional drought-tolerance into forages, for example, as well as possibly impact the cell wall digestibility.

NMR of isolated kenaf bast fiber lignins suggested well over 50% lignin acetylation, almost entirely of the sidechains' primary aliphatic alcohols. Our "Derivatization Followed by Reductive Cleavage" ("DFRC") method, which cleaves ether linkages in lignins and releases analyzable monomers and dimers, leaves such esters intact. A modification (termed DFRC′), using propionyl analogs of the normal acetyl reagents, allowed us to establish that the native lignin was about 60% 9-acetylated and, more revealingly, that syringyl (3,5-dimethoxy-4-hydroxyphenyl) units were significantly acetylated whereas only traces of acetylated guaiacyl (4-hydroxy-3-methoxyphenyl) units could be detected.

How is it possible to establish whether monolignols are acetylated prior to the polymerization steps of lignification? Even finding acetylated monomers in lignifying tissues will not rigorously establish that they are involved in lignification. Structural analysis of the lignin polymer can provide a reasonably definitive answer. There is one lignification pathway that is significantly altered by pre-acetylation of the monolignols. That is the pathway in which the 9-OH on the monolignol becomes involved in post-coupling reactions, i.e. the pathway normally leading to 8–8-coupled (resinol) units **3**, Fig. 1. The key concept is the following. With the 9-position acetylated, 8–8-coupling can still presumably occur (the 9-OH is not required for the radical coupling step; the propenyl analog isoeugenol G-CH=CH-CH_3, for example, will also undergo 8–8-coupling), but the re-aromatization reactions following the radical coupling step can no longer be driven by the internal attack of the 9-OH on the quinone methide intermediate **QM2**, Fig. 1. The 9-acetylation prevents such a reaction. Other pathways must therefore be in effect producing other products. The important point is that the acetyl group can remain attached in non-resinol 8–8-coupling products, products that could not have arisen from post-coupling acetylation reactions.

Results and Discussion

In seeking preliminary evidence for sinapyl acetate incorporation, it didn't appear necessary to elucidate the full coupling and cross-coupling pathways for sinapyl acetate. All that was required was to show that sinapyl acetate **2**, in coupling and cross-coupling reactions, would give acetylated products that would produce DFRC′ 8–8-linked products identical to those that are released from

kenaf (lignins) and not from plants having non-acetylated lignins. Oxidation of sinapyl alcohol"**1** with H_2O_2/peroxidase or metal oxidants typically gives the lignan syringaresinol **3** as the predominant dehydrodimeric product, along with small amount of the 8–O–4-coupled product. In this study, sinapyl alcohol was oxidized with H_2O_2/peroxidase in a 20 mM buffer solution containing 20% acetone; syringaresinol **3** was the only 8–8-product, produced in over 90% yield. DFRC′ treatment of syringaresinol **3** yielded aryltetralin **6a**, Fig. 2, as a major product. Similar compounds are produced following thioacidolysis of 8–8-linked lignin units. Oxidation of sinapyl acetate **2** (Fig. 1) under similar conditions yielded a mixture of currently uncharacterized compounds retaining acetate groups. DFRC′ treatment of the mixture yielded **6c**, the diacetate analog of **6a** (from sinapyl alcohol). The structural analogy, from MS spectra (Fig. 2), indicates that sinapyl acetate also undergoes 8–8-coupling, and that at least one of the products **5** (Fig. 1, although the exact structure has not yet been determined) produces the aryltetralin **6c** following DFRC′ treatment. Oxidation of a mixture of sinapyl alcohol **1** and sinapyl acetate **2** must result in cross-coupling reactions to produce crossed 8–8-coupled structures **4**, since DFRC′ degradation now produces mono-acetylated aryltetralins **6b** in addition to the non- and di-acetylated analogs **6a** and **6c** (as evidenced by GC-MS).

It would be reasonable to expect that substructures **4** and **5** exist (in phenol-etherified form) in kenaf if sinapyl acetate participates in formation of its lignin. Selected ion chromatograms of TLC-fractionated DFRC′ products from kenaf lignin (Fig. 2) or whole kenaf cell walls clearly show the presence of all three DFRC′ products, compounds **6a-c**. Comparison of GC retention times and mass spectra of DFRC′ products with those from the *in vitro* coupling reactions of sinapyl alcohol and sinapyl acetate indicates that compounds **6b** and **6c** derive from sinapyl acetate. Compound **6a** derives from normal lignins, but the acetylated analogues **6b** and **6c** do not.

Although a great deal remains to be done to detail the coupling reactions, authenticate the nature and stereochemistry of the products, and fully elucidate their DFRC′ products, the preliminary data presented here appears to us to provide rather compelling evidence that sinapyl acetate is involved in lignification in kenaf, and is the likely source of the high 9-acetylation observed in kenaf bast fiber lignins. Detection of 9-acetates in syringyl 8–8-coupled DFRC′ products **6b-c** from kenaf suggests the existence of substructures which are likely formed from dehydrogenative coupling of sinapyl acetate itself or cross-coupling with sinapyl alcohol during the lignification process and provides evidence that acetates on kenaf lignin are formed through incorporation of sinapyl acetate, as a lignin precursor, into lignin macromolecules by radical coupling. Sinapyl acetate therefore appears to be an authentic lignin monomer in kenaf.

Conclusions

The preliminary evidence provided here that acylation is at the monolignol stage allows researchers to seek the substrates and presumed transferases involved in the specific acylation of monolignols, and to identify the genes responsible, allowing the process to be genetically manipulated.

The complete manuscript is available from our website at:
http://www.dfrc.ars.usda.gov/DFRCWebPDFs/2002-Lu-JCSCC-90.pdf

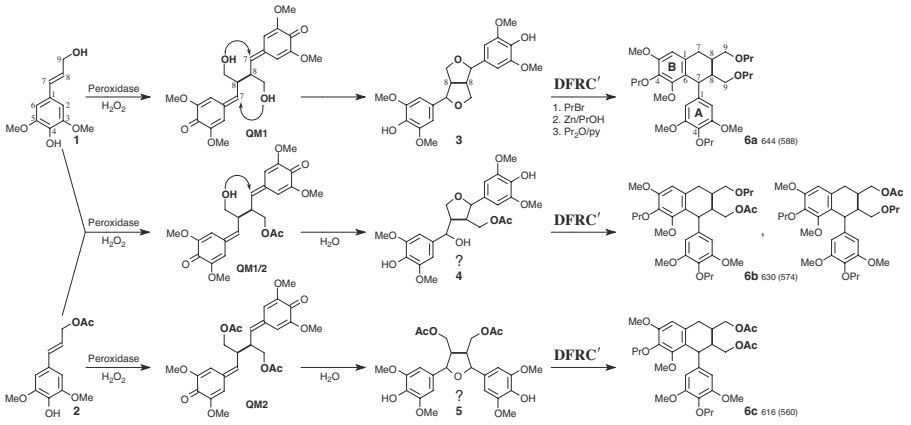


Fig. 1. The key to establishing whether monolignols are pre-acylated lies with the 8–8-coupling products (the resulting 8–8-bonds are bolded for emphasis). The traditional monolignol sinapyl al cohol **1** will dehydro-dimerize initially forming the 8–8-coupled bis-quinone methide intermediate **QM1**, which re-aromatizes by internal 9-OH attack on each quinone methide electrophilic 7-carbon to produce syringaresinol **3** as the overwhelmingly major product. When acylated sinapyl alcohol **2** dimerizes, it forms an analogous bis-quinone methide intermediate—**QM2**. However, **QM2** can not be re-aromatized by internal trapping. The products have not yet been characterized but it is logical that structure **5** would arise from water attack on one quinone methide moiety with the resulting 7-OH attacking the other quinone methide to form a tetrahydrofuran; analogous products have been found in ferulate dehydrodimers. When **1** and **2** radicals cross-couple, the intermediate bis-quinone methide **QM1/2** now has one quinone methide moiety which can be internally trapped by the single 9-OH to form a single tetrahydrofuran ring, but the other quinone methide can only be re-aromatized by attack of an external nucleophile. The resulting product, presumably **4**, therefore retains at most a single acetate. Products **6** result from DFRC′-degradation (the DFRC modification using only propionate reagents) of the non-, mono-, and di-acetylated 8–8-coupled units. See text for details. The MWs of DFRC′ products are given beside the compound numbers, with the MWs of the base peaks (used for Fig. 2) in bracket

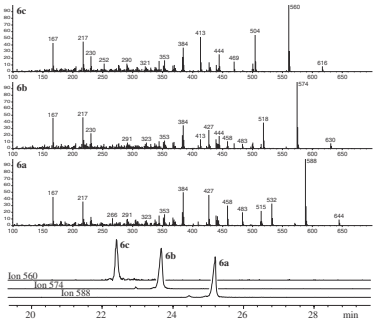


Fig. 2. Mass spectra and selected-ion chromatograms of DFRC products from kenaf showing the presence of aryltetralin products containing 0, 1, and 2 acetates.

Lignin Concentration in Plant Samples Determined by the Acetyl Bromide Soluble Lignin Spectrophotometric Method

R. S. Fukushima and R. D. Hatfield

Introduction

Lignin, a polyphenolic compound, inhibits the digestion of plant cell wall components, and this effect becomes more pronounced as the forage matures. For the understanding of how mechanistically lignin acts upon the cell wall carbohydrates it is imperative to determine its concentration with acceptable accuracy and precision. Most of the current methods for quantifying lignin are gravimetric. An alternative method has been proposed, which is based upon solubilization of lignin into a solution of 25% acetyl bromide in glacial acetic acid and reading it at 280 nm, the acetyl bromide soluble lignin (ABSL) method. The objective of this project was to compare lignin results obtained from the Klason lignin and ABSL methods of lignin quantification.

Materials and Methods

Plant materials included a range of forage plants and pine as a woody plant comparison (see Table 1). For the ABSL method procedure samples were incubated with 25% acetyl bromide in glacial acetic acid for 2h at 50° C, before diluting 20 fold in acetic acid and reading the absorbance at 280 nm. Isolated lignin used as standard was obtained through extraction with acidic dioxane (0.2NHCl-Dioxane). Klason lignin (KL) was the acid insoluble residue remaining after total hydrolysis with concentrated sulfuric acid of cell wall polysaccharides.

Results and Discussion

Lignins were extracted from the respective plants using HCl-dioxane and used as standards to develop regression equations to calculate lignin concentration in the original plant cell wall material. Table 1 shows the regression equation for each plant analyzed and the corresponding ABSL values.

As a general rule, regression slopes were similar at different maturity stages. The only exception was corn stalk that had a higher slope than all other samples (Table 1). It would appear that exchanges among some DL regression equations might be possible, therefore only one type of lignin (DL) isolate is necessary to use as a standard for determining lignin concentration in wide range of plants, regardless of origin, anatomical part or maturity stage. Once a regression curve is developed calibration will be required only occasionally. All intercepts but the one from corn stalk were close to zero.

Alfalfa and red clover lignin values determined through the ABSL method were lower than those obtained by the Klason's method. Pine showed higher concentration with the ABSL procedure (Table 1). The ratio between ABSL and KL were from 0.81 to 1.58 with legumes typically showing ratios below 1.0. This observation could be attributed to the hypothesis that Klason lignin method for legumes has some protein contamination and the ABSL method may account for any acid soluble lignin that otherwise would be lost during the strong acid digestion in Klason type lignin procedures.

Conclusion

The spectrophotometric procedure reported here employing dioxane-HCl lignin as a standard appears to be applicable to quantifying lignin concentration in a wide range of forage samples.

Table 1. Regression equations obtained from standard curves of DL preparations and lignin concentration (g kg^{-1} DM)[1] obtained through two analytical procedures

Sample name	Regression equation	ABSL	KL	ABSL/KL
Corn stalk	X = (Y' + 0.1244)/20.48	81.3	76.7	1.06
Alfalfa Y	X = (Y' + 0.0702)/17.20	99.7	123.0	0.81
Alfalfa M	X = (Y' + 0.0502)/17.15	127.9	130.4	0.98
Bromegrass	X = (Y' + 0.0709)/18.63	112.7	102.2	1.10
Bromegrass M1	X= (Y' +0.0981)/17.98	115.7	100.4	1.15
Bromegrass M	X = (Y' + 0.0981)/17.98	132.2	109.8	1.20
Red clover	X = (Y' + 0.0154)/16.13	63.1	71.2	0.89
Pine	X = (Y' + 0.0955)/17.747	404.7	256.0	1.58

[1]Values are means of two observations. Y – young; M – mature (1 and 2 refer to two different maturity stages); X – Concentration of ABSL (mg/mL); Y' – absorbance readings; ABSL – acetyl bromide soluble lignin; KL – Klason lignin.

Spectral Characteristics of Lignins Isolated with Acidic Dioxane

R. S. Fukushima and R.D. Hatfield

Introduction

Lignin is a complex phenolic polymer, composed of phenylpropanoid units and is present in forage cell walls. It is generally believed that lignin and its cross-linking to structural polysaccharides are the major impediments to fiber digestion. Spectrophotometric methods (e.g. the acetyl bromide soluble lignin method, ABSL) require isolation of lignin from the cell wall. Scanning of isolated lignins in a given spectral range provides clues for identifying variability in lignin composition and concentration. Also, the spectra are useful for evaluating the acetyl bromide soluble lignin method to determine if side reactions have occurred such as excessive degradation of carbohydrates or lignin.

Materials and Methods

Dioxane lignins were extracted from the cell wall of following plants; corn stalk, alfalfa, bromegrass, and loblolly pine using acidic dioxane (0.2 NHCl-dioxane). Dioxane lignins (DL) were dissolved in 25% acetyl bromide solution and heated for 2 hours at 50 °C before determining the spectral characteristics in the wavelength range of 250 to 350 nm. For comparison cell wall materials were treated with acetyl bromide (ABSL) method and the soluble lignin analyzed in the same spectral range.

Results and Discussion

Acetyl bromide soluble lignin spectral characteristics of DL extracts were similar to those of the original cell walls (Figure 1A and B). One would expect lignin to give similar spectra irrespective of the original source from which it is extracted since the major components that make up lignin macromolecules are the same (i.e., coniferyl and sinapyl alcohols). Acetyl bromide solubilized lignin should produce an absorbance maximum at 280 nm. If the lignin is unusually rich in syringyl units the maximum can be shifted slightly to 275 nm. The shoulders at 300 nm, evident in the grass samples, are due to hydroxycinnamates attached to the lignin.

Generally spectra from the different DL were similar. One exception was corn stalk DL standard that had a higher tracing than all other samples (Figure 1A). The deviation of the corn sample may be due to the unusually high levels of hydroxycinnamates, especially *p*CA attached to the lignin. This can be seen in the nitrobenzene products (data not shown) where *p*CA is the second largest component recovered. Such high levels of *p*CA could alter the acetyl bromide lignin spectrum. Although *p*CA has an absorption maximum 300-310 nm, it also has a strong shoulder at 280nm that would add to the lignin absorption maximum at 280 nm. Earlier work with milled-wood-enzyme lignins isolated from corn revealed that approximately 20% of this lignin fraction was made up of *p*CA. Bromegrass in this study had *p*CA levels that were one third those of corn.

Lignins isolated from cell walls using HCl-dioxane have nearly identical spectral characteristics as the lignin in the original cell walls. Such lignins provide good standards for calibrating spectrophotometric methods of determining lignin concentrations.

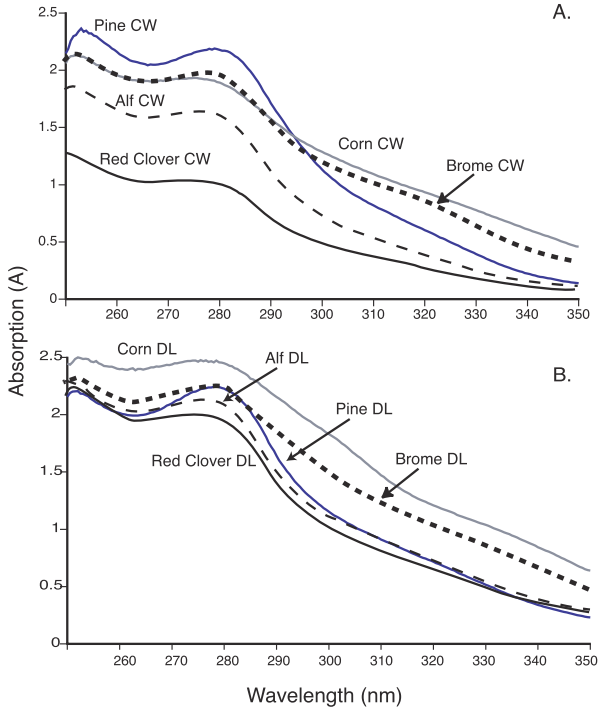


Fig. 1 Spectral characteristics of acetyl bromide soluble lignins. A. Acetyl bromide soluble lignin spectra obtained from isolated cell wall samples. B. Spectra obtained from dioxane-HCl extracted lignins used to produce standard curves of acetyl bromide soluble lignins.

Carbohydrate Profile in Two Isolated Lignins

R. S. Fukushima and R.D. Hatfield

Introduction

Lignin is a complex phenolic polymer forming an integral part of forage cell walls that cannot be digested by ruminants and often limits digestion of other wall components (i.e., carbohydrates and protein). Lignin concentrations must be determined with acceptable precision and accuracy in order to be useful as a predictor of nutritional value of forages. A method for measuring lignin in forages has been proposed which is based upon solubilization of lignin into a solution of 25% acetyl bromide in glacial acetic acid, the acetyl bromide soluble lignin (ABSL) method. Lignin is read at 280 nm; however, as with any spectrophotometric method, a reliable standard is needed to develop calibration curves. Among several options to extract and isolate lignin there is the acetyl bromide lignin (AcBrL) which employs the same acetyl bromide solution for ABSL and acidic dioxane lignin (DL) which employs dioxane acidified with 2 N HCl. This experiment was conducted to assess the type and amount of contaminants present in these two forms of lignin in order to asses their suitability as a standard.

Materials and Methods

The following plant materials were examined: corn stalk; alfalfa; bromegrass and loblolly pine. To prepare the cell walls, ground samples were sequentially extracted with water, ethanol, chloroform and acetone in a Soxhlet apparatus, until no color leached from the walls. Acetyl bromide reagent (25% AcBrin acolic acid, w/w) was used to solubilize lignin from plant cell walls. Lignin was recovered after neutralization and filtration. For comparison, lignin was isolated using 0.2 NHCl-dioxane reflux method. Neutral sugars, total uronosyls and total N were determined by general procedures.

Results and Discussion

AcBrL extraction method for obtaining lignin. Leaf tissue had higher concentrations of neutral sugars than the stems (Table 1). Total sugar concentration in the AcBrL varied from 1.44 to 36.92%. With the exception of pine, sugar contamination in AcBrL was high. Composition of individual neutral sugars was similar among the palnts with glucose (presumably from cellulose) being the highest. Protein content of AcBrL was considerable (Table 1).

DL extraction method for obtaining lignin. Utilization of acidic dioxane to extract and isolate lignin was tested to determine if this procedure would reduce the level of contamination, particularly carbohydrates. Uronosyl content of DL showed no differences among plants with the exception of pine. Neutral sugar concentration in DL showed a drastic reduction in sharp contrast to lignins obtained through extraction with acetyl bromide. The most prevalent sugar in the DL was xylose followed by arabinose and glucose which was a different profile from the AcBrL; sugar composition resembled that of hemicelluloses. Xylose was detected at higher concentration in grasses in comparison to alfalfa and pine. Other sugars exhibited no substantial trend to any species probably because the extraction conditions removed most of sugars such that quantity of the remaining sugars would be little enough to have any significant biological meaning. Apparently, there was no maturity effect on sugar composition. DL samples showed levels of protein varying from 9.5 to 71.5 g kg^{-1}

lignin; grasses exhibited lower concentration of protein than legumes and pine had the lowest content (Table 2).

Conclusions

These findings indicate that lignin extracted with acidic dioxane would be a better reference standard for lignin analysis by the acetyl bromide soluble lignin method.

Table 1. Total uronosyls, neutral sugars, individual composition of neutral sugars and protein content in the AcBrL (g kg^{-1} lignin)[1]

Sample name	Total uronosyls	Neutral sugars	Ara	Rha	Gal	Glc	Xyl	Man	Protein
Corn stalk	5.2	212.4	6.0	0	0.5	198.1	7.8	0	15.6
Alfalfa leaf	11.7	357.5	0.4	1.3	2.0	348.5	3.2	2.0	26.9
Alfalfa stem	18.8	158.6	0	0.4	0.4	156.5	1.3	0	33.6
Bromegrass leaf	7.3	352.3	7.5	0.2	0.5	340.8	3.3	0	18.0
Bromegrass stem	6.0	97.7	7.7	0	0	84.6	5.4	0	36.5
Pine	3.7	10.7	0	0	0	9.4	0	1.3	2.3

[1]Ara – arabinose; Rha – rhamnose; Gal – galactose; Glc – glucose; Xyl – xylose; Man – mannose.

Table 2. Total uronosyls, neutral sugars, individual composition of neutral sugars and protein in the dioxane lignin (g kg^{-1} lignin)[1]

Sample name	Uronosyls	Neutral sugars	Ara	Rha	Gal	Glc	Xyl	Man	Protein
Corn stalk	16.9	38.3	7.6	0.4	0.7	3.9	25.7	0	22.5
Alfalfa Y	12.4	22.8	1.9	0.5	1.4	2.4	16.6	0	71.5
Alfalfa M	12.5	19.6	0.9	0.5	1.2	2.6	14.4	0	41.7
Bromegrass Y	16.7	40.3	12.2	0	0.5	3.2	24.4	0	26.4
Bromegrass M1	14.5	34.1	11.5	0	0.2	2.7	19.7	0	34.2
Bromegrass M2	15.3	36.6	11.9	0	0.5	2.8	21.4	0	37.2
Pine	3.7	18.4	1.1	0	3.3	3.6	4.9	5.5	9.5

[1] Y – young; M – mature (1 and 2 refer to two different maturity stages).

Rumen Microbiology

The Bacteriocins of Ruminal Bacteria and Their Potential as an Alternative to Antibiotics
J.B. Russell and H.C. Mantovani

Introduction

Simple stomached animals lack enzymes that can degrade cellulose or hemicellulose, and fibrous materials are poorly utilized. Ruminant animals do not synthesize fiber digesting enzymes, but they have formed a symbiotic relationship with ruminal microorganisms that can. The ruminant provides the microorganisms with a habitat for their growth, the rumen, and microorganisms supply the animal with fermentation acids, microbial protein and vitamins. However, ruminal fermentation also produces methane and ammonia, and these end-products are a loss of energy and nitrogen, respectively. When methane is inhibited, acetate production declines, the fermentation is diverted towards propionate, and energy retention increases. If proteins can be protected from ruminal deamination, ammonia declines and the animal has more amino acids for its nutrition. Some ruminal bacteria produce lactic acid at a rapid rate, and this acid can cause pronounced declines in ruminal pH, founder, and in severe cases, even death of the animal. Ruminant nutritionists, farmers and ranchers have used ionophores and other antibiotics to modify ruminal fermentation and increase the efficiency of feed digestion. However, there has been an increased perception that antibiotics should not be routinely used as feed additives. Some bacteria produce small peptides (bacteriocins) that inhibit gram-positive bacteria, and the bacteriocin, nisin, had effects on ruminal fermentation that were similar to the ionophore, monensin. However, preliminary results, indicated that mixed ruminal bacteria degraded nisin and became resistant. A variety of ruminal bacteria produce bacteriocins, but the effect of these peptides on ruminal fermentation had not been examined.

Materials and Methods

Ruminal fluid (diluted 1 to 10 or 1 to 100 in basal medium) was streaked onto agar-plates and incubated anaerobically at 39˚C. Colonies that developed after 18 h were picked, transferred to broth. Isolates (n = 90) were then spotted onto basal agar and the plates were incubated at 39˚C. After 24 h of incubation, the plates were overlayed with soft agar seeded with approximately 10^5 *S. bovis* JB1 cells ml^{-1}. The plates were re-incubated for another 24 h at 39˚C, and each isolate was scored for its ability to create a distinct zone of clearing ($\geq$ 3 mm) in the agar overlay. HC5 (the isolate that produced the largest zone of clearing, see above) was grown overnight in basal medium. Samples were diluted 10-fold and subjected to a heat treatment to lyse the cells. 16S rDNA was amplified using universal 27F and 1492R eubacterial primers. PCR products were cloned. Clones containing the 16S rDNA insert were sequenced. Sequences were assembled and aligned to known *Strepcococcus* 16S rRNA sequences using ClustalX.

Stationary phase *S. bovis* JB1 cells were harvested by centrifugation, washed anaerobically in basal medium lacking ammonia, and re-suspended in 10 ml of the same medium. The washed cell suspensions were energized with glucose and some suspensions were treated with either nisin or partially

purified *S. bovis* HC5 bacteriocin. Samples were centrifuged through silicon oil. The microcentrifuge tubes were frozen, and the bottom of the tubes containing the cell pellets were removed with a pair of dog nail clippers. Potassium in cell pellets was determined with a flame photometer.

Stationary phase *S. bovis* HC5 cells were re-suspended in acidic sodium chloride (100 mM, pH 2.0, 2 h , 4 ˚C). The cell suspensions were re-centrifuged to remove cells, and the cell-free supernatant was lyophilized. The lyophilized material was re-suspended in sterile water. The bacteriocin extract was then applied to an SP Sepharose column. The final purification of the active peptide was completed by re-injecting the active fractions onto a C16 column using ethanol as a carrier solvent. The active fractions were then collected and lyophilized. Purified bacteriocin from *S. bovis* HC5 was subjected to Edman degradation analysis.

Results

A freshly isolated *S. bovis* strain (HC5) had antimicrobial activity against a variety of *S. bovis* strains and other of gram-positive ruminal bacteria. This activity was primarily associated with the cells, but it could be removed with acidic NaCl. The activity was purified by HPLC. The purified peptide had an N-terminal that was VG-RYAS-PG-SWKYV-F. Amino acid residues that did not correspond to amino acids commonly found in proteins had approximately the same position as dehydroalanines found in some lantibiotics. The N-terminal amino acid sequence of bovicin HC5 showed similarity to a lantibiotic precursor of *S. pyogenes* SF370, but the identity was only 55%. Further work will be needed to locate the gene, but bovicin HC5 appears to be a novel bacteriocin. Because the purified extract caused potassium efflux from glycolyzing *S. bovis* JB1 cells, it appears to be a pore forming bacteriocin.

Discussion

Because some ruminal bacteria can produce bacteriocins, there were speculations that these compounds might provide "effective alternatives to ionophore antibiotics as feed supplements." Because the rumen is a highly diverse bacterial ecosystem that is inhabited by many different species (and strains within a species) and bacterial competition is very intense, inoculation would not necessarily increase the amount of a bacteriocin in the rumen. However, cattle are often fed silages, and silage fermentation is a batch culture system that favors rapidly growing lactic acid bacteria. When inoculated silages, "*S. bovis* grew faster than any of the commercial species tested and resulted in the most homolactic fermentation." Given the observation that some *S. bovis* produce very potent bacteriocins, silage fermentation could be a vehicle for delivering bacteriocins to the rumen..

In Vitro Fermentation of Polydextrose by Bovine Ruminal Microorganisms
P.J. Weimer and S.M. Abrams

Introduction

Polydextrose (PD) is a water-soluble condensation polymer of dextrose (D-glucose) that is used as a bulking and texturizing agent for the manufacture of low-calorie foods. This use is based on the fact that PD provides physical and flavor characteristics of fat, yet it is only partially metabolized in monogastric animals. Unsold commercial PD is occasionally available as a ruminant feed. However, no data are available on the ruminal utilization of PD, and nutritionists have no information on which to evaluate informal claims that PD can serve as a starch substitute in ruminant rations. This study was carried out to compare PD with two starches and two fiber sources in terms of fermentation kinetics and product formation by mixed ruminal microbes in vitro, and by pure cultures of carbohydrate-fermenting ruminal bacteria.

Methods

PD was obtained from Bower's Feed and Grain, (Wrightsville, WI), which obtained the material as a manufacturer's overrun. Purified corn starch (CS), potato starch (PS) and Sigmacell 50 microcrystalline cellulose (SD50) were obtained from Sigma. Barley (BH; field-grown, first cut, 55.5% NDF, 35.8% ADF); was air-dried and then ground through a Wiley mill having a 1-mm screen.

In vitro fermentations were conducted using a gas pressure transducer-based measurement system having 40 experimental channels, plus 4 channels dedicated to relative barometers and 2 channels dedicated to absolute barometers. Two separate fermentations runs were conducted in Goering-Van Soest buffer. Each experiment was carried out using an inoculum from a different fistulated, lactating Holstein cow fed a total mixed ration (corn silage, corn grain, alfalfa hay, soybean meal, and supplemental vitamins and minerals). For each run, fermentations of each substrate were conducted in quadruplicate in serum vials (nominal 60 ml). Vials contained 80 mg of dry matter (PD, CS, PS, SC50, BH, all weighed to 0.1 mg). Blank vials, prepared in the same manner but lacking added substrate, were also run to permit subsequent calculation of net gas production from experimental samples. End products of the fermentation were determined by HPLC.

Growth experiments with pure cultures were performed in a modified Dehority medium in microtiter plates inside an anaerobic glovebag (5% H_2/95% CO_2), with eight replicate wells per culture/substrate combination. Culture turbidity was determined using a microtiter plate reader contained within the glovebag. Plates were incubated in the instrument's heated chamber at 39 ^{o}C, and were automatically subjected to gyratory shaking at maximal speed for 30 s prior to each optical density (600 nm) reading, taken at 15-30 min intervals. Growth rates were calculated from the slope of the linear region of plots of ln OD_{600} versus time.

Model coefficients, and the data from fermentation, microbial growth and enzymatic hydrolysis experiments were analyzed using the ANOVA protocol of the GLM procedure of the SAS statistical software package (SAS Institute 1986), and comparisons among treatment means were conducted using Duncan's Multiple Range test at a significance level of $P<0.05$.

Results and Discussion

The monosaccharide composition (molar basis) of the the PD sample, analyzed following hydrolysis (2 *N* trifluoroacetic acid, 120 °C, 2h), was: glucose, 0.961; mannose, 0.017; xylose, 0.014; arabinose, 0.007. PD did not contain detectable NDF or measurable levels of N when assayed by combustion analysis. In general, PD was hydrolyzed by starch-degrading enzymes to an extent similar to that of potato starch, but less than that of corn starch.

Time courses of gas production from the polysaccharides are shown in Figure 1. PD was incompletely fermented by the mixed ruminal microbial culture. The fermentation proceeded without a significant lag, even in cultures not previously exposed to PD, suggesting that PD is readily depolymerized by hydrolytic enzymes produced by the indigenous microflora (Fig. 1). Gas production from PS or CS was adequately described by a single-pool exponential model (Table 1), with lag times of about 2 h, and first-order rate constants that averaged 0.43 h^{-1} and 0.17 h^{-1} respectively . By contrast, gas production from PD, SC50, or barley hay was best described by a two-component digestion model. The relatively small, more rapidly digesting pools of these substrates displayed little or no lag, while the larger and more slowly digesting pool displayed lag times of 2 – 10 h. For PD, one pool, which contained 20-47 per cent of the substrate, was fermented without a lag period and at rates similar to that of purified PS or CS. The second pool was fermented considerably more slowly than was cellulose. Both the total amount of gas produced and the concentrations of VFA after 48 h were about one-third lower from PD than from starch. The molar ratios of acetate to propionate ranged from 2.7 to 3.5 and were generally higher for PD than from the other purified polysaccharides

The growth rate of *Selenomonas ruminantium* D was higher on PD than on starches, while the reverse was true for *Streptococcus bovis* JB1, *Prevotella ruminicola* $B_1$4, and *Butyrivibrio fibrisolvens* H17c (Table 2). For all four strains, the low final culture densities obtained suggest that only a fraction of PD was utilized. Neither PD nor either of the starches supported growth of the fiber-digesting strain *Fibrobacter succinogenes* S85. Separate experiments in tube cultures (rather than in microtiter plates) revealed that PD did not support the growth of two other fiber-digesting strains, *Ruminococcus albus* 7 and *Ruminococcus flavefaciens* FD-1.

PD is produced by thermal processing of starch under vacuum in the presence of certain reactive cosubstrates, resulting in a product having a monosaccharide composition similar to that of starch, but with altered physical properties and all possible glycosidic linkages, with α-1,6-linkages predominating. The altered properties of PD are reflected in its reportedly poor digestibility in nonruminant animals. While the postruminal degradability of PD has not been examined, experiments with nonruminant animals suggests minimal digestibility. On the whole, PD appears to be unsuitable as a starch substitute for ruminants under production conditions.

Conclusions

The fermentation properties of PD suggest that this material, even if available at competitive prices, is a not an effective substitute for starch in ruminant feeds.

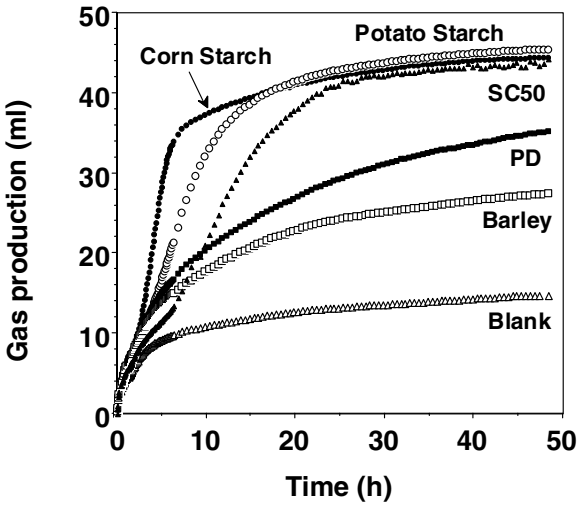


Fig. 1. Time course of in vitro gas production by mixed ruminal microorganisms fed polydextrose (PD), corn starch, potato starch, cellulose, or barley hay. Blank vials contained inoculum and carryover substrates, but no other fermentable substrate

Table 1. Kinetic constants for gas production from rapidly and slowly digesting pools of substrate during in vitro fermentations by mixed ruminal microflora.[a]

		A		k_1		L_1		B		k_2		L_2	
		(ml gas/g substrate)		(h^{-1})		(h)		(ml gas/g substrate)		(h^{-1})		(h)	
		Expt 1	Expt 2	1	2	1	2	1	2	1	2	1	2
Substrate	Model [b]												
Polydextrose	2	153.0 [e]	62.6 [d]	0.110 [f]	0.387 [e]	0.09 [e]	0 [d]	167.3 [d]	245.6 [d]	0.021 [e]	0.034	9.58 [c]	2.62 [d]
Corn starch	1	362.0 [d]	341.8 [c]	0.384 [c]	0.481 [d]	2.21 [c]	0.86 [c]	-	-	-	-	-	-
Potato starch	1	429.6 [c]	361.6 [c]	0.141 [e]	0.193 [f]	2.64 [c]	1.17 [c]	-	-	-	-	-	-
Cellulose	2	74.9 [f]	38.3 [d]	0.137 [ef]	0.087 [g]	0.68 [d]	0 [d]	271.4 [c]	337.0 [c]	0.125 [c]	0.112 [c]	7.85 [c]	8.14 [c]
Barley hay	2	117.8 [e]	64.3 [d]	0.225 [d]	1.82 [c]	0 [e]	0 [d]	107.0 [e]	180.0 [e]	0.052 [d]	0.060 [d]	8.03 [c]	2.15 [d]

[a] Kinetic parameters: A and B, gas production from rapidly and slowly digesting pools, respectively; k_1 and k_2, first-order rate constants for rapidly and slowly digesting pools, respectively; L_1 and L_2, lag time prior to initiation of gas production in rapidly and slowly digesting pools, respectively. Results are mean values of four replicates for each sample.

[b] Kinetic model: 1, single pool; 2, dual pool . For single pool model, only coefficients A, k_1 and L_1 are used. Values for goodness of fit of data to the models, calculated as in Weimer et al. (2000) and averaged within treatments for a given experiment, ranged from 0.9909 to 0.9991.

[cdefg] Means within a column having different superscripts differ ($P< 0.05$)

Table 2. Maximum specific growth rate constants and optical density increases during growth of pure cultures of ruminal bacteria on glucose, maltose, potato starch and polydextrose. Results are mean values from eight replicate cultures grown in microtiter plates.

	μ_{max} (h^{-1})				OD_{600} increase			
Bacterial strain	Glucose	Maltose	Potato Starch	PD	Glucose	Maltose	Potato Starch	PD
Butyrivibrio fibrisolvens H17c	0.26[b]	0.24[b]	0.35[a]	0.13[c]	0.93[b]	1.43[a]	1.46[a]	0.19[c]
Fibrobacter succinogenes S85	0.23[a]	0[b]	0[b]	0[b]	1.38[a]	0[b]	0[b]	0
Lachnospira multipara 40	0.66[a]	0[b]	0[b]	0[b]	1.87[a]	0[b]	0[b]	0
Prevotella ruminicola $B_1$4	0.59[a]	0.47[b]	0.36[c]	0.18[d]	1.39[a]	1.14[c]	1.21[b]	0.20[d]
Selenomonas ruminantium D	0.61[a]	0.56[b]	0.12[d]	0.25[c]	0.59[b]	1.11[a]	0.12[d]	0.30[c]
Streptococcus bovis JB-1	1.27[a]	1.02[b]	0.92[c]	0.43[d]	1.11[b]	1.23[a]	1.19[a]	0.30[c]

[abcd] Means within a row having different superscripts for a each parameter (μ_{max} or OD_{600} increase) differ (P<0.05).

Growth of Ruminal Bacteria in Culture Media Reduced by Photocatalytic Interaction of Resazurin and Cysteine

R.S. Fukushima, P.J. Weimer, and D.A. Kunz

Introduction

Many ruminal bacteria are strict anaerobes that require pre-reduced culture media for growth in the laboratory. Removal of oxygen and establishment of reducing conditions requires gassing with CO_2 or other O free gas, and addition of a chemical reducing agent. Cysteine (Cys) is a preferred reducing agent because of its low toxicity to the bacteria, but reduction of the medium by Cys is very slow, requiring preparation of the medium and addition of Cys well in advance of the medium's use. We have developed a simple means of accelerating the reduction by using light to exploit the photocatalytic reactivity of resazurin, a dye normally added to anaerobic culture media to assess oxygen status.

Material and Methods

Modified Dehority Medium (MDM) containing the standard concentration (2 mg/L) of resazurin was prepared under a CO_2 gas phase, without the evacuation and flushing of the gas phase that was normally performed to decrease the O_2 concentration in the medium. Cys was typically added at 1.25 g/l, and the tubes were exposed to ordinary laboratory illumination (10 $\mu E/cm^2/s$) or to quartz/tungsten light source (360 $\mu E/cm^2/s$). For uninoculated culture media, bleaching of resazurin was measured spectrophotometrically at 540 nm. For growth experiments, media were inoculated with the microorganisms as soon as RNO was reduced ($A_{540} < 0.05$), media. Microbial growth was measured turbidimetrically (540 nm) with a Milton-Roy Spectronic 21, at various intervals following inoculation (2% v/v) with pure culture of ruminal bacteria. The effect of light intensity on O_2 removal in medium solutions was determined with a Clark-type O_2 electrode.

Results and Discussion

The rate of reduction of MDM, as measured by bleaching of the redox dye resazurin, was enhanced by light in an intensity-dependent manner (Fig.1), and the time required for medium reduction was decreased from several hours under normal light, to a few minutes under high intensity light. O_2 electrode experiments (Fig.2) revealed that the photoinduced bleaching of resazurin was related to light-enhanced removal of O_2 from the medium (i.e., was not due to a reaction of RNO unrelated to its status as a redox dye), although partial removal of O_2 prior to Cys addition was required. Media that had been rapidly reduced by photocatalysis supported growth of five strains of ruminal bacteria in an identical fashion to the same media reduced with Cys over a period of hours under typical laboratory illuminaation (Table 1). Similar results were obtained with four strains of *Clostridium* and one strain of *Thermoanaerobacter* in another culture medium (CM5) under a N_2 gas phase (data not shown).

Conclusion

Photocatalytic reaction between cysteine and RNO accelerates the reduction of anaerobic culture media, permitting almost immediate use of the medium for cultivation of ruminal bacteria and other strict anaerobes. Medium reduced photocatalytically supports growth of ruminal bacteria in a manner quantitatively identical to that of media reduced more slowly at low or zero light.

Table 1. Growth parameters for five strains of ruminal bacteria in media reduced at low or high light intensity [a]

Culture	Substrate [b]	Lag time (h)		Growth rate (h^{-1})		MaximumOD$_{540}$	
		Low	High	Low	High	Low	High
Lachnospira multipara 40	Glucose	3.25 ± 0.47	3.04 ± 1.20	0.49 ± 0.06	0.46 ± 0.06	1.18 ± 0.05	1.17± 0.03
Prevotella ruminicola $B_1$4	Glucose	0.08 ± 0.03	0.25 ± 0.30	0.56 ± 0.02	0.56 ± 0.03	1.41 ± 0.01	1.42.±0.03
Ruminococcus flavefaciens FD-1	Cellobiose	1.76 ± 0.93	0.93 ± 0.72	0.27 ± 0.01	0.27 ± 0.01	0.70 ± 0.04	0.79. ±0.06
Selenomonas ruminantium D	Glucose	1.22 ± 0.24	1.09 ± 0.06	0.61 ± 0.01	0.61 ± 0.03	1.19 ± 0.03	1.21. ±0.04
Streptococcus bovis JB1	Glucose	1.57 ± 0.18	1.33 ± 0.16	1.23 ± 0.03	1.23 ± 0.03	1.41 ± 0.04	1.30. ±0.03

[a] Media were reduced at low (10 □E/cm^2/s) or high (360 □E/cm^2/s) light intensity. Results are mean values of four replicate cultures ± S.E.M.

[b] Cultures were grown in MDM + 1 g of yeast extract /l, plus glucose (10 g/l) or cellobiose (4 g/l), under a CO_2 gas phase.

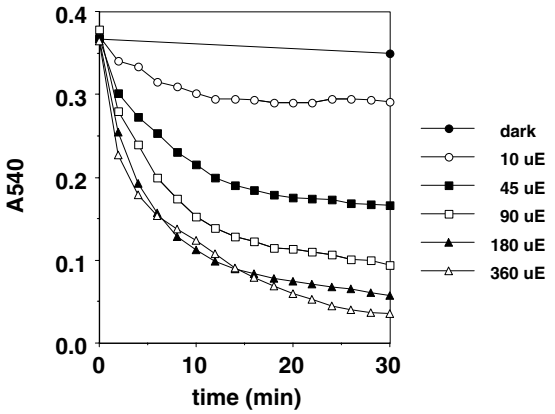


Fig.1. Effect of light intensity on bleaching of RNO in MDM.

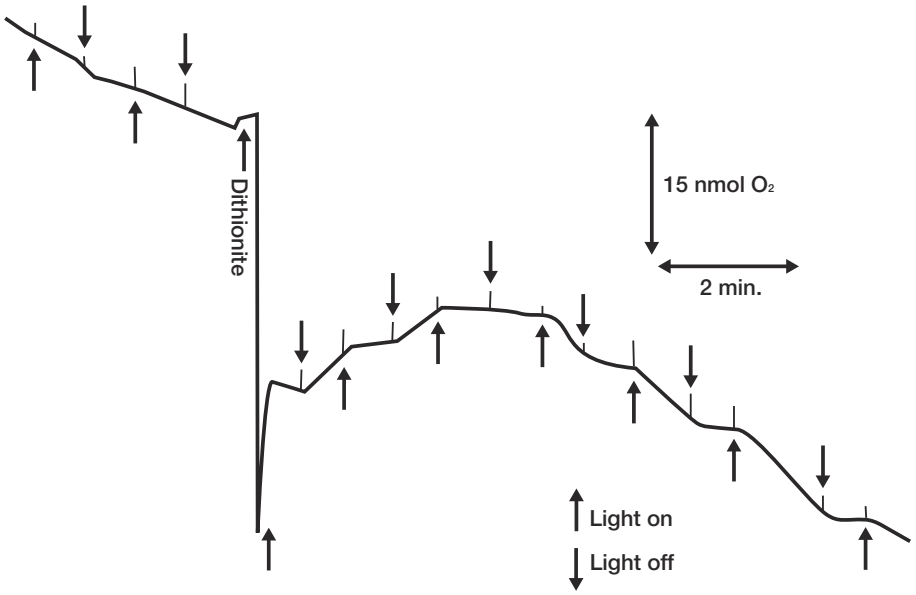


Fig.2. Effect of illumination (arrows) on O_2 removal from MDM containing RNO (2 mg/l) and Cys (1.25 g/l). RNO-photocatalyzed O_2 removal required prior removal of air, achieved in the air-saturated O_2 electrode chamber by addition of dithionite. In experiments performed in culture tubes (see text), air was removed by gassing tubes with CO_2 prior to Cys addition and illumination, and no dithionite was used in the medium. Control incubations lacked RNO or Cys, and did not result in O_2 removal (data not shown).

Isolation and Characterization of a *Trichoderma* Strain Capable of Fermenting Cellulose to Ethanol

D. M. Stevenson and P.J. Weimer

Introduction

For several decades the production of fuel ethanol from biomass has been considered a laudable goal because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity. In most of the processes currently under investigation, cellulosic biomass is first enzymatically broken down into sugars (often utilizing fungal cellulases) which microorganisms (usually yeast) then ferment to ethanol. To lower capital and opeating costs, it is desirable to accomplish the fermentation of cellulose to ethanol in one step. Few species of filamentous fungi are capable of this conversion, and all require rich (and thus expensive) culture media. In this study environmental samples were screened for organisms capable of producing ethanol from cellulose on minimal medium. We isolated a filamentous fungus capable of this conversion, and developed for this organism a genetic system based on parasexuality.

Methods

Rich medium (yeast-peptone-malt extract; YPM) consisted of (per 1) 3.0 g yeast extract, 5.0 g peptone, 0.3 g malt extract, and 5.0 g glucose. Minimal medium (MM) was yeast nitrogen base

(YNB) without added vitamins. Unless otherwise noted, the pH of MM was adjusted to 5.0 and carbon sources were added at 5 g/1 for aerobic growth and 50 g/1 for anaerobic fermentation. Sigmacell 20 microcrystalline cellulose was used as the cellulose source. Growth on soluble substrates was measured as dry weight after filtration onto tared glass fiber filters.

Anaerobic fermentation of cellulose and other substrates was tested using two growth methods. In the first (sealed flask) method, MM (20 ml in a 25 ml Erlenmeyer flask) was simply inoculated with conidia (~ $1x10^5$) and the culture vessel was sealed with either a butyl stopper or screw-cap. Thus approximately 20% of the flask volume was left as air space. The oxygen in this space allowed mycelia to develop before the flask became anaerobic and fermentation began. In the second (vented flask) method, the culture flask was prepared as in the first method but the stopper used to seal the flask was vented through an inserted 26 gauge needle capped with a 3 ml syringe barrel packed tightly with cotton. Ethanol and other fermentation products were analyzed in culture supernatants by HPLC.

Mutants were generated by exposure of conidia (washed off the surface of 7 d-old YNB glucose plates) to UV irradiation (5 cm from a 256 nm, 4W lamp for 10 min). Potential cross-feeding between auxotrophic mutants was tested in a U-tube apparatus constructed from two 10 ml syringe barrels, separated by a short length of tubing with or without an intervening 0.2 μm membrane filter disc. Each of syringe barrel contained MM, and was inoculated with an agar plug of an individual mutant strain. After 48h incubation, syringe pressure was used to force liquid (and, in the absence of the membrane filter, mycelial fragments) back and forth across the tubing, and growth was allowed to continue for an additional 7 d.

Results and Discussion

Fifty-six strains of fungi were isolated from environments rich in cellulose, such as the dung of ruminants, rotting wood, etc. These strains were all isolated on MM with either cellobiose or cellulose as the carbon source. When cellobiose was used, chloramphenicol was often added (10 mg/ml) to suppress bacterial growth. Each strain was either collected from a different source or was determined to be morphologically distinct from all other strains isolated from the same sample. Forty-one of the strains were found to use cellulose aerobically on MM, and two of these were found to produce measurable amounts of ethanol upon anaerobic incubation in MM plus cellulose. The more active strain, originally isolated from cow dung on cellulose medium, was selected for further study and was designated strain A10. This strain grew well on cellulose in both liquid and solid medium.

Strain A10 was found to grow rapidly and, when mature, to produce dark green conidia on MM plus glucose, and yellow-green to dark green conidia on rich medium (YPM), often in concentric rings as a response to light. The conidiophores (conidia bearing hyphae, Figure 1A) bore bottle-shaped cells (phialides) from which the conidia arose singly, and phialides were generally paired along the conidiophores. Little accumulation of conidia was seen on the conidiophores, except in very old cultures. Under light microscopy, conidia formed on solid medium were smooth-walled and nearly spherical with a mean diameter of 3.0 mm (Figure 1B) (Figure 1A). These observations suggest that A10 is a strain of *Trichoderma.* Sequencing of the intergenic spacer region ITS1 (GenBank Accession No. AY094141) and subsequent BLAST search indicated 100% sequence identity to several strains of *T. harzianum*. The culture has been deposited at the National Center for Agricultural Utilization Research as strain NRRL 31396. .

Maximum growth was observed at around 30-35°C, and the best growth occurred at the lowest pH range tested, pH 3.2 to 3.8. The strain grew well on cellulose in both liquid and solid medium, although no zone of cellulose clearing could be seen on solid medium suggesting that large amounts of extracellular cellulase were not secreted by this strain. Numerous carbohydrates were shown to support growth in YNB within 5 d under aerobic conditions. Growth under strict anaerobic conditions was tested using. No growth was observed on cellulose, cellobiose, glucose or xylose under anaerobic conditons; other carbohydrates were not tested.

When initially isolated, strain A10 produced only about 0.4 g ethanol/l when fermentation was by the sealed flask method in MM with 50 g added cellulose/l. Ethanol accumulation was eventually improved, by selection and the use of a vented fermentation flask, to 2 g/l when the fermentation was carried out in submerged culture. The highest levels of ethanol, >5.0 g/l, were obtained by the fermentation of glucose. Little ethanol was produced by the fermentation of xylose, although other fermentation products such as succinate and acetate were observed. Increasing the size of the inoculum for anaerobic incubations by several stages of pregrowth (obtained by removing spent medium from the aerobic culture and refillling with fresh media) did not increase the final concentration of ethanol.

Following mutagenesis, three auxotrophic and two morphological mutants were obtained. The auxotrophic strains required arginine (*arg*$^-$, strain A217), adenine or hypoxanthine (*ade*$^-$/*hpx*$^-$, strain A277), or inositol (*ino*$^-$, strain B429). The two morphological mutants (strains A28 and B462) both produced extremely compact colonies, and the latter strain produced a soluble extracellular yellow pigment. To test for parasexuality, plugs of the *arg*$^-$ and *ade*$^-$/*hpx*$^-$ mutants (strains A217 and A277, respectively) were placed next to each other on rich medium (YPM), as in Figure 2. After three days the mycelia had grown together, and three plugs were taken from the area of contact and placed on MM/glucose. Controls were also performed in which each mutant was grown separately. Growth occurred only from mycelia taken from the area of mutant contact, and none from the control (Figure 2A). Subsequently, nearly identical results were obtained using mutants A277 (*ade*$^-$/*hpx*$^-$) and B429 (*ino*$^-$), and also strains A217 (*arg*$^-$) and B429 (*ino*$^-$). This suggests the probable formation of heterokaryons in the manner of parasexuality. Heterokaryons thus formed were allowed to form conidia which were then subsequently diluted and plated. It was found that nearly all the resultant conidia germinated into mycelia that had phenotypes like one or the other parental strains. Only a few prototrophs were found, and these could be explained as having originated from either mycelial fragments of the original heterokaryon or perhaps as cases of anastomosis occuring between adjacent germinating conidia (these few colonies were always seen at the lowest dilution and therefore the most crowded plates). Only after 6-8 rounds of subculturing did strains develop that produced nearly 100% prototrophic conidia, indicating that diploidization or recombination had occurred. This procedure was also used to complement two morphological mutants (data not shown), as well as the three crosses of auxotrophs described above.

A U-tube experiment (see Methods) was carried out to test the possibility that cross-feeding was responsible for the complementation between strains A217 (*arg*$^-$) and A277 (*ade*$^-$/*hpx*$^-$). Complementation occurred only when hyphae came into contact, ruling out cross-feeding as a possibility.

Conclusions

The ability of this new fungal strain to use a wide variety of carbohydrates (including crystalline cellulose) combined with its minimal nutrient requirements and the availability of a genetic system suggests that the strain merits further investigation of its ability to convert biomass to ethanol.

Acknowledgments
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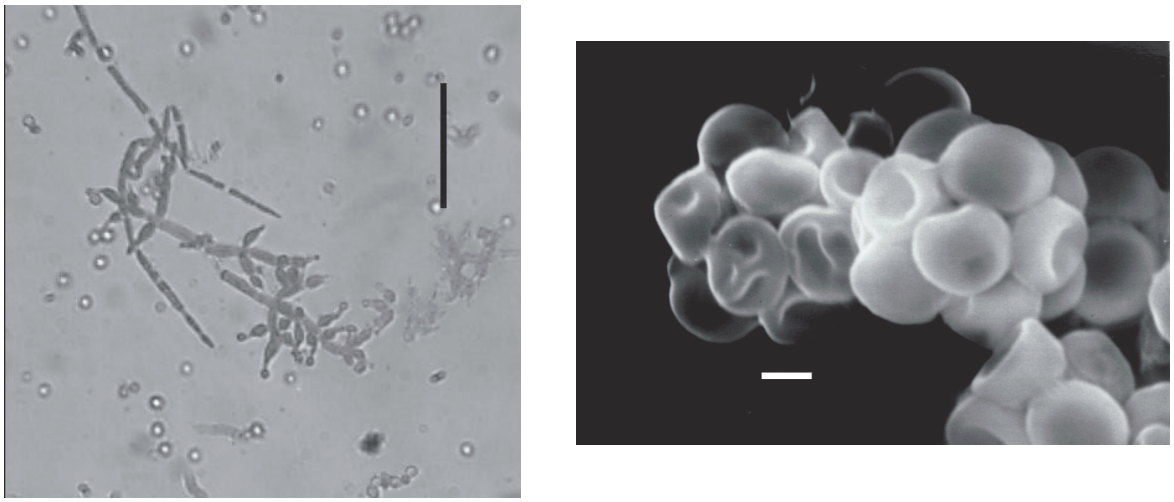

Fig. 1. Left panel: Conidiophores of isolate A10 showing arrangement of conidia and phialides (conidia bearing hyphae) from a 3 d old culture on aerobic solid glucose minimal medium. The bar represents approximately 50 mm. Right panel: SEM on non-fixed, air-dried conidia. The bar represents 1.0μm.

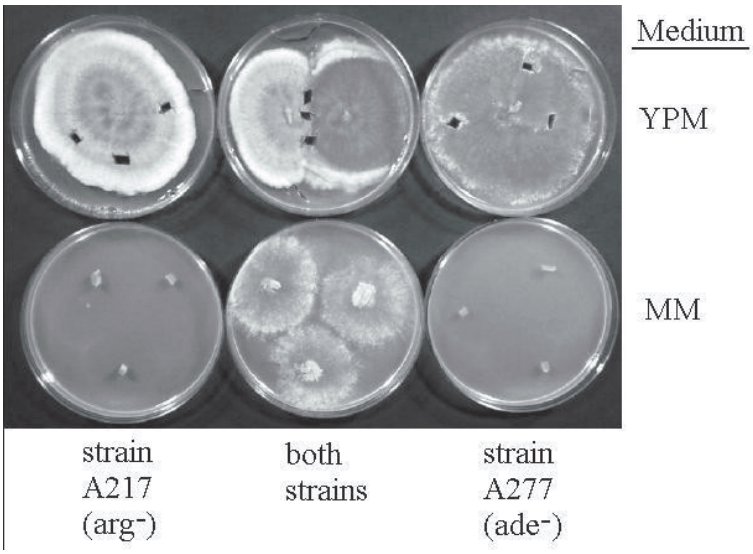

Fig. 2. Complementation of auxotrophs of A10. Top row shows 3 day old plates of rich medium, with an *arg*$^{-}$ mutant (A217) on the left and an *ade*$^{-}$/*hpx*$^{-}$ mutant (A277) on the right. In the center both mutants grew in proximity and can be seen to have grown together. The bottom row shows plate of minimal medium inoculated with plugs taken from the plates above, and clearly shows growth only from plugs taken from the region of contact between the two mutants.

Forage Quality

Nutritive Value of Silage Corn Harvested at Two Heights Above Ground for Lactating Cows
Z. Wu, F. Kanitz, and L. D. Satter

Introduction

The use of corn silage is increasing in dairy diets in some regions of the United States. Corn is increasingly being grown on land that is subject to soil erosion. Can we leave more of the lower stalk in the field to reduce soil erosion by providing surface cover? Also, can we improve the nutritive value of silage that is cut at a greater height to compensate for the loss of DM yield with the higher cutting height?

Materials and Methods

Forty-six Holstein cows (15 primiparous and 31 multiparous) averaging 112 days in milk were utilized in a crossover design experiment. The two treatments were corn silages harvested at two different heights (14" or 28" above the ground, respectively). The experiment included two periods, each lasting 4 wk. At the beginning of the trial, cows were divided into two groups based on similarity in parity, milk yield, and days in milk. The two groups were each assigned to a treatment during period 1. During the second period, cows received the opposite treatment. Cows were administered bST (Posilac; Monsanto Co., St. Louis, MO) every 2 wk beginning at the first week of the trial.

Each group of cows was housed in a free-stall barn and offered a TMR once daily ad libitum (5 to 10% refusal). Both diets contained 40% corn silage, either low or high cut. Actual amounts of feed offered and refused by each group were recorded daily to obtain net DM intake. However, only the last three weeks' records in each period were used for analysis; the first week was considered as a transition time.

Data on milk yield and milk composition were analyzed by the general linear model procedure of SAS (1985) using a model that included cow, period and treatment as the independent variables.

Results and Discussion

Cutting at 28" rather than 14" had a large effect on nutrient analyses of the silage (Table 1). Dry matter content of the ensiled material increased from 36.4 to 42.8%. The lower portion of the corn stalk is very low in DM content; about half as much as the average for the rest of the plant. Cutting at a greater height would have the practical benefit of yielding a drier silage. This could be advantageous in widening the window for harvesting, and/or in getting silage in a more desirable DM range for storage in tower silos. Quality of the silage was also markedly improved with higher chopping height. NDF and ADF were reduced by about 7 and 4 percentage points, respectively. Crude protein was not affected.

Table 2 shows the nutrient content of the total mixed diets for the two treatments. Nutrient content of the total mixed diets reflected the nutrient content of the treatment corn silages.

Dry matter intake, milk production and milk composition is shown in Table 3. Since the cows were group fed, statistical analyses on dry matter intake was not possible. It did appear that dry matter intake was slightly lower with the high cut silage. This might be expected if digestibility of the silage was increased.

Milk production was 1.2 kg per cow per day greater ($P < .01$) with the high cut silage, however 3.5% FCM was not different. Milk fat percent was reduced with the high cut silage, most likely reflecting the lower fiber content of the high cut forage. Milk protein was not different. If corn silage is cut at a greater height, it will be important to adjust the ration formulation to reflect the lower fiber content.

Accurate silage yield measurements were not made in this experiment. From other observations we have made, however, it would appear that leaving an additional 14" of stalk would reduce DM yield by 5-8%. Yield of wet silage would be reduced by about twice that amount because of the low DM content of the lower stalk. Loss of digestible nutrients were not measured in this study, but would probably be around 3-4%. These estimates apply to corn silage where the grain represents about 50% of total plant DM. Losses, expressed as a percent of plant DM, will be greater in situations where grain content is appreciably less.

Visual observation of chopped stubble indicates considerably more ground cover with the high cut silage. It remains to be seen how effective this can be in reducing soil erosion.

Conclusion

Cutting height can have a marked effect on moisture and fiber content of the resulting silage. Higher milk production was achieved with the high cut corn silage. Lower fat test was observed with the high cut silage, indicating the need to balance diets for the lower fiber content. Cutting at 28" can leave residue in amounts that may be effective, when chopped, in providing ground cover.

Table 1. Nutrient analyses of corn silage cut at two different heights.

Item	Low cut (14" above ground)	High cut (28" above ground)
	----------(%)----------	
DM	36.4	42.8
NDF	40.9	34.0
ADF	25.4	21.3
CP	8.4	8.3

Table 2. Nutrient content of diets containing corn silage cut at two different heights.

Item	Low cut (14" above ground)	High cut (28" above ground)
	----------(%)----------	
DM	53.9	56.4
CP	17.8	17.8
NDF	29.4	26.6
ADF	19.1	17.4

Table 3. Performance of cows fed diets containing corn silage cut at two different heights.

Item	Low cut (14" above ground)	High cut (28" above ground)	SEM	*P*
DMI, kg/d	20.8	19.8	...	...
Milk, kg/d	33.9	35.1	0.2	0.01
3.5% FCM, kg/d	35.0	34.5	0.4	0.42
Milk fat, %	3.74	3.39	0.06	0.01
Milk protein (true),%	3.11	3.12	0.02	0.44

Improving Use of Near Infrared Reflectance Spectroscopy Calibrations Among Laboratories of a Network

Neal Martin, Paolo Berzaghi, and Dan Undersander

Introduction

Forages represent about 50 % of the diet in lactating dairy cattle and larger percentages of other ruminant animals. Information about the chemical composition of forages is necessary to correctly balance nutrients in the diet. However, chemical and nutritional composition of forages is highly variable. Sources of variation include botanical composition, stage of maturity at harvest, harvest and storage method and climatic conditions. Near-infrared reflectance spectroscopy (NIRS), a rapid nonconsumptive method of analyzing forage crops was first identified by Karl Norris, USDA-ARS.

Many commercial forage testing laboratories use NIRS to analyze forage samples for dairymen, commercial hay producers and other livestock owners. Farmers are concerned about accuracy of forage test results and repeatability of sample analyses from lab to lab. Computerized transfer of NIRS calibration equations from one instrument to another, laboratory to laboratory, has been established as a satisfactory solution to reducing laboratory-to-laboratory variation. We propose a model NIRS network to support development of universal calibration, which can be distributed by a master instrument to slave instruments within the network.

Material and Methods

A network of commercial and public laboratories, NIRS Forage and Feed Testing Consortium, http://www.uwex.edu/ces/forage/NIRS/home-page.htm has been in operation since 1992. Experiments conducted with laboratories will be used to demonstrate to establish criteria of a model NIRS network to test forages for farmers in North America.

Laboratories have scanning monochrometer instruments, which collect reflectance information over 400 to 2500 nm, using NIRSystems instruments, models 4500, 5000, and 6500. NIRSytems WinISI software is utilized to run network calibrations. The network instrument operator utilizes ISI software for instrument diagnostics, instrument monitoring, and calibration monitoring. Each instruments spectra output is matched to a master instrument. A 30-cell characterization and 14-cell forage system are used to match instruments, which spectra trimmed to 1300-2500 nm, the region of the least expansive instrument. Routine samples are microwave dried and ground through cyclone grinders fitted with 1 mm screens. Samples are mixed before spectra and reference method analysis. National Forage Testing Association (NFTA), recommends reference methods utilized within the network. Network calibrations are developed using protocol established by National NIRS network. Calibrations are monitored using the network protocol.

Results and Discussion

Use of a network of NIRS instruments designed to use universal calibration equations has potential to deliver accurate forage test results among laboratories across North America. Such a network requires standardized drying and sampling processing, a method of monitoring accuracy of reference methods, harmonization of the instrument network, and calibration monitoring and development.

Standardization of drying and sample processing. Experience of operating our network has supported the recommendation that samples are dried alike, cyclone mills using 1 mm screens, and uniform sample processing, adequate mixing of samples before packing into cups for NIRS analysis as well as before reference method analysis. Large samples often dictates two separate grinds; grinding in a Willey mill using a 6 mm screen to break the large sample into smaller pieces rapidly followed by the cyclone mill. A segment of our network, alfalfa plant breeders have investigated elimination of the later grind using only one grind to a using a 2 mm screen to save time and labor. The network calibration monitoring statistics shows this is possible: %CP, 0.83 and 0.77; % ADF, 1.66 and 0.85; % NDF, 1.76 and 0.85; and % IVDDM, 1.60 and 0.78 for SED © and R^2, for 1mm vs. 2 mm grind, respectively.

Accurate results are dependent on drying method for various parameters. The accuracy of ruminal degradable protein (RUP) of legume and grass silages is dependent on method of drying. When comparing silage samples freeze-dried to those either oven-dried or microwave-dried, Hoffman and associates found oven-dried samples were similar to freeze-dried, but microwave samples were different. A repeatability file was prepared using hay and silage samples split, dried with microwave and oven-dried. Spectra are collected of each drying method samples to prepare a repeatability file, a file, which allows spectra adjustment for drying treatment. The adjusted calibration equations compared to original calibrations for RUP and dNDF are shown in Table 1. Improvements can be made with the repeatability file; however, drying methods must remain consistent with the repeatability treatment to remain effective.

Accuracy of reference methods. Reference method accuracy must be as good as possible for NIRS calibrations to be accurate. A method of maintaining accurate reference methods is for network laboratories to participate in National Forage Testing Association proficiency program. Using spectra from samples, which have been tested, by laboratories receiving proficiency grade is a must.

Harmonization of the instrument network. Software is available to match spectra output from scanning NIRS instruments. Ten years experience matching instrument spectra output to a master instrument for forage crop calibrations using a 30-cell characterization set and 14-cell forage set is available. The standardization set must be scanned by the master instrument, scanned by the slave instrument and rescanned by the master. For several years our network relied on successful standardizations using a 30-cell set. However, a test of the set determined the 95 % of check cells were leaking. Testing the original standardization made from the original standardization set collected at 7 laboratories against standardizations developed with a new forage set showed marked improvement in agreement among instruments, Table 2. (Standard deviation between 7 labs was improved by as much as 72 percent for % NDF).

Monitoring instrument performance via diagnostics is a must. Instrument manufactures recommend daily diagnostics with a check cell test being saved weekly. Our recent experience using a web-based diagnostics-monitoring program has been very successful. The program shows instrument operators there instrument performance statistics and also alters the network operator of instrument failures.

Calibration monitoring and development.
The final, but essential component of NIRS network operation is monitoring calibration performance. Selection of samples tested by laboratories within the network, selection based on monitoring instrument spectra supplied to network operator, provides the network validation of performance of the calibration used by the network. The network hay calibration, n = 1013, was updated using 654 samples from the network and 10 NFTA proficiency samples. Validation statistics of 2 updated

calibrations and a new calibration developed using "local" a new calibration model developed to be used with large data sets by 4 laboratories is shown in Table 3. In most cases the local calibration improved the calibration performance.

Conclusions

The potential to produce small deviations between laboratories testing forage crops with NIRS has been demonstrated. Standardizing drying and processing methods, monitoring reference method accuracy, harmonizing standardized instrument performance via web based programs, and monitoring calibration equations are keys to providing accurate forage tests between laboratories.

Table 1. The effect of drying method on ruminal undegradable protein and digestible neutral detergent fiber calibration performance.

	Original		Adjusted		Original		Adjusted	
Lab	SED	Bias	SED	Bias	SED	Bias	SED	Bias
	-----RUP, % of CP------				--------dNDF, % of DM----------			
Hays								
1	6.40	5.40	2.20	1.02	3.76	-2.56	1.81	0.97
2	6.60	-6.25	1.75	-1.15	2.75	-2.22	1.26	0.04
3	4.63	-3.35	1.75	-0.69	2.81	-0.87	1.39	0.04
Haylages								
1	2.14	1.16	1.12	0.38	1.41	-0.15	1.14	0.15
2	2.58	-2.20	1.17	-0.91	1.12	0.27	1.15	0.25
3	2.14	0.11	1.65	-0.10	1.73	-0.55	1.46	-0.41

Table 2. Improvement of laboratory network instrument performance from replacing defective standardization sample set.[1]

	Old standardization set				New standardization set			
Lab	DM	CP	ADF	NDF	DM	CP	ADF	NDF
	----------% of DM----------							
1	90.74	21.48	32.09	37.84	90.97	20.84	32.13	38.89
2	91.07	21.08	32.32	38.94	90.97	20.84	32.13	38.89
3	91.63	21.26	31.95	39.01	91.63	21.28	32.06	38.69
4	91.35	21.40	31.54	38.62	91.50	21.07	31.97	38.61
5	90.72	21.35	31.76	39.22	90.80	21.05	32.03	38.84
6	91.61	21.34	31.98	38.14	90.90	21.12	31.83	38.78
7	91.73	21.26	31.09	38.14	91.64	21.20	32.27	38.85
Mean	91.26	21.31	31.82	38.62	91.20	21.06	32.06	38.79
SD	0.43	0.13	0.40	0.49	0.37	0.17	0.14	0.11

[1]Monitored over 6-month period.

Table 3. Calibration monitoring statistics from 3 different updates including evaluation of a new local concept, 'Local'.

Method	SEP	Bias	SEP–C	R^2
	----------% of dry weight----------			
Crude protein				
LH0801	.80	.17	.78	.90
LH1097	.90	.48	.80	.89
LOCAL	.69	-.02	.69	.92
Acid detergent fiber				
LH0801	1.83	-.36	1.79	.86
LH1097	1.98	-.58	1.89	.84
LOCAL	1.48	.09	1.48	.90
Neutral detergent fiber				
LH0801	2.05	-.27	2.03	.89
LH1097	1.97	-.54	1.89	.90
LOCAL	1.52	.09	1.52	.94

Improving the Nutritive Evaluation of Corn Silage: I. Variability in Chemical and Physical Characteristics and Their Interrelationships.

D. R. Mertens and G. F. Ferreira

Introduction

Evaluation of corn silage provides a unique challenge because it contains variable proportions of grain and vegetative matter each of which can differ in availability due to chemical composition and physical form. When animals consuming corn silage do not perform as expected based on fiber level, it is uncertain if the discrepancy is due to altered proportion of grain in the silage, energy availability of the grain or stalk, or a combination of factors. Although chemical composition ultimately determines the availability of energy at the cellular and molecular level, physical properties of forages can impact digestibility by altering access to tissues and surface area available for microbial colonization and fermentation. Compared to other forages corn silage is more susceptible to the impact of physical properties because corn kernels may be inadequately chewed by dairy cows and pass out of the digestive tract before digestion is completed.

Summative equations, which add the digestible amounts of neutral detergent fiber(NDF), protein, fat, starch and non-starch soluble matter, may improve the nutritive evaluation of corn silage. Some summative equations also alter nonfibrous carbohydrate or starch digestibility when kernel processors are used during chopping of corn silage. The (CPM) net protein and carbohydrate model uses digestion kinetics of various fractions of protein and carbohydrates to estimate the energy value of corn silage. However, little is known about the relationships among chemical composition and physical properties of corn silage that might impact feed evaluation systems. Furthermore, there is no quantitative system for estimating the extent of grain damage that occurs with different levels of chopping or kernel processing. The objective of this research was to determine interrelationships among chemical and physical characteristics of corn silage, including a physical method for assessing kernel damage.

Methods

Thirty-two corn silages were obtained from a commercial feed analysis laboratory based on diversity in DM, CP, ADF, NDF, starch and visual appraisal of particle size. For each characteristic, materials were selected to represent the mean of the population of all corn silages analyzed by the commercial laboratory and to represent materials that were plus or minus two standard deviations from the mean for each characteristic while keeping other characteristics close to their respective means. Ash, CP, ADF, and acid detergent lignin (ADL-72% sulfuric acid method) were determined by AOAC procedures. Dry matter was determined by oven drying at 55°C for 48h. The amylase-treated NDF (aNDF) was determined using both amylase and sodium sulfite. Starch was measured using a YSI Biochemistry analyzer after enzymatic hydrolysis (Dairyland Laboratories). Mean particle size was determined on dried samples using a vertical shaker and sieves with apertures of 19.00, 13.20, 9.50, 6.70, 4.75, 2.36 and 1.80 mm.

It was observed in a preliminary experiment that whole kernels and fragments >1/4 of a kernel were retained on sieves with apertures > 4.75 mm. Each silage (233 ± 89 g of wet material) was sieved undried for 15 min with a vertical shaker using sieves with apertures of 19.00, 13.00, 9.50, 6.70 and 4.75-mm, in addition to the pan. After sieving, kernels and kernel fragments on the sieves were manually collected and then dried (48 h at 55°C) and analyzed for starch content.

Results and Discussion

Average DM, aNDF, ADF, and ADL (table 1) of our corn silages were similar to the DM (35%), NDF (45%), ADF (28.1%) and lignin (2.6%) reported in the 2001 Dairy NRC. However, average CP was lower than that reported in the Dairy NRC (8.8%) and average ash was higher than NRC (4.3%). The standard deviation and range in minimum and maximum values indicate substantial variation occurs in the chemical and physical characteristics of corn silage and illustrates the importance of feed analysis in determining nutritive value.

Table 1. Chemical and physical characteristics of 32 corn silages.

	Mean	SD	Minimum	Maximum
DM, %	34.68	7.81	19.23	48.10
CP, %	7.80	1.36	5.68	12.49
Ash, %	5.07	1.52	3.09	9.62
Starch, %	25.23	5.68	12.23	36.17
NFC[a], %	40.92	6.93	27.36	56.60
aNDFom[b], %	43.01	6.12	29.36	54.12
aNDF, %	44.18	6.40	29.98	56.30
ADF, %	26.94	4.42	17.67	34.68
ADL, %	2.26	0.59	1.16	3.50
Starch, %	25.23	5.68	12.23	36.17
MPS[c], cm	4.18	1.40	2.05	7.26

[a] NFC = 100 - CP - aNDF - Ash - EE, where EE = 3.20.
[b] ash-corrected, amylase-treated neutral detergent fiber organic matter.
[c] Geometric mean particle size was determined with a vertical shaker.

Although R^2 were typically <0.30, there were significant relationships between corn silage DM (as an indicator of corn maturity) and aNDF, ADF, ADL, starch, grain, and ash. These relationships indicate that a corn silage containing 25% DM would contain 8.1% CP, 5.8% ash, 21.9% starch, 34.9% grain, 48.0% aNDF, 29.9% ADF, and 2.5% ADL. They also indicate that a corn silage containing 45% DM would contain 7.4% CP, 4.3% ash, 28.8% starch, 43.6% grain, 40.1% aNDF, 23.7% ADF, and 2.0%

ADL. Some of these values differ from those reported in the latest Dairy and Beef NRC. The decrease in ADL and NDF with increased maturity probably reflects the diluting effect of starch during grain filling

For this diverse set of corn silages good relationships were obtained between grain and silage DM, between ADF or ADL and aNDF, and between starch and NFC (Table 2). If grain DM is an important factor related to starch utilization, it appears that corn silage DM can be used to indicate grain DM and starch availability. The relationship between ADF and aNDF indicates that ADF is about 60% of the aNDF in corn silage, which is lower than for other forages. Although lignin increased with increased aNDF, the proportion of lignin in NDF did not increase with maturity as expected, in this diverse set of corn silages. (This may be related to the fiber characteristics of grain, which contains some aNDF but very little ADL.) The Beef NRC indicates that 100% of the NFC, which they call NSC, is starch. Our results indicate that NFC in corn silage contains a significant non-starch component as indicated by the negative intercept and slope less than 1.0. It appears that starch is less than 75% of the NFC in corn silage.

Table 2. Linear relationships between chemical characteristics (n = 32).

		Intercept (b_0)		Slope (b_1)			
Y	X	Coef.	P <	Coef.	P <	R^2	SE
Grain DM	CS DM	36.71	.01	0.561	.01	.770	2.43
ADF	aNDF	-1.01	.66	0.633	.01	.840	1.79
ADL	aNDF	-0.82	.10	0.070	.01	.575	0.39
CS Starch	NFC	-5.95	.02	0.762	.01	.865	2.12
CS Starch	aNDF	59.98	.01	-0.787	.01	.786	2.67

About 20% of corn silage DM consists of whole and large fragments of corn kernels (Table 3). If these kernels and fragments are inadequately chewed and digested by lactating dairy cows, they can represent a significant loss of energy value from corn silage. The proportion of grain in corn silage, which was calculated by dividing corn silage starch concentration by the concentration of starch in manually collected kernels and large fragments, represented about 40% of corn silage DM and varied from 20 to 50%.

Table 3. Proportions and characteristics of kernel and kernel fragments in 32 corn silages

	Mean	SD	Min	Max
Kernels and fragments[a], %CS DM	20.49	9.66	4.18	48.35
Starch, % grain DM	64.32	3.40	56.96	70.27
Grain DM[b], % CS DM	39.09	7.86	20.41	52.96
Starch >4.75[c], % CS DM	13.27	6.64	1.83	33.02
Starch >4.75[c], % CS starch	52.18	20.93	8.73	100.0

[a] Retained on sieves with >4.75-mm apertures (as a percentage of corn silage DM).
[b] Determined by dividing total starch in each corn silage by its grain starch concentration.
[c] Starch in kernels and kernel fragments retained on sieves with >4.75-mm apertures.

Utilization of starch can impact the utilization of energy in corn silage as evidenced by the improvement in performance when kernel processors are used. The proportion of total starch in corn silage that is in kernels or fragments retained on sieves with apertures >4.75 mm, averaged about 50% and

varied from 10 to 100%. It appears that the proportion of starch > 4.75 mm can be used as a quantitative estimate of kernel damage due to chopping and kernel processing (Figure 1). The upper left and lower right borders of the data in figure 1 indicate the extremes in kernel damage. Although information about chopping and processing of these silages was unavailable, it is logical to speculate that the upper left border represents corn silages that were chopped without additional processing. The line fitting these silages indicates that 100% of starch is in kernels and large fragments when silages are chopped to attain a geometric mean particle size of >4.5 cm. The line fitting the lower right border of the data in figure 1 represents the maximum extent of processing that can be attained when chopped to various geometric mean particle sizes. Data between these two borders represent variable processing effectiveness and indicate the proportion of starch in kernels and large fragments.

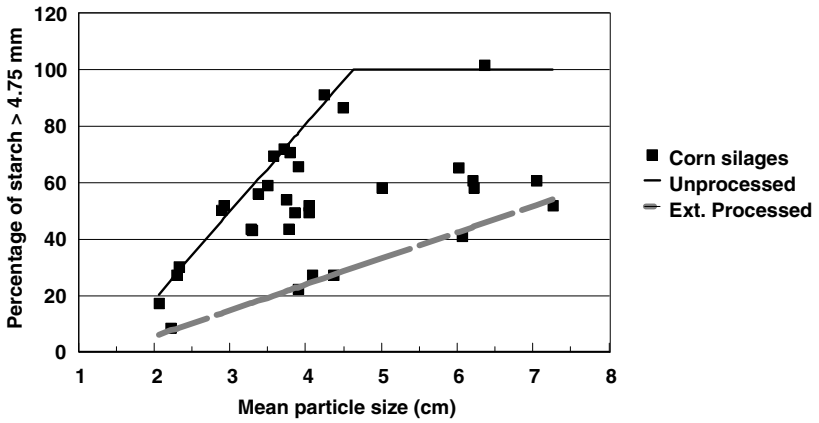

Fig. 1. Graph of the percentage of starch in kernels and large fragments (starch >4.75 mm) versus the geometric mean particle size of the silage with lines indicating unprocessed corn silage and extensively processed silages.

Summary and Conclusions

Average composition of a diverse set of corn silages agreed with values reported by the Dairy NRC. However, analyses of immature (25% DM) and mature (45% DM) differed from those reported in the Beef and Dairy NRC. Regression of starch versus NFC indicated that corn silage contains a significant non-starch fraction and that NFC is only about 75% starch in corn silage. Percentage of total starch in whole kernels and large fragments retained on sieves with apertures >4.75 mm provides a quantitative measure of kernel damage in corn silage, and may provide information useful in estimating processing adjustment factors for altering starch or NFC utilization when determining the energy value of corn silages.

Acknowledgement

Appreciation is extended to Dave Taysom and staff at Dairyland Laboratories, Arcadia, WI for donating samples and collaborating in this project.

Improving the Nutritive Evaluation of Corn Silage: II. Identifying Factors Affecting In Vitro Fiber and Soluble Matter Digestibility.

D. R. Mertens and G. F. Ferreira

Introduction

In vitro "artificial rumen" methods are receiving renewed interest as approaches for improving forage evaluation. These methods were refined in the 1960's and made useful as research tools for comparing forages and understanding ruminal fermentation. The two-stage Tilley & Terry (T&T) method (or modifications of it) became the standard in vitro method for evaluating forages. This method involves a 48 h fermentation with ruminal inoculum followed by incubation in acid pepsin. In vitro dry matter digestibilities (IVDMD) of 1-mm ground materials measured by the T&T method correlate well with in vivo DM digestibilities measured at maintenance levels of intake. In the 1970's Van Soest replaced the second stage of the T&T method with neutral detergent (ND) extraction. Because ND solubilizes microbial debris, this method can be used to measure in vitro dry matter true digestibility (IVDMTD).

Evaluation of corn silage using in vitro methods is complex because a significant portion of its ND soluble matter (NDS) is starch. It appears that starch fermentation and digestion is affected by particle size. Grinding corn silage to 1 mm may result in IVDMD or IVDMTD that do not represent in vivo digestion, especially at production levels of intake when dairy cows may not chew corn kernels completely. A macro in situ method of measuring digestibility of corn silage has been proposed in which the fresh, whole silage is fermented with minimal physical disruption. This method probably underestimates in vivo digestion of corn silage because the effects of mastication are not mimicked.

The 2001 Dairy NRC indicated that a 48 hr in vitro fermentation could be used to estimate NDF digestibility when calculating TDN at 1X maintenance intake (TDN_{1X}). The TDN_{1X} is then reduced as a function of intake to estimate net energy value at production levels of intake. The objectives of this research were to assess the difference in IVDMD and IVDMTD of corn silages in various physical forms and to identify factors affecting the digestion of NDS and NDF fractions of DM.

Methods

The 32 corn silages selected to provide a diversity of chemical and physical characteristics were described previously. Dried silages were whole or ground through 4- or 1-mm screens and fermented in in situ (IS) bags. Sample amounts and bag dimensions varied to obtain about 9 mg/cm^2 of bag surface: Whole, 3.6 g in 10X20 cm IS (Wh-L) or 1.8 g in 10X10 cm IS (Wh-H); 4-mm, 0.90 g in 5X10 IS (4-mm); and 1-mm, 0.45 g in 5X5 cm IS (1-mm). Materials were fermented for 24 h in a rotating jar system using a media containing 400 mL strained ruminal fluid composited from 3 cows, 400 mL of buffer blended with ruminal solids from 3 cows, and 1200 mL of Van Soest media. After 24 h, pH and temperature of each jar were recorded and the bags removed. Bags were rinsed twice manually in ice water to stop fermentation, and once in a washing machine for 3 minutes. Twenty-four hours of fermentation was selected to minimize end product inhibition in the fermentation jars and to maximize differences among treatments. Washed bags were dried (24 h at 55 °C) and weighed to measure IVDMD without the second stage pepsin treatment.

In vitro residues from the Wh-L, Wh-H, and 4-mm materials were ground through a 1-mm screen before fiber analysis. All residues were extracted in neutral detergent using the filter bag system to determine true (IVDMTD) and in vitro aNDF digestibility (IVNDFD). Four replicates of digestibilities were measured in four in vitro runs with two incubators each containing four jars within each run. Samples were blocked by run, incubator, and jar location within incubator. Replicates of blanks and a corn silage standard were included in each jar within incubator within run. Digestibilities were corrected for blanks and means of replicates were used in statistical analyses.

Results and Discussion

The pH of media at the end of fermentation was not different among runs, incubators, or jar location within incubator (average pH=6.34). However, temperature of the media was different among days and jar location within incubator and did not agree with controller settings. Run 2 was cooler (37.6 °C) than runs 3 or 4 (38.1 °C), and we have no explanation for this difference. Jar location within incubator were numbered from 1 to 4, clockwise, starting in the upper left corner. Temperatures were 37.5, 37.9, 38.8, and 37.7 °C for locations 1 to 4, respectively. Location 3 was warmer and location 1 was cooler than other locations. This probably is due to the location of the heat source in the incubators and the direction of airflow.

Both IVDMD and IVDMTD of the corn silage standard differed between incubators (P>.09 and .04, respectively), but did not differ among runs or jar location within incubator. The pooled standard error for replication of corn silage standard IVDMD and IVDMTD were 1.73 and 1.57, respectively. Although there was no statistical difference in digestibilities among jar locations, there were high correlations (>.95) between jar temperature and IVDMD or IVDMTD. In vitro digestibility percentage increased 0.37 for each increase in degree centigrade.

A preliminary study indicated no difference in IVDMD between wet and dried silages and all digestibilities in this experiment were determined using dried samples. In vitro digestibilities of all components (DM, NDF, and NDS) were lower for whole material compared to 4- or 1-mm ground materials, but there was no difference in whole material digestibility when fermented in large or half-sized IS bags (table 1). The IVDMTD and IVNDFD of 4-mm ground materials were lower than that of the 1-mm ground silages, but IVDMD and IVNDS were not different between 4- and 1-mm grinds. The difference in IVNDSD between whole and ground silages, but lack of difference between 4- and 1-mm ground materials suggests that reducing particle size from whole to a 4-mm grind may destroy the physical effects of starch that inhibit its fermentation in vivo.

Table 1. In vitro[a] dry matter digestibility (IVDMD), true dry matter digestibility (IVDMTD), aNDF digestibility (IVNDFD) and neutral detergent solubles digestibility (IVNDSD) of 32 corn silages after 24 h of fermentation.

	IVDMD, %	IVDMTD, %	IVNDFD, %	IVNDSD, %
Whole-Large IS	61.1[b]	72.3[c]	37.4[c]	79.6[b]
Whole-Half IS	61.4[b]	71.7[c]	35.6[c]	81.2[b]
4-mm ground	70.8[a]	75.7[b]	44.9[b]	90.9[a]
1-mm ground	72.6[a]	77.4[a]	48.7[a]	91.2[a]

[a,b,c] Means with different superscripts within columns are different (P>.05).

Average correlation coefficients for IVDMD of 4- and 1-mm grinds were -.84, -.82, -.80 and +.75 for ADF, ADL, aNDF, and NFC, respectively. The correlations were similar for IVDMTD. Although the

summative equation approach includes a variable digestion coefficient for NDF, it implies that DM digestibility should be a function of NDF concentration, especially when physical limitations are minimized by grinding. The relationship between IVDMD and aNDF was improved when ADL was included in the equation:

IVDMD (1-mm) = 97.0 - .34(aNDF) – 4.14(ADL); R^2 = .68 and SEreg = 3.06.

Stepwise regression was used to determine which variables in addition to aNDF and ADL would explain variation in the IVDMD of whole and 4-mm ground materials. For whole material, dry matter concentration of corn grain (CG_DM) and starch >4.75 mm as a percentage of corn silage DM (StGT_DM) were the first additional variables included in regressions models:

IVDMD (Wh-L) = 136.6 - .54(aNDF) – 9.79(ADL) - .79(StGT_DM) - .34(CG_DM); R^2 = .67 and SEreg = 5.32 and

IVDMD (Wh-H) = 135.3 - .51(aNDF) – 7.02(ADL) - .42(StGT_DM) - .51(CG_DM); R^2 = .54 and SEreg = 5.49.

The regression coefficients for StGT_DM (an indicator of starch physical limitation) and CG_DM (an indicator of starch availability) indicate that these variables have a negative impact on digestion of whole silages. When these variables were include in the regression model for IVDMD of 1- and 4-mm ground materials they were not significant. This is additional evidence that grinding through a 4-mm screen eliminates the physical impact of starch on in vitro digestion. The corresponding equation for 4-mm ground material was:

IVDMD (4-mm) = 93.7 - .29(aNDF) – 4.44(ADL); R^2 = .82 and SEreg = 1.99.

The only variable that was consistently related to IVNDFD was the percentage of ADL in aNDF (ADL_NDF). Although regression coefficients were highly significant, lignin did not explain a large proportion of the variation in IVNDFD. The variables of mean particle size (a possible factor affecting whole material fiber digestion), corn silage DM (an indicator of maturity) or NFC (a potential depressor of fiber digestion) did not affect NDF digestibility:

IVNDFD (Wh-L) = 65.0 – 5.45(ADL_NDF), R^2 = .27 and SEreg = 8.05;
IVNDFD (Wh-H) = 61.2 – 5.05(ADL_NDF), R^2 = .16 and SEreg = 10.51;
IVNDFD (4-mm) = 60.5 – 3.08(ADL_NDF), R^2 = .24 and SEreg = 4.86; and
IVNDFD (1-mm) = 62.4 – 2.69(ADL_NDF), R^2 = .20 and SEreg = 4.84.

These equations suggest that the upper limit of 24 h IVNDFD for these corn silages was 60 to 65% and that lignin has a greater negative impact on the digestion of whole compared to ground material.

Regressions of digestible NDS versus NDS or NDS organic matter yielded high R^2 and regression coefficients (estimates of true digestibilities) near 1.00. Although the R^2 were low because the true digestibility of NDS is relatively constant, the proportion of total starch that was <4.75 mm (StGT_TS) had a significant negative impact on IVNDSD if whole materials:

IVNDSD (Wh-L) = 99.7 - .39(StGT_TS); R^2 = .49 and SEreg = 8.40, and
IVNDSD (Wh-H) = 90.6 - .18(StGT_TS); R^2 = .19 and SEreg = 8.00.

Summary and Conclusions

There were differences in temperature of the media at the end of fermentation among runs and locations within the incubator. Although in vitro digestibilies were not statistically different among locations within incubators, there was a significant relationship between temperature and IVDMD among the locations of jars. Whole corn silages have lower in vitro digestibilities compared to 4- or

1-mm ground materials. The IVDMTD and IVNDFD differed between and 4- and 1-mm ground materials. In vitro DM digestibility was related to aNDF and ADL concentration for all physical forms. The variables of dry matter concentration of corn grain and starch >4.75 mm as a percentage of corn silage DM improved the prediction of IVDMD for whole materials.

In vitro NDF digestibility was related to the percentage of ADL in aNDF in all physical forms. The true digestibility of NDS was near 100% for 4-mm and 1-mm ground materials. The proportion of total starch that was <4.75 mm had a negative influence on the IVNDSD of whole materials. It appears that particle size affects the digestibility of fiber and soluble matter in corn silages and this effect is nearly eliminated when silages are ground through a 4-mm screen. The negative impact of lignin on NDFD is largest in whole silages and the proportion of starch in whole kernels and fragments >4.75 mm affects NDSD.

Collaborative Study Demonstrates that the aNDF Method is Reproducible Among Laboratories

D.R. Mertens

Introduction

Analysis for NDF has the reputation for being more difficult and variable than determining ADF or CF. Differences in NDF methodology and poor laboratory technique are the most important and controllable sources of variation in NDF results among laboratories. Both problems can be minimized by following a standard NDF method exactly. Although the concept of fiber is based on nutritional criteria, the chemical measurement of fiber is defined by the laboratory method that is used. Modifications of the NDF method affect the "fiber" being measured, cause values to be different among laboratories, and give the mistaken impression that NDF cannot be measured accurately or precisely. Poor laboratory technique compounds these problems by increasing filtration difficulties and decreasing the effectiveness of washing fiber residues.

The original NDF method used a boiling detergent solution with sodium sulfite to remove protein and EDTA to chelate calcium and remove pectin. However, this procedure did not adequately remove starch from concentrates or silages that contained grains. The neutral detergent residue (**NDR**) method, which uses a heat and detergent-stable amylase to assist in the removal of starch, was developed to improve fiber determination in concentrates, but this modification of NDF eliminated the use of sodium sulfite because it might remove phenolic compounds thought to be lignin. Our laboratory developed a NDF method that can be used on all feeds and is both repeatable within laboratories and reproducible among laboratories. This method uses both amylase and sodium sulfite and is called the amylase-treated NDF (**aNDF**) method to distinguish it from other modifications of the NDF method. The objective of this study was to determine repeatability and reproducibility of the aNDF method for approval by the Association of Official Analytical Chemists International (AOAC) as an Official Method.

Methods

A detailed description of the method is too long to be included in a research summary. A copy can be obtained by contacting the author. In brief, the method includes sodium sulfite and uses two additions of heat-stable amylase to hydrolyze starch, one after bringing the reagents to boiling and one at the first residue-soaking step. The amylase solution is standardized to contain adequate activity in hot neutral detergent solutions. Fiber residue washing is described as a soaking procedure, blanks are included in each run, and residues may be ashed to obtain ash-free aNDF organic matter (aNDFom). Samples >10% fat are extracted with acetone prior to aNDF determination and modifications for difficult samples are described.

Twelve laboratories representing, research, feed company, regulatory and commercial feed testing laboratories analyzed 11 materials as blind duplicates. The materials represented a wide range of feed matrices including animal products, high protein feeds, high fat feeds, high pectin feeds, oil seeds, grains, heated byproduct feeds, and legume and grass hays and silages. Materials were selected to vary in chemical composition and contained 0 to 90% aNDF, 1 to 16% ash, 1 to 20% crude fat, 1 to 40% crude protein, and 0 to 50% starch.

Results and Discussion

Results of aNDF analyses were calculated four different ways (aNDF, aNDF blank-corrected, ash-free aNDF organic matter – aNDFom, and aNDFom blank-corrected) and the results for aNDFom blank-corrected are given in table 1. Outliers were detected using statistical tests recommended by AOAC. The laboratory ranking test indicated that Lab 12 was an outlier because it had an average bias of +1.99%-units of aNDF for all materials. Lab 12 indicated that they used medium porosity Gooch crucibles with ceramic fiber as a filter aid, which were not indicated in the aNDF method and their results were removed. Remaining outliers occurred mainly in two laboratories and in each case only one of the blind duplicates was suspect. This suggests that the aNDF method and its description may not be at fault because most remaining collaborators produced acceptable results and even the two laboratories with problems generated one acceptable result for all materials. The outlying results were eliminated from evaluation of the aNDF method.

Table 1. Statistical data for ash-free aNDF organic matter (aNDFom) that were blank-corrected.

Sample ID	n[a]	Mean (%)	s_r[b]	s_R[c]
Alfalfa silage	12	39.09	0.91	0.91
Brewer's grains	11	47.88	1.82	2.24
Citrus & beet pulp	12	27.36	0.75	1.08
Corn grain with cob	11	21.30	0.34	0.46
Corn silage	12	36.29	0.60	0.82
Corn stalks	11	69.27	0.98	1.46
Dairy mixed feed	12	12.09	0.67	0.89
Grass hay	11	55.83	1.16	1.38
Milk replacer	11	0.11	0.21	0.37
Roasted soybeans	11	13.38	0.59	1.63
Sawdust	10	89.01	1.74	1.74

[a] Number of laboratories.
[b] Standard deviation of repeatability within laboratories.
[c] Standard deviation of reproducibility within and among laboratories.

Blanks may account for systematic weighing variation among runs. The 95% confidence interval of blank variation by collaborating laboratories was about 10 mg. Because the weights of fiber residues are small, the effect of adjusting for weighing variation using blanks should be greater for materials with low aNDF. If a material contains 10% aNDF, the fiber residue from a .5 g test sample would weigh only 50 mg and the variation attributed to blank-correction could be 20%. When aNDF was blank-corrected, the average reproducibility standard deviation for materials containing <25% aNDF decreased from 1.30 to 1.01. Thus, blank-correction increases analytical precision for materials with <25% aNDF.

Analytically, ashing fiber residues and expressing results as aNDFom improves the reproducibility of results for materials with <50% aNDF. However, it decreases analytical reproducibility slightly for materials with >50% aNDF. Ashing fiber residues requires an additional step in the procedure, which incurs extra time and expense. For routine fiber analysis of forages, measuring aNDFom may not be cost effective, but there are nutritional benefits for measuring aNDFom. When nonfibrous carbohydrates (NFC) or neutral detergent soluble carbohydrates (NDSC) are calculated as dry matter - crude protein - crude fat - ash – aNDF, the ash included in aNDF is subtracted twice. Using aNDFom to calculate NFC would correct this error.

The average reproducibility standard deviation for all materials was 1.29, 1.24, 1.20, and 1.18 for aNDF, aNDF (blank-corrected), aNDFom, and aNDFom (blank-corrected), respectively. Although adjusting results for both ash and blanks improves precision, the small differences among results do not indicate a strong preference for one method of expressing the results over another. Thus, it is suggested that the method for expressing the data be left to the discretion of the laboratory and calculations for all four results are given in the aNDF method. It will be incumbent on the laboratory to state clearly which method for calculating results was used. The exception to this general rule of allowing analysts the option for choosing the method of expressing aNDF results is for feeds containing <25% aNDF. The average reproducibility standard deviation for these materials is 1.30, 1.01, 0.90 and 0.84 for aNDF, aNDF (blank-corrected), aNDFom, and aNDFom (blank-corrected), respectively. There is a clear advantage to ash- and blank-correction when materials contain <25% aNDF and this is the recommended method for these feeds and for regulatory laboratories.

Table 2 provides expected performance parameters for the aNDF method determined on categories of feeds. The standard deviation of repeatability within laboratories (s_r) was 50 to 80% of the total reproducibility within and among laboratories. This suggests that much of the variation in aNDF results is related to differences among test subsamples that are selected for analysis. The coefficient of variation or relative standard deviations were small for forages and concentrates with > 30% aNDF. The $2.8*s_r$ provides an estimate of acceptable differences between replicate analyses within a laboratory. Replicates outside this value should be reanalyzed. When performing single analyses, 19 out of 20 results among laboratories should be within ±R from table 2 (approximate 95% confidence interval).

Table 2. Performance parameters for the aNDF and aNDFom blank-corrected method.

Feed	Fiber	Mean (%)	s_r[a]	s_R[b]	RSD_r[c] (%)	RSD_R[d] (%)	r[e]	R[f]
Forages	ANDF	52.2	0.84	1.08	1.61	2.08	2.36	3.03
Forages	aNDFom	50.1	0.93	1.16	1.85	2.32	2.60	3.26
Concentrates <10% fat	ANDF	33.2	1.25	1.57	3.76	4.72	3.50	4.38
Concentrates <10% fat	aNDFom	32.2	1.14	1.47	3.55	4.56	3.20	4.11
Concentrates >10% fat	ANDF	8.7	0.79	1.24	9.06	14.26	2.21	3.47
Concentrates >10% fat	aNDFom	8.5	0.53	1.10	6.25	12.94	1.49	3.09
All materials	ANDF	38.6	1.02	1.28	2.64	3.32	2.86	3.59
All materials	aNDFom	37.4	1.00	1.24	2.67	3.32	2.80	3.48

[a] Standard deviation of repeatability within laboratories.
[b] Standard deviation of reproducibility within and among laboratories.
[c] Relative standard deviation ($100*s_r$/mean) for repeatability within laboratories.
[d] Relative standard deviation ($100*s_R$/mean) for reproducibility within and among laboratories.
[e] $2.8*s_r$ = approximate 95% confidence interval.
[f] $2.8*s_R$ = approximate 95% confidence interval.

Conclusions

Fiber is an important constituent of animal feeds because it represents the portion of feeds that is bulky and difficult to digest. The amylase-treated NDF (aNDF) method was developed as an accurate and precise method of measuring total insoluble fiber in feeds. A collaborative study was conducted to evaluate the repeatability and reproducibility of the aNDF method over the full range of feeds. Twelve laboratories representing, research, feed company, regulatory and commercial feed testing laboratories analyzed 11 materials as blind duplicates. The materials represented feed matrices including animal products, high protein feeds, high fat feeds, high pectin feeds, oil seeds, grains, heated byproduct feeds, and legume and grass hays and silages. Correcting results for changes in blanks and reporting results as ash-free aNDF organic matter improved the repeatability and reproducibility of results when aNDF was <25%. The within laboratory repeatability standard deviation for percentage aNDFom in feeds varied from 0.21 to 1.82 and the standard deviation of reproducibility among and within laboratories varied from .37 to 2.24. Both are within limits accepted by the Association of Official Analytical Chemists International and currently aNDF is being considered for Official Method - First Action status.

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Feed Utilization by Cattle

Availability of Phosphorus in dairy feeds
M.J. Aguerre, S. Marcot, H. Henselmeyer, and L.D. Satter

Introduction

Dairy diets in the U.S. typically contain more phosphorus (P) than is required, resulting in added cost (~$100 million annually in the U.S.) and increased risk of environmental damage. One reason dairy diets are formulated to contain excess P is due to uncertainty regarding the P requirement. One aspect of the uncertain requirement relates to availability of dietary P in the gastrointestinal tract. This study was conducted to determine availability of P in soybean meal and cottonseed.

Methods and Procedures

Three trials were conducted. In trial 1 and 2, 10 and 9 cows in mid to late lactation were fed for 3 wks a low P basal diet (BD) containing 0.17-0.19%P (dry basis). During the last 3-d of the third wk 12 fecal samples (dispersed through the 24-hour day) were collected. Ytterbium was used as an external marker for estimating DM digestibility. Following this three wk period, all cows were assigned to a trt diet where the test feed (soybean meal and corn gluten feed for trials 1 and 2) was inserted to provide a source of P. The test feed replaced P-free starch in the BD and increased P content of the test diets to approximately 0.3%. Fecal samples were obtained during the last 3 days of the two-wk test period. This cycle was repeated in trial 1 and 2, this time with cottonseed and corn distillers grain. Dry matter digestibility estimates from the two BD periods were averaged for calculating P availability of the two test feeds for trial 1. Marker problems prevented DM digestibility estimates in trial 2, so values of 67 and 65% were assumed for BD diets. The incremental increase in fecal P excretion due to feeding of the test feed was considered as unavailable P. Trial 3, utilizing 10 cows, was conducted in the same way, except there was only one BD period sandwiched between two test feed periods (porcine meat and bone meal and dicalcium P).

Results and Discussion

The basal diet was clearly deficient in phosphorus. When the test feed was included in the diet, dietary phosphorus would have been very close to meeting the requirement. As shown in the table, availability of phosphorus ranged between 64% for meat and bone meal to 85% for dicalcium phosphate. Meat and bone meal probably contained larger pieces of bone than typical bone meal preparations, so this availability value should not necessarily be extended to bone meal.

Conclusion

The NRC "Nutrient Requirements of Dairy Cattle" (2001) uses availability values of 64 and 70% for forages and concentrates, respectively. The availability of P, as measured in this experiment, ranged between 73 and 83% for concentrate. Availability of P in bone meal and dicalcium phosphate was 64 and 85%, respectively. These slightly higher availability values for concentrations suggest that there may be some margin of safety implied in calculating P requirements when using NRC (2001) recommendations for formulating dairy diets.

Phosphorus availability in soybean meal, cottonseed, gluten feed, corn distillers grain, and dicalcium phosphate

	DMI (kg/day)	DM Digestibility(%)	P intake (g/day)	P excreted (g/day)	P Availability (%)
Trial 1 (n=10)					
BD	19.2	64	33.0	19.8	
BD+soybean meal	20.7	63	51.3	26.0	74
BD	20.0	64	34.5	23.8	
BD+cottonseed	20.2	66	48.1	24.4	81
Trial 2 (n=9)					
BD	18.5	67	33.3	20.9	
BD+corn gluten feed	20.7	65	66.1	29.7	73
BD	17.0	67	30.6	19.0	
BD+corn distiller grain	17.0	65	49.2	22.4	83
Trial 3 (n=10)					
BD+meat and bone meal	21.2	70	61.4	31.0	64
BD	20.2	74	40.5	23.0	
BD+dicalcium phosphate	21.7	68	65.1	26.9	85

Measuring Volatile Nitrogen Losses from Dairy Farms in Wisconsin

V.R. Moreira and L.D. Satter

Introduction

Nitrogen losses from livestock excreta are due primarily to ammonia volatilization before manure is fully incorporated into the soil. Mass balances, i.e., calculating nutrient excretion as the difference between intake and product amounts is considered reasonably accurate. Measuring N disappearance from open-dirt feedlots in Nebraska, found losses up to 60 to 70% of excreted N. Volatile N losses were greater in summer than in winter. There have been reported differences in N losses from different practices in European dairy farms. In the US, an inventory of N losses is still lacking for most species, especially dairy operations.

In this survey we intended to measure N disappearance from manure of lactating dairy cows. The nitrogen to phosphorus ratio (N/P) in manure sampled from commercial dairy farms is being used to estimate volatile N losses.

Materials and Methods

Thirteen farms were initially selected for manure collection in the Spring of 2001. Due to numerous reasons, we were able to use the results of only five farms hereafter identified as 4, 5, 6, 9, and 10. Lactating cows at farm 4 were kept in a sand bedded free stall barn (Table 1). Manure was scraped twice daily, and top loaded into a concrete walled pit located under the free stall barn roof. This was emptied about every two to three weeks. Mixing occurred only before hauling the approximately 18 loads needed to empty the manure pit. This farm was surveyed twice for that season. On farm 5, lactating cows were held on a slatted floor, sawdust bedded free stall. Approximately 340,000 gallons of manure from the previous six months were pumped through a hose to the fields from the storage located beneath the slatted floor. This storage was well mixed throughout the entire emptying time.

Lactating cows on farm 6 were maintained in a sand bedded free stall barn. The barn was scraped 3x daily. Three to four loads were hauled daily from a top loaded, covered cement manure storage located under the barn floor. Manure was not mixed in this daily haul system. On farm 9, there were primarily lactating cows in a sand bedded free stall barn. The barn was scraped 3x/d and manure was hauled with minimum of mixing. On farm 10, cows were housed in a free stall with sand for bedding. Manure was scraped 3x/d, and loaded from the bottom of the storage, but was never completely emptied.

The ratio between N and P (N/P ratio) was chosen in this survey, using P as a marker that was assumed to have a recovery of 100%. Manure samples were collected throughout the emptying of manure storages. Sampling rates varied from every two loads for the smaller storages to twice daily in the larger one. Temperature and pH were measured at time of sampling. Samples of feeds as well as manure were analyzed for total nitrogen and total phosphorus.

Results and Discussion

Results presented in this report are preliminary. Dietary N and P levels were calculated from individual feed analyses and are presented in Table 1. Excreted N/P ratios were calculated based on the difference between daily nutrient intakes and amounts secreted in milk. Milk CP and phosphorus were assumed to be 3.15% and 0.09%, respectively.

The NRC (2001) model was used to estimate DM intake from body weight, milk production, DIM and CP values. Chemical composition of lactating cows' diets varied relatively little among farms.

Nutrient content of manure samples and calculated N disappearance are presented in Table 2. The ratio between manurial N and P (X=6.01; sd=0.52) only varied 8.74%, but resulted in 45.5% variation in the percentage of losses among the systems (X=15.92%; sd=7.24). As expected, lower losses occurred with the three times daily scraping, daily haul protocol (4.0%). Scraping three times a day and bottom loading was intermediate (16.9 and 16.2%), while scraping twice a day and top loading had the highest losses (19.0 and 23.5%) of the excreted N.

Conclusion

There are differences among dairy systems in N disappearance from manure. There is need for more quantitative data relating manure handling systems to volatile nitrogen losses.

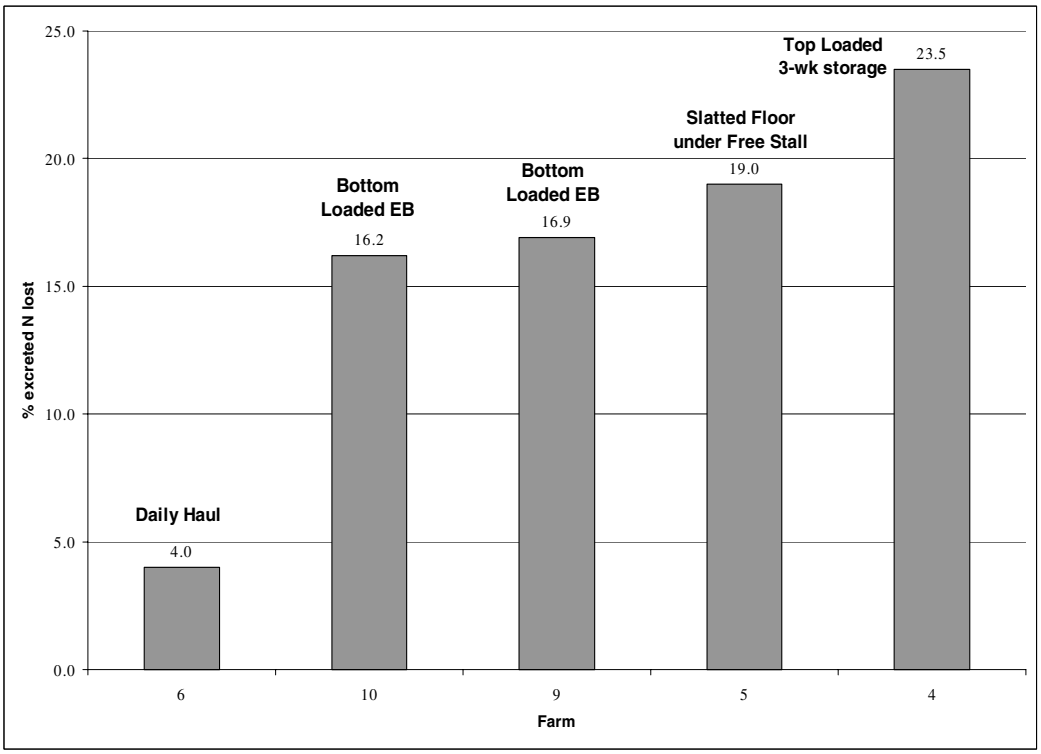
Table 1. Dietary composition, milk yield and composition, and nutrient excretion of five farms investigated in Wisconsin.

Farms	4	5	6	9Lact	9Dry	10
Average Dietary TN (%DM)	2.98	2.84	2.59	2.81	2.29	2.76
Average Dietary P (%DM)	0.45	0.44	0.41	0.38	0.36	0.41
DMI (kg/d)	24.9	24.9	28.2	23.6	5.4	25.8
Milk yield (kg)	34.1	35.8	42.2	29.9	0	36.3
Milk N (%)	0.49	0.49	0.49	0.49	0	0.49
Excreted N/P	7.05	6.80	6.72	7.82		7.40

Table 2. Manure characteristics and calculated losses.

Farms	4	5	6	9	10
pH	7.49	7.48	8.52	7.12	6.85
T (^{o}C)	13.60	10.45	10.50	20.94	18.41
DM (%)	14.85	7.85	9.07	17.75	15.87
TN (%DM)	2.88	4.34	3.65	2.65	2.51
Ash (%DM)	47.20	23.48	43.09	53.99	57.30
P (%DM)	0.52	0.81	0.59	0.40	0.39
Manure N/P	5.39	5.51	6.45	6.49	6.20
% N disappearance	23.5	19.0	4.0	16.9	16.2

[1] High due to sand bedding.



EB= Earthan Basin

Effect of Feeding Brown Midrib Corn Silage or Conventional Corn Silage Cut at Either 9" or 28" on Milk Yield and Milk Composition

D.D. Dominguez, V.R. Moreira and L.D. Satter

Introduction

Brown midrib (bm3) corn silage has been shown to increase milk production over control corn silage, due most likely to its lower lignin content and greater digestibility. Another approach to improving digestibility of corn silage is to cut corn silage at a greater height at harvest time, thus leaving behind the relatively indigestible lower stalk. The objective of this experiment was to measure milk production when bm3 corn silage or conventional corn silage cut at either 9" or 28" above ground level was fed to lactating dairy cows.

Materials and Methods

Thirty lactating Holstein cows averaging 113 days in milk and 37.3 kg milk daily were randomly assigned (n=5) to one of six treatment groups (table 1) for use in a 6x6 Latin Square design. The treatments were designed to give two levels of dietary NDF (28 and 33%), and three corn silage sources- bm3, low cut (9"), or high cut (28"). The bm3 was Cargill 657, and the control corn silage for both low and high cut was Dekalb 520 RR. The length of each of four experimental periods was three weeks, with the first week used for adaptation.

The corn silages were cut at a theoretical cut length of 3/8", and were not processed. Milkline at harvest was about .65 for the control corn silage, and .83 for the bm3.

Cows were housed in a tie stall barn, and fed once daily. Feed refusals and samples of the TMR and individual forages were collected daily and composited weekly. Cows were milked twice daily. Milk samples were taken on one day during each of the last two weeks of each period (am and pm) and measured for milk components.

Statistical analysis was done using an unbalanced and incomplete 6x4 Latin square, using the general linear models procedure in SAS.

Results and Discussion

Dry matter, CP, NDF, and ADF content of LC and HC corn silages were (%): 38.4, 6.7, 38.6, and 23.9; 40.9, 7.0, 33.9, and 20.5. Cutting corn silage at 28" rather than 9" increased dry matter content and decreased NDF and ADF content.

Table 2 contains performance information. Dry matter intake was higher for the bm3 treatment. Milk production tended to be higher with the bm3 treatment. The high-cut corn silage treatments supported the same amount of milk production as the low-cut treatments, but did so with diets containing greater amounts of forage. Milk composition was not different.

Conclusion

The bm3 treatment tended to have more milk production, but feed intake was increased with this treatment, so feed efficiency was reduced. Cutting corn silage higher results in an increase in DM content of the harvested forage and a decrease in NDF and ADF content. Lactation diets formulated to an equal NDF content enable diets with greater forage content when corn silage is harvested at a greater height, and it appears that milk production and milk composition will not be changed with these higher forage diets.

Table 1. Ingredients and chemical composition of experimental diets (% DM)

	28% NDF			33 % NDF		
	LC[1]	HC[2]	Bm3[3]	LC[1]	HC[2]	Bm3[3]
Alfalfa Silage	19.9	20.9	20.4	25.3	26.5	24.8
High cut CS	0.00	42.60	0.00	0.00	54.0	0.00
Low cut CS	40.6	0.00	0.00	51.7	0.00	0.00
Bm3	0.00	0.00	41.6	0.00	0.00	50.7
High moisture shelled corn	20.0	17.5	18.1	4.07	2.20	5.37
Roasted soybeans	12.7	12.7	12.7	12.7	12.7	12.7
Soybean meal	5.07	4.57	5.50	4.50	2.87	4.70
Mineral and vitamin mix	1.73	1.73	1.73	1.73	1.73	1.73
Diet composition						
DM%	55.0	55.0	52.7	49.6	49.5	47.9
CP%	16.8	16.8	17.0	17.2	16.8	17.2
NDF%	28.8	27.6	29.2	33.5	32.0	33.0
ADF%	18.7	17.9	18.7	22.6	21.5	21.8

[1,2,3] Correspond to low cut, high cut and bm3 corn silages, respectively

Table 2. Performance and Milk Composition

	28% NDF			33% NDF			P value			
	NC	HC	Bm3	NC	HC	Bm3	NDF	Bm3 vs HC	Bm3 vs NC	HC vs NC
DMI (kg/d)	19.81[a]	20.28[a]	22.56[b]	19.71[a]	20.18[a]	23.21[b]	.6423	<.0001	<.0001	.2640
Milk Yield (kg/d)	34.11[a]	34.37[a]	35.04[a]	30.97[b]	31.50[b]	32.62[b]	<.0001	.2607	.0968	.6084
3.5% FMC	34.60[a]	34.81[a]	35.26[a]	32.40[b]	32.50[b]	33.75[a]	.0026	.3168	.2159	.8309
Fat (%)	3.61[a]	3.60[a]	3.57[a]	3.81[b]	3.69[b]	3.73[b]	.0433	.9659	.5469	.5182
Fat (kg)	1.22[a]	1.23[a]	1.24[a]	1.17[a]	1.15[b]	1.21[a]	.0695	.3656	.4135	.9134
Protein (%)	3.05[a]	3.07[a]	3.06[a]	3.01[a]	3.0[a]	3.0[a]	.044	.7873	.9731	.7566
Lactose (%)	4.69[a]	4.74[a]	4.73[a]	4.71[a]	4.75[a]	4.71[a]	.9589	.6011	.8193	.4453
Kg milk/kg feed DM	1.73[a]	1.74[a]	1.58[b]	1.58[b]	1.58[b]	1.45[c]	<.0001	<.0001	<.0001	.8395

Means in rows with different superscripts are different (P<.05)

Use of Brown Midrib 3 Corn Silage (Cargill's Fulltime Forage 657) as the Major Forage for Transition Cows.

H. Santos, V.R. Moreira, Z. Wu and L.D. Satter

Introduction

Brown midrib 3 (bm3) is a natural mutant that has been incorporated into a modern corn hybrid. This mutant has a defective step in the synthetic pathway of lignin, and presence of the mutant results in lower lignin content of plant tissue. Corn silage with the mutant gene is normally more digestible, and has been shown to increase milk production in a number of studies. The purpose of this study was to evaluate a bm3 corn silage hybrid (Cargill Fulltime Forage 657) as a major source of forage for cows in the last three weeks of the dry period and the first four weeks of lactation.

Materials and Methods

Cows (29 primiparous and 83 multiparous) were divided into three groups and balanced according to lactation number and 305d ME milk (multiparous). Two of the three groups were combined for one of two prepartum trts. Cows were placed in trt groups 3-4 wk (mean=23 days) before their projected calving date. The two prepartum diets contained 65% forage and 35% concentrate, with corn silage (CS) providing 60% and alfalfa silage (alf) 40% of the forage (DM basis). The control CS (Dekalb 512 RR) was stored in two tower silos, and the bm3 (Cargill F657) stored in a bag silo. After calving the three groups, two of which were fed control CS prepartum, were assigned to three postpartum diets for 4-5 wk (mean=33d). The control diet (control 55 F) contained 55% forage and 45% concentrate, with 58% of the F as control CS and the balance as alf. The second and third postpartum diets contained 65% F, 58% of which was CS and 42% alf. One of these was the control CS (control 65 F) and the other was bm3 CS (bm3 65F). Cows fed control CS prepartum were fed control CS postpartum. Cows were fed a TMR once daily in a tie stall barn.

Results

The dry matter (%) and crude protein, NDF and ADF (% of DM) of the control and bm3 corn silages averaged: 35.8, 39.6; 8.2, 7.7; 41.5, 37.2; 26.2, 23.0. Dry matter intake for primiparous and multiparous cows during the 3 week prepartum period were 9.6 and 13.5 kg/day for the control corn silage, and 10.1 and 13.8 kg/day for the bm3 silage. These differences were not significant.

Dry matter intake and yield of milk and milk components for the four wk post-partum period are shown in Table 1. Results are presented separately for primiparous and multiparous cows. No differences were noted between treatments in terms of DMI. Multiparous cows, with their higher milk production, responded positively to the bm3 65 F treatment, producing about 2 kg more milk per day than either of the other two treatments. This response was even larger in terms of 3.5% FCM because of the higher milk fat test with the bm3 65 F treatment. Milk protein content was not different between treatments for primiparous cows, but it was lower in multiparous cows for the bm3 65 F treatment.

The advantage of the bm3 treatment over the other two treatments increased progressively over the four week postpartum period, and averaged 1.28, 1.33, 2.32, and 2.67 kg per cow per day for weeks 1, 2, 3, and 4, respectively. This is illustrated further in Figure 1 which shows milk production for

the three treatments during the 4 wk post-partum period as well as milk production for treatment groups after leaving their treatment diets and being placed on a common lactation diet. As pointed out previously, not all cows were available for continuing on with the post-experimental period, but the majority were. It is very interesting that the difference established between the bm3 treatment and the two control treatments during the first four weeks was sustained for at least an additional 9 wk. Milk production values for this post-experimental period were summarized only for the additional 9 wk because too few cows were available beyond this time.

Conclusion

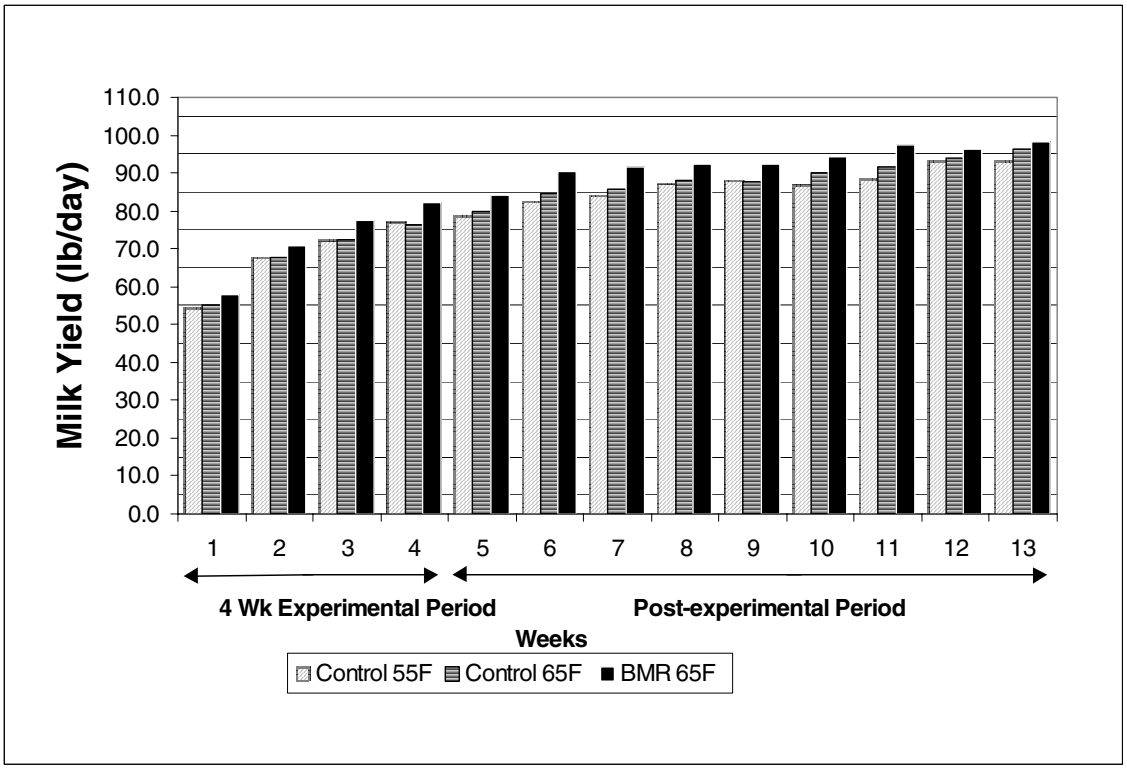
Feeding bm3 corn silage as a major part of the forage to cows in the last three weeks of the dry period and the first four weeks of lactation resulted in about two kg more milk during the first four weeks of lactation compared to cows fed a conventional corn silage hybrid. This difference tended to persist beyond the four week period during which bm3 was fed.

Table 1. Performance of cows during the first 4 wk postpartum.

	Treatment				*P*	
Item	Control 55 F	Control 65 F	bm3 65 F	SEM	Corn silage source[a]	Forage level[b]
DMI, kg/d						
Primiparous	13.0	13.1	12.8	1.1	0.84	0.91
Multiparous	16.7	16.9	17.1	0.6	0.78	0.85
Milk, kg/d						
Primiparous	25.0	24.5	24.3	1.6	0.93	0.82
Multiparous	33.4	32.9	35.2	1.0	0.09	0.75
3.5% FCM, kg/d						
Primiparous	27.9	28.1	29.2	2.1	0.73	0.94
Multiparous	38.9	39.4	42.2	1.2	0.10	0.75
Milk fat, %						
Primiparous	4.25	4.41	4.80	0.25	0.29	0.65
Multiparous	4.57	4.81	4.81	0.14	0.99	0.24
Milk protein, %						
Primiparous	3.29	3.24	3.28	0.11	0.84	0.74
Multiparous	3.35	3.47	3.26	0.06	0.02	0.20

[a]Control 65 F vs. bm3 65 F.
[b]Control 55 F vs. control 65 F.

MILK PRODUCTION BY WEEK POST-PARTUM



Milk Production, Phosphorus Excretion, and Bone Characteristics of Dairy Cows Fed Different Amounts of Phosphorus for Two or Three Years.

Z. Wu, A.J. Blohowiak, R.H. Stauffacher, J.H. Wilson, and L.D. Satter

Introduction

Although the data relating milk production to dietary phosphorus (P) content are substantial and convincing, more information is needed on the status of bone P as a function of dietary P. Bone serves as an important reservoir of P that can be mobilized to meet P requirements. A significant amount of bone P is made available in the first weeks of lactation when calcium is mobilized from bone to support lactation. The objective of this experiment was to obtain information on bone strength and bone P content after cows were fed low P diets for two or three lactations.

Materials and Methods

Thirty-seven multiparous Holstein cows were used in a 308 d lactation trial. Diets (Table 1) containing 0.31, 0.39, or 0.47% P (DM basis) were assigned to groups of 10, 14, and 13 cows at parturition. Molasses and beet pulp were included as diet ingredients because of their low P content, and this

enabled formulation of a basal diet containing 0.31% P. Diets containing 0.39 and 0.47% P were obtained by adding monosodium phosphate to the low P diet. The 37 cows used in the present trial included 14 and 19 cows that were fed similar dietary P concentrations for one or two previous lactations, respectively. The remaining four cows (three in the 0.31% P group and one in the 0.39% P group) were new to this trial.

At the end of lactation, or in some cases, after cows were removed from the experiment but still milking and receiving the same diet, surgery was conducted to remove part of the 12th rib bone. Bone strength was tested, and bone ash and P content measured.

Results

Averages of dry matter intake over the lactation were similar among treatments (Table 1), indicating that varying P from 0.31 to 0.47% of the diet had no effect on ad libitum feed intake. Cows in all groups milked well, averaging > 11,900 kg/308 d. Milk production for the 0.31% P group was higher ($P < 0.10$) than that of the 0.39% P group, and appeared the highest of the three groups during the entire lactation. Interpretation of the milk production data from his trial needs caution. This trial was a continuation of trials carried out in the previous 1 or 2 yr, and cows remained on similar levels of dietary P without being randomly reallocated to treatments. We did this for the purpose of evaluating bone characteristics after long-term feeding of different amounts of P.

In Table 3 are each year's milk yield for just the cows that had been fed similar amounts of P for 2 or 3 yr. The yields for yr 1 and 2 were obtained from previous reports, and those for yr 3 from the current trial. Compared with 0.48 to 0.49% dietary P, feeding 0.31 or 0.38 to 0.40% P did not reduce the cow's milking capacity after 2 to 3 yr.

The concentration of inorganic P in blood serum was lower ($P < 0.01$) for cows fed the lowest P diet than for those fed the other diets; the overall means during the lactation were 5.7, 6.1, and 6.5 mg/dl (SE 0.1) for the 0.31, 0.39, and 0.47% P groups, respectively. The concentrations were similar toward the end of lactation. Serum P concentration can reflect P intake in ruminants, but it is not always a good indicator of P status. Only extremely low serum concentrations (<4 mg/dl) may indicate deficiency of P.

In a previous paper, we showed that the concentration of total P in milk appears to be related to milk protein concentration. A trend for this relationship again was observed in the present study using cows from all treatments ($r^2 = 0.21$, Figure 1). The average concentration of P in milk from this data set that involved 705 measures was 0.094% (SD 0.009). Extrapolation of the regression in Figure 1 to 0% protein indicates that P content of protein-free milk is 0.0487%, about half of the P present in milk containing 3.00 to 3.25% crude protein. This agrees with other observations that about half of the total P in milk is complexed with casein and the other half exists as diffusible ions or in milk serum (Farrell, 1988; Jenness, 1985). A cow having milk testing 4% rather than 3% crude protein would have about 0.0146 percentage units more P in the milk, or approximately 16% more total P (Figure 1). Since approximately 60% of the P requirement for a cow milking 40 kg/d is partitioned to milk production, and assuming the P maintenance requirement for the cow is independent of milk protein content, then a cow producing 4% protein milk (crude protein) should require about 10% more dietary P than a cow producing 3% protein milk. Our feeding standards need to reflect this.

No differences were found among treatments in the sheer stress the bone endured before rupture or the amount of energy required to deform the bone to the point of fracture (fracture energy) (Table 4). Wall thickness of the bone was 5.1 mm for all treatments. Bone specific gravity tended ($P<0.1$) to be lower for the 0.31% P treatment than for the other two treatments, with the difference being about 4%. The ash content of the bone, expressed on dry weight, wet weight, or wet bone volume, was slightly lower ($P<0.06$ to 0.13) for the 0.31% P group. The P content of bone was similar among treatments when expressed on an ash or dry weight basis, averaging 17.6 (SE 0.3) and 9.5% (SE 0.2), respectively. When expressed on a wet weight or volume basis, however, P content was lower ($P<0.06$ to 0.13) for the 0.31% P treatment compared to the 0.47% P treatment. The average decrease in ash and P content, based on measurements in dry weight, wet weight, and wet bone volume, was 4.8 and 6.0%, respectively, between the 0.31% and 0.47% P treatments.

Summary

Figure 2 is a summary of what we consider the status of P nutrition of lactating dairy cows producing >9000 kg/305 d lactation. The bare minimum of dietary P consistent with normal or near normal animal performance is 0.30%. At this amount, signs of P deficiency may be just appearing. At the other extreme of the continuum in Figure 2 is what most dairy producers in the United States are actually feeding. Several surveys show that dairy producers are feeding 0.46% to 0.50% dietary P. We consider the NRC recommendation as being more than adequate, but a reasonable level for the dairy industry to quickly move to. Feeding 0.35% P will provide a margin of safety above what might be considered a borderline deficient diet containing 0.30% and may be the choice for dairy producers facing serious nutrient management problems. If dairy producers reduce dietary P from current amounts to NRC recommended amounts, P excretion in manure will be reduced 25 to 30%, and P supplementation costs reduced by $10 to $15 per cow per lactation.

Table 1. Performance of cows fed diets differing in P content.

	Dietary P content(%)			
Item	0.31	0.39	0.47	SEM[a]
Number of cows	10	14	13	...
DMI, kg/d	25.0	25.0	24.6	0.6
Milk, kg/308-d	13,038	11,909	12,126	407[b]
3.5% FCM, kg/d	43.4	39.4	40.3	1.4[b]
Milk fat, %	3.64	3.50	3.64	0.12
Milk protein, %	3.16	3.13	3.10	0.05
Body weight during lactation				
Initial[1], kg	663	623	609	20[c]
Ending[1], kg	735	718	701	22
Change, g/d	277	345	320	76

[a]No treatment by month interaction for lactational measurements ($P > 0.10$).
[b]0.31% P > 0.39% P ($P < 0.10$).
[c]0.31% P > 0.47% P ($P < 0.10$).
[1]Initial weight was taken on an average DIM of 15 (SD 9), and ending weight at 290 DIM (SD 10).

Table 2. Strength measurements of the 12th rib bone from cows fed diets differing in P content for 2 to 3 yr.

	Dietary P content (%)			
Item	0.31	0.39-0.40	0.47-0.49	SEM
Number of cows[1]	9	9	11	...
Shear stress, N/mm^2	26.5	28.1	27.5	2.2
Fracture energy[2], N-m	66.6	60.5	65.0	4.2
Wall thickness, mm	5.1	5.1	5.2	0.1
Bone specific gravity[g]	1.50	1.57	1.55	0.02
Ash, % of dry weight	53.9[c]	56.2[a]	55.6[ab]	0.8
Ash, % of wet weight	46.0[c]	47.4	48.1[a]	0.7
Ash, g/cc, wet bone	0.69[c]	0.74[a]	0.74[a]	0.01
P, % of ash	17.7	17.3	17.9	0.3
P, % of dry weight	9.5	9.7	9.9	0.2
P, % of wet weight	8.1[c]	8.2	8.6[b]	0.2
P, g/cc, wet bone	0.122[c]	0.129	0.133[a]	0.003

[a, b, c]c < a (P <0.06), c < b (P < 0.13).
[g]Linear and quadratic (P < 0.14) effects.
[1]The nine cows sampled from the 0.31% P group included three cows that were fed this amount of P for 1 yr; all other cows sampled in this trial had been fed similar amounts of P for 2 or 3 yr.
[2]Area under the force (N) and deformation (m) curve. It is an expression of the amount of energy the bone absorbs before fracture.

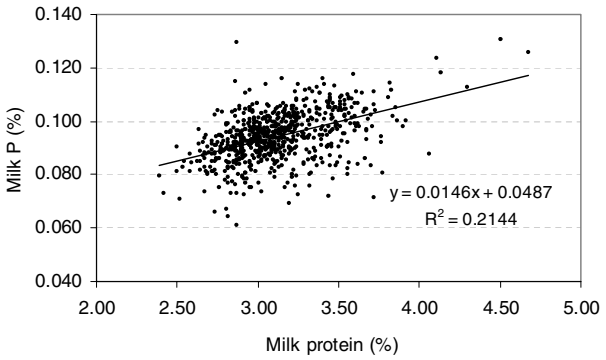


Fig. 1. Relationship between milk P concentration and milk crude protein content.

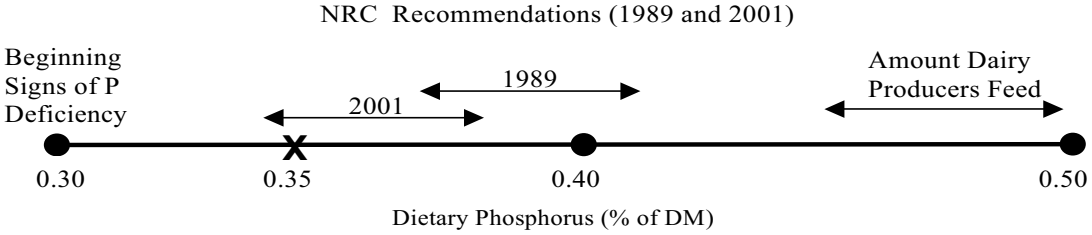


Fig. 2. Status of phosphorus nutrition of lactating dairy cows milking more than 9000 kg/305 d of lactation.

Effects of Replacing Dietary High Moisture Corn With Dried Molasses on Production of Dairy Cows

G.A. Broderick and W.J. Radloff

Introduction

An earlier feeding study (Broderick, Luchini et al., USDFRC Res. Summaries, 2001) indicated that replacing dietary corn starch with sugar (sucrose) increased DM intake and fat yield in cows fed an diet in which two-thirds of the forage was alfalfa silage. Although there were trends in ruminal concentrations of protein degradation products suggesting increased microbial protein formation, there were no significant changes in milk or protein yield in that study. Dried molasses is a practical source of dietary sugars for dairy cows. Therefore, we repeated our earlier trial, except that dried molasses was added to an alfalfa silage-based diet at the expense of high moisture shelled corn, the major concentrate component. Again, our objective was to determine if sugar supplementation would improve milk production and N utilization in dairy cows fed a diet containing alfalfa silage as the major forage.

Materials and Methods

Eight primiparous and 40 multiparous (8 with rumen cannulae) Holsteins were fed TMR containing (DM basis) 40% alfalfa silage, 20% corn silage, and 40% concentrate without molasses (Table 1) for a 2-wk standardization period. Production of milk and milk components during this period was used as a covariate adjustment for production during the subsequent experimental period. After standardization, cows were blocked by parity and days in milk to 12 blocks of four (two blocks with rumen cannulae) and within each group were randomly assigned to one of four TMR ranging from 29 to 17% high moisture shelled corn and from 0 to 12% dried molasses (Table 1). Cows were fed their assigned experimental diets for 8-wk. All cows received biweekly injections of BST. Milk yield was measured at each milking; DM intake was determined daily. Yield of milk components was determined from milk samples taken at both daily milkings one day during the covariate and every 2-wk during the experimental period. Spot urine and fecal samples were collected from all cows on the last day of wk-4 and wk-8. Ruminal metabolites also were measured on the last day of wk-4 and wk-8 in the eight cannulated cows. Statistical analysis was done using proc GLM in SAS. The statistical model for each milk component included block (n = 12) and covariate value, and for urinary and fecal traits, for ruminal metabolites, and for apparent digestibility included block and sampling period.

Results and Discussion

The four experimental diets averaged 18.0% CP (Table 1), somewhat higher than anticipated, because the alfalfa silage contained about 22% CP. Experimental diets also contained an average 29% NDF and 42% nonfiber carbohydrates, indicating that there was sufficient energy content to support about 40 kg/d of milk production. Nonstructural carbohydrate analysis of the diets indicated that replacing high moisture corn by adding from 0 to 12% dried molasses (in 4% increments) increased dietary sugars by 4.6%. However, there was a 8% decrease in dietary nonstructural carbohydrates over this range due to starch declining more than total sugars increased (Table 1). This was because the dried molasses fed in this trial was produced using a fibrous carrier and contained about 22% ADF. Thus, unlike the earlier study where sucrose replaced equal amounts of corn starch, results

from the present trial were confounded by a small step-wise increase in dietary fiber with each increment of dietary sugar. Despite this confounding effect, there was a linear increase in DM intake, and significant quadratic responses in the yields of FCM and fat, with increasing dietary sugar (Table 2). The maximum of the quadratic responses for FCM and fat occurred at an average of 3.5% dried molasses added to the diet. There were trends ($P \leq 0.11$) for linear increases in yield of protein and true protein, and a trend ($P = 0.11$) for a quadratic response in protein yield, with increasing dietary sugar. However, because significant increases in milk or protein yield did not accompany the increased DM intake, there were significant linear declines in milk/DM intake and milk N/N intake with increasing dietary sugar. The linear decreases in urinary excretion of urea N and total N were not consistent with the observed reduction in N efficiency with added dietary sugar.

There was a significant quadratic effect of sugar addition to the diet on ruminal ammonia (Table 3); the minimum ammonia concentration occurred at 6.1% added dried molasses—corresponding to about 2.3% added total sugars. Feeding dried molasses also gave a linear decline in branched-chain VFA. Reduced ruminal concentrations of both ammonia and branched-chain VFA suggested that sugar feeding stimulated microbial protein formation. Adding sugar to the diet had no effect on total VFA, or on molar proportions of acetate, propionate, or acetate: propionate ratio. However, molar proportion of butyrate was increased with sugar addition; this may explain our finding of increased fat secretion (Table 2). Although no effect was detected on ruminal butyrate in our previous study (Broderick, Luchini et al., USDFRC Res. Summaries, 2001), increased butyrate was observed in a number of trials in which sugar feeding also increased fat yield in lactating cows. Adding dried molasses to the diet gave linear increases in apparent DM and OM digestion, which indicated that, although dietary fiber was elevated, energy availability also was increased.

Summary and Conclusion

Incremental replacement of high moisture corn with dried molasses resulted in a linear increase in DM intake. Moreover, there were significant quadratic responses in yields of FCM and fat; maxima were found at about 3.5% dried molasses or about 1.4% added total sugars in the dietary DM. Significant reductions in urinary N excretion and in ruminal concentrations of ammonia and branched-chain VFA suggested there was improved microbial protein formation with sugar supplementation; however, there were no effects on the yield of milk, protein, lactose, or SNF under the conditions of this trial. Overall, the major responses to supplementing dietary sugar as dried molasses were improved feed intake and fat production.

Table 1. Composition of diets[1].

Item	Covariate	Diet A	Diet B	Diet C	Diet D
Dried molasses, % DM	. . .	0	4	8	12
HMSC, % DM	31	29	25	21	17
	% of DM				
Alfalfa Silage	40.0	40.0	40.0	40.0	40.0
Corn Silage	20.0	20.0	20.0	20.0	20.0
High moisture shelled corn	30.6	29.0	25.0	21.0	17.0
Solvent soybean meal	4.0	8.0	8.0	8.0	8.0
Roasted soybeans	4.4	0.0	0.0	0.0	0.0
Fat (Energy Booster®)	0.0	2.0	2.0	2.0	2.0
Sodium bicarbonate	0.4	0.4	0.4	0.4	0.4
Dicalcium phosphate	0.2	0.2	0.2	0.2	0.2
Salt	0.3	0.3	0.3	0.3	0.3
Trace minerals and vitamins[2]	0.1	0.1	0.1	0.1	0.1
Dried Molasses	0.0	0.0	4.0	8.0	12.0
Chemical composition					
CP	18.1	18.1	17.9	17.9	18.1
NDF	30.5	28.2	29.1	29.2	29.3
ADF	20.5	18.3	19.3	19.6	20.0
NFC	39.6	42.8	41.4	40.7	41.2
Fat	4.1	4.3	4.2	4.7	3.8
NSC[3]	31.6	33.9	32.6	30.8	30.4
Starch[3]	29.4	31.3	28.4	25.2	23.2
Sugar[3]	2.2	2.6	4.2	5.6	7.2
Sugar increment[4]	. . .	0.0	1.6	3.0	4.6

[1]HMSC = High moisture shelled corn; NFC = nonfiber carbohydrate; NSC = nonstructural carbohydrate.

[2]Provided 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E/kg of DM.

[3]Nonstructural carbohydrates determined by Tammy Miller Webster of West Virginia University.

[4]Incremental sugar addition to diets B, C, and D determined by chemical analysis.

Table 2. Effect of replacing dietary high moisture corn with dried molasses on intake, weight gain, production, and urinary excretion in lactating dairy cows.

	Diets					Probability[2]	
Item	A	B	C	D	SE[1]	Linear	Quadratic
Dried molasses, % DM	0	4	8	12			
HMSC, % DM	29	25	21	17			
DM intake, kg/d	25.1	25.8	26.2	26.0	0.3	0.04	0.16
Weight gain, kg/d	0.41	0.16	0.36	0.19	0.09	0.27	0.65
Milk, kg/d	38.0	37.5	38.9	36.7	0.6	0.34	0.16
Milk/DM intake	1.51	1.46	1.48	1.43	0.02	0.03	0.99
Milk N/N intake	0.255	0.244	0.254	0.231	0.006	0.02	0.29
3.5% FCM, kg/d	41.4	42.0	43.4	39.5	1.0	0.33	0.04
Fat, %	4.07	4.26	4.11	4.06	0.10	0.65	0.24
Fat, kg/d	1.54	1.59	1.63	1.47	0.04	0.34	0.03
Protein, %	3.12	3.09	3.11	3.04	0.04	0.29	0.61
Protein, kg/d	1.19	1.14	1.23	1.09	0.03	0.10	0.11
True protein, %	3.00	2.98	2.98	2.92	0.04	0.20	0.55
True protein, kg/d	1.14	1.09	1.17	1.05	0.03	0.11	0.19
SNF, %	8.85	8.84	8.85	8.80	0.04	0.48	0.62
SNF, kg/d	3.38	3.30	3.49	3.18	0.08	0.25	0.18
Milk urea N, mg/dl	15.3	14.4	15.0	14.7	0.3	0.52	0.37
Urine volume, l/d	32.5	33.0	32.1	33.1	0.9	0.83	0.78
Urinary urea-N, g/d	175	154	171	146	5	< 0.01	0.69
Urinary total-N, g/d	324	295	321	288	8	0.04	0.80
Urea-N/total-N	0.554	0.535	0.546	0.519	0.015	0.17	0.82

Least square means.
[1]SE = Standard error.
[2]Probability of a significant linear or quadratic effect of the dietary level of dried molasses.

Table 3. Effect of replacing dietary high moisture corn with dried molasses on ruminal pH and metabolites and on nutrient digestibility in lactating dairy cows.

	Diets					Probability[2]	
Item	A	B	C	D	SE[1]	Linear	Quadratic
Dried molasses, % DM	0	4	8	12			
HMSC, % DM	29	25	21	17			
Rumen metabolism							
pH	5.81	5.88	5.79	5.91	0.09	0.59	0.79
Ammonia-N, mg/dl	11.33	9.12	10.71	10.67	0.46	0.86	0.05
Total AA, mM	1.80	1.34	2.19	2.01	0.42	0.46	0.76
Total VFA, mM	129.4	129.4	135.8	131.6	4.1	0.50	0.63
VFA molar proportions, mol/100 mol							
Acetate	62.2	63.6	62.0	64.1	1.0	0.38	0.74
Propionate	22.0	20.8	21.9	19.8	1.0	0.26	0.67
A: P ratio	2.90	3.13	2.88	3.28	0.18	0.30	0.63
Butyrate	11.5	11.3	11.9	12.0	0.2	0.10	0.52
Branched-chain VFA	2.62	2.83	2.49	2.52	0.06	0.05	0.19
Valerate	1.74	1.55	1.73	1.64	0.07	0.69	0.52
Digestibility, %							
DM	57.7	58.9	60.0	61.9	0.7	< 0.01	0.65
OM	58.5	60.2	61.2	63.2	0.8	< 0.01	0.80
NDF	39.2	38.7	38.7	40.5	0.8	0.27	0.17
ADF	41.8	39.9	38.9	39.9	0.8	0.07	0.09
N	56.9	55.5	57.8	57.6	1.2	0.41	0.65
Fecal excretion, kg/d							
N, g/d	316	323	328	307	12	0.71	0.24

[1]SE = Standard error.

[2]Probability of a significant linear or quadratic effect of the dietary level of dried molasses.

Effect of Feeding Alfalfa or Red Clover Silage to Lactating Dairy Cows With or Without Corn Silage

G.A. Broderick and R.P. Walgenbach

Introduction

The large amounts of NPN in alfalfa silage (AS) substantially reduce its protein efficiency when fed to lactating dairy cows. Researchers at the Dairy Forage Center (Jones et al., J. Sci. Food Agric. 67:329-333, 1995) found that an enzyme, polyphenol oxidase, acts in red clover silage (RCS) to produce a forage with less NPN than AS. A previous feeding study (Broderick and Maigan, 1996 USDFRC Res. Sum.) showed that, although DMI was greater on AS, milk yield was similar on AS and RCS, and N-efficiency greater on RCS. Other consistent findings have been lower CP content and greater DM and fiber digestibility for RCS. The present trial assessed the effects of feeding AS versus RCS, with or without dietary corn silage (CS).

Materials and Methods

Alfalfa silage was harvested from first cutting beginning on May 20 and ending on May 28, 1998; RCS was harvested from two cuttings taken on June 8, 1998 (first cutting) and July 14, 1998 (second cutting). Forages were field-wilted, chopped, and ensiled in two bunker silos (AS) or in two plastic bags (RCS). Forages were cut using a conventional mower conditioner, wilted to about 35% DM

(range 26 to 41% DM), chopped to a theoretical length of 2.9 cm, and ensiled without additives. No forage was rained on during harvest. Diets (Table 1) were formulated to about 60 or 48% of DM from either AS or RCS; diets with 48% AS or RCS contained 12% CS. Half of the RCS DM on both RCS diets came from each cutting. Diets with 60% AS or RCS contained equal soybean meal (SBM); sufficient SBM was added to the 48% RCS diet to give an CP content equal to that in the 60% RCS and 48% AS diets. Twenty-four multiparous cows were randomly assigned to diets in six balanced 4 X 4 Latin squares. Diets were fed for 4-wk periods before switching (total 16 wk); production and composition data were collected in the third and fourth wk of each period. Apparent nutrient digestibility was estimated from fecal grab samples using indigestible ADF as internal marker. Statistical analysis was done using proc GLM in SAS.

Results and Discussion

Red clover silage contained less DM, NDF, and ADF than did AS but there was only a trend for CP in AS to be higher than in RCS (Table 1). As was observed in every trial comparing these two forages, NPN content was lower in RCS than AS: 40 versus 64% NPN in total N. Proportions of total N present as ammonia and free AA also were lower in RCS. The RCS contained about 2 percentage units more ADIN than AS (Table 1). As expected, greater CP in AS resulted in the 60% AS diet containing more CP than the other three diets, which averaged about 16.5% CP (Table 1).

Results from the lactation study are in Table 2. Cows ate more when fed AS than when fed RCS, with or without CS or supplemental SBM in the diet. Yields of milk, total protein, true protein, and SNF were not different among cows fed diets containing forage from AS, RCS, or AS + CS, regardless of CP level or DMI. Cows fed forage as RCS + CS (with additional SBM) actually yielded more milk, protein, true protein, and SNF than when fed the other three diets, despite lower feed intake than on either AS diet. This observation was at variance with the reduced yields that accompanied lower intakes on RCS in some of our earlier work (Broderick and Sterrenburg, 1996 USDFRC Res. Sum.). The RCS diets fed in those previous trials, although containing forage DM equal to that of the AS diets, contained less CP. Additional SBM in the RCS + CS diet provided both RDP and RUP and the greater protein yields may have reflected improved protein status. Earlier, we found that equalizing dietary CP by adding SBM gave rise to similar production on RCS as on AS (Broderick and Maigan, 1996 USDFRC Res. Sum.). Feed DM and N efficiencies were greater on both RCS diets than on the AS diets (Table 2). Although confounded by dietary CP level, MUN concentration was higher on the diet containing 60% AS than on the other three diets. We noted that MUN was lower on 60% RCS than on AS + CS or RCS + CS, despite its numerically greater CP (Table 1). However, DMI and N intake also were lowest on the RCS diet; earlier, excess N intake (total N intake - milk N secretion) was found to be nearly as well correlated to MUN as dietary CP concentration. Additionally, milk fat content averaged about 0.25 unit lower on the two RCS diets (Table 2). Previously, we observed lower milk fat content and yield when RCS replaced AS as dietary forage (Broderick and Sterrenburg, 1996 USDFRC Res. Sum.). However, milk fat content was in excess of 4%, quite high for Holsteins, and there was no effect of diet on fat yield ($P = 0.32$). Differences in feed efficiency were associated with large effects ($P < 0.01$) of diet on nutrient digestibility (Table 2). Apparent digestibility of DM, OM, NDF, ADF, and hemicellulose were highest on RCS, intermediate on RCS + CS, and lowest on the two AS diets. Replacing RCS with CS reduced apparent digestibilities but there was no effect when CS replaced dietary AS, suggesting that apparent digestibility of RCS nutrients exceeded that of CS but that digestibilities of AS and CS were comparable. Despite differences in DMI, intake of digestible OM was about equal across all diets, ranging from 14.5 to 14.9 kg/

d. This indicated that lower DMI on the RCS was due to its greater energy digestibility and cows ate to constant energy supply.

Summary and Conclusion

Relative to AS, RCS averaged two or more percentage units lower in CP, NDF, and ADF but had only 62% as much NPN (proportion of total N). When fed at equal proportions of diet, cows consumed less DM on RCS but similar digestible OM. Yields of milk, FCM, protein, and SNF were equal when RCS replaced AS at 60% of the diet, despite lower DMI. Supplementing the RCS + CS diet (in which CS replaced one-fifth of the forage) with enough SBM to increase CP to the level in the AS + CS diet increased yields of milk, total protein, true protein, and SNF. Replacing AS with RCS increased apparent digestibility of dietary nutrients and increased feed and N efficiencies. Utilization of both energy and CP in RCS exceeded that of AS. Greater nutrient digestibility and lower NPN content suggested that feeding RCS may result in lower environmental N losses than feeding AS.

Table 1. Composition of alfalfa and red clover silages and diets[1].

	Forage			
Item	AS	RCS	SE	$P > F$
DM, %	36.6	28.9	0.4	< 0.01
CP, % of DM	21.7	19.1	0.7	0.08
Ash, % of DM	10.5	11.5	0.3	0.13
NDF, % of DM	44.6	42.6	0.4	0.04
ADF, % of DM	35.6	32.9	0.4	0.02
Hemicellulose, % of DM	9.0	9.7	0.4	0.40
pH	4.70	4.54	0.09	0.35
NPN, % of total N	64.0	39.6	1.6	< 0.01
NH_3-N, % of total N	13.2	11.1	0.3	0.01
Total AA-N, % of total N	26.7	16.6	0.6	< 0.01
ADIN, % of total N	3.1	5.2	0.2	< 0.01

	Diet			
Item	AS	RCS	AS + CS	RCS + CS
	% of DM			
Alfalfa silage	60.5	. . .	48.3	. . .
Red clover silage	. . .	60.7	. . .	48.5
Corn silage	. . .	. . .	12.3	12.3
High moisture shelled corn	35.7	35.6	35.6	32.8
Solvent soybean meal	2.9	2.9	2.9	5.6
Dicalcium phosphate	0.5	0.5	0.5	0.5
Salt	0.3	0.3	0.3	0.3
Trace minerals and vitamins[2]	0.1	0.1	0.1	0.1
Chemical composition				
CP	18.4	16.6	16.5	16.3
NDF	32.0	31.3	32.1	31.7
ADF	24.1	22.7	22.7	21.8

[1]AS = alfalfa silage, RCS = red clover silage, CS = corn silage.

[2]Provided 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E/kg of DM.

Table 2. Effect of feeding diets containing alfalfa silage (AS), red clover silage (RCS), or AS or RCS plus corn silage (CS), on production and digestibility in lactating dairy cows.

Item	Diets				SE[1]	$P > F^2$
	AS	RCS	AS+CS	RCS+CS		
DMI, kg/d	23.5[a]	21.8[c]	23.8[a]	22.8[b]	0.2	<0.01
BW gain, kg/d	0.30	0.56	0.46	0.60	0.12	0.27
Milk, kg/d	30.4[b]	30.4[b]	30.3[b]	31.7[a]	0.3	0.02
3.5% FCM, kg/d	31.6	31.1	32.0	32.4	0.5	0.27
Fat, %	4.30[a]	4.06[b]	4.33[a]	4.08[b]	0.07	<0.01
Fat, kg/d	1.15	1.11	1.16	1.15	0.02	0.32
Protein, %	3.33	3.30	3.39	3.36	0.03	0.29
True protein, %	3.13	3.14	3.20	3.18	0.03	0.41
Protein, kg/d	0.89[b]	0.90[b]	0.91[b]	0.95[a]	0.01	<0.01
True protein, kg/d	0.83[b]	0.86[b]	0.86[b]	0.90[a]	0.01	<0.01
SNF, %	8.81	8.87	8.89	8.95	0.08	0.64
SNF, kg/d	2.35[b]	2.43[b]	2.39[b]	2.54[a]	0.03	<0.01
Milk/DMI	1.30[b]	1.40[a]	1.28[b]	1.40[a]	0.01	<0.01
Milk N/N intake	0.204[c]	0.249[a]	0.228[b]	0.256[a]	0.003	<0.01
Milk urea, mg N/dl	13.6[a]	9.2[d]	11.2[b]	10.1[c]	0.3	<0.01
Apparent digestibility, %						
DM	64.5[c]	71.5[a]	65.1[c]	68.4[b]	0.7	<0.01
OM	66.7[c]	73.3[a]	67.4[c]	70.0[b]	0.6	<0.01
NDF	44.1[c]	55.3[a]	44.3[c]	49.8[b]	0.7	<0.01
ADF	44.2[c]	54.2[a]	43.4[c]	49.1[b]	0.7	<0.01
Hemicellulose	43.9[c]	58.0[a]	46.3[c]	51.3[b]	1.1	<0.01
N	62.3	59.7	60.7	58.2	1.1	0.06

[a,b,c,d]Means within the same row without a common superscript differ ($P < 0.05$).
[1]SE = Standard error.
[2]Probability of a significant effect of diet.

Alfalfa Versus Red Clover Silage—Summary of Results From Five Feeding Studies With Lactating Dairy Cows

G.A. Broderick

Introduction

When ensiled, 50 to 60% of the CP in alfalfa typically is broken down to NPN. High levels of NPN depress protein utilization by lactating dairy cows. Red clover, a forage legume similar to alfalfa, forms less NPN in the silo. However, widespread use of red clover is limited by its lower yield per acre, poorer stand persistency, and slower field drying rates. If there were consistent advantages in efficiency of nutrient utilization for red clover, then research could be directed toward improving the agronomic characteristics of this forage. Over a number of years, five lactation trials were conducted at the Dairy Forage Center to determine the relative feeding value of alfalfa silage (AS) and red clover silage (RCS) for dairy cows. Although the trials had several different dietary treatments, each contained at least one direct comparison of AS to RCS fed at equal dietary DM. This report summarizes results of an analysis of the relative performance of cows fed AS or RCS at equal proportions of the diet over all five studies.

Materials and Methods

Results were from five Latin square feeding studies that are already published (Broderick et al., J. Dairy Sci. 83: 1543-1551, 2000; Broderick et al., J. Dairy Sci. 84:1728-1737, 2001). Data were obtained by feeding AS and RCS harvested at various cuttings during 1992 through 1998. Generally, the forages were cut using conventional mower conditioners, field wilted to 30 to 50% DM, chopped to a theoretical length of 2.9 cm, and ensiled without additives in either upright concrete stave silos or in plastic bags. Diets (Table 1) were formulated to contain about 60% DM from either AS or RCS; overall mean dietary compositions were weighted for the number of dairy cows used in each trial. A total of 104 animal observations were made on each diet. Mean soybean meal contents on the two diets were not the same because more soybean meal was fed with RCS in one of the five trials to equalize dietary CP. Data from all five trials were analyzed using proc GLM in SAS with a model that included period(trial), trial, forage, and forage by trial interaction. Least square means are reported.

Results and Discussion

Consistently, red clover silage contained less CP and ADF, and more hemicellulose, than did AS (Table 1). There was a trend for forage pH to be lower in RCS, suggesting this silage fermented more rapidly. The NPN content of RCS was lower than AS: 36 versus 53% of total N; proportions of total N present as ammonia and free AA also were lower in RCS. These differences were observed in all five trials. The RCS contained 1.6 percentage units more ADIN than AS; formation of ADIN may be related to the action of polyphenol oxidase, the plant enzyme that accounts for the lower NPN formation in RCS. As expected, the greater CP in AS resulted in those diets containing more CP than the RCS diets (Table 1).

Least square means from the five lactation studies are in Table 2. Cows consistently ate more when fed AS than when fed RCS; overall DMI was 1.5 kg/d lower on RCS. Yield responses due to silage source were variable among trials. For example, milk yield was greater on AS in two trials, not different in two trials, and greater on RCS in one trial. However, there were no significant overall effects of silage source ($P \geq 0.16$) on yields of milk, FCM, protein, or SNF despite the differences in feed intake. There were trends ($P \sim 0.10$) for greater weight gain and lower fat yield on RCS than AS; additionally, milk fat content averaged about 0.15 unit lower ($P = 0.05$) on RCS diets. These results suggested that replacing dietary AS with RCS may result in the redirecting of fat synthesis away from mammary secretion to body deposition. Blood glucose concentration was unaffected by forage source, indicating there were no overt differences in insulin secretion on the two diets. Efficiency of capture of feed N in milk was greater ($P < 0.01$), and both milk and blood urea concentrations were lower ($P < 0.01$), on RCS diets. Although these factors were confounded by higher dietary CP on AS (Table 1), they suggested that N losses to the environment may be reduced through feeding of RCS or other low NPN silages. A lactation in which a cow produces 10,000 kg of milk with 3.2% protein would result in secretion of (320/6.38) = 50 kg of milk N. Increasing N efficiency from 0.236 to 0.271 would reduce N excretion from 162 to 135 kg N/lactation. Feed DM efficiency also tended to be greater (P = 0.10) on RCS. The difference in feed DM efficiency was associated with large effects of forage on nutrient digestibility. Apparent digestibility of DM, OM, NDF, ADF, and hemicellulose all were higher ($P < 0.01$) on RCS than on AS. Moreover, replacing AS with RCS reduced ($P < 0.01$) estimated excretion of fecal DM by 19%. A reduction of this magnitude, if milk yield were not altered, would have the positive effect of substantially decreasing the amount of manure solids that

would have to be handled on the dairy farm.

The NEL requirements for maintenance, BW gain, and milk output (based on observed fat and SNF content) were used to compute forage energy values (Table 3). The NEL requirements for mean production were nearly identical (average 32.4 Mcal/d). Subtracting the NEL contributed from the concentrate portion of the diet yielded estimates of NEL supplied by AS and RCS. Per unit DM, AS was computed to have 1.14 Mcal/kg, versus 1.29 Mcal/kg for RCS, indicating that RCS contained 13% more NEL than AS, despite the two forages having equal NDF contents (Table 1). An NEL content of 1.88 Mcal/kg for the concentrate portion of the ration (Table 3) corresponds to a TDN value of 76% (NRC, 2001). If TDN is assumed equal to OM digestibility, OM digestibilities of 55.3 and 63.0% may be computed, respectively, for the AS and RCS forages in these diets, indicating a relative value for RCS that is 14% greater than AS. Despite differences in DMI, intake of digestible OM was nearly identical on the two silages: 14.4 kg/d on AS and 14.5 kg/d on RCS. This indicated that the lower DMI on RCS diets was due to greater energy digestibility and cows ate to constant energy supply.

Summary and Conclusion

Over five different lactation trials, RCS averaged 3.0 and 1.7 percentage units lower in, respectively, CP and ADF and contained only 67% as much NPN (as a proportion of total N). When fed at equal amounts of the diet, cows consumed less DM on RCS than on AS but equal amounts of digestible OM. Yields of milk, FCM, protein, and SNF were equal when RCS replaced AS, despite the lower DMI. Milk fat content was lower, and there were tendencies for greater weight gain and lower fat yield, when RCS replaced AS in the diet. Replacing AS with RCS increased apparent digestibility of dietary DM, OM, NDF, ADF and hemicellulose and N efficiency and tended to increase feed efficiency. Utilization of both energy and CP in RCS exceeded that of AS. Greater nutrient digestibilities and lower NPN content suggested that feeding RCS would result in improved nutrient efficiencies and lower environmental N losses than feeding AS.

Table 1. Composition of alfalfa and red clover silages and diets.

Item	Forage			
	Alfalfa silage	Red clover silage	SE[1]	$P > F^2$
DM, %	41.3	40.3	1.2	0.55
CP, % of DM	20.9	17.9	0.4	<0.01
Ash, % of DM	11.2	10.8	0.3	0.37
NDF, % of DM	43.3	43.0	0.5	0.63
ADF, % of DM	33.8	32.1	0.4	<0.01
Hemicellulose, % of DM	9.5	10.8	0.2	<0.01
pH	4.62	4.49	0.05	0.07
NPN, % of total N	53.1	35.8	1.0	<0.01
NH_3-N, % of total N	29.5	17.7	0.7	<0.01
Total AA-N, % of total N	9.5	7.6	0.4	<0.01
ADIN, % of total N	3.5	5.1	0.2	<0.01

Item	Diet	
	Alfalfa silage	Red clover silage
	% of DM	
Alfalfa silage	62.7	. . .
Red clover silage	. . .	62.4
High moisture corn	34.6	34.0
Solvent soybean meal	1.6	2.4
Dicalcium phosphate	0.6	0.6
Salt	0.3	0.3
Trace minerals and vitamins[3]	0.2	0.2
Chemical composition		
CP	17.7	15.8
NDF	33	32
ADF	24	22

[1]SE = Standard error.

[2]Probability of a significant effect of forage source.

[3]Three different but typical trace mineral and vitamin supplements were fed over the course of the five trials.

Table 2. Effect of feeding diets containing either alfalfa silage or red clover silage on production and digestibility in lactating dairy cows.

Item	Diet		SE[1]	$P > F^2$
	Alfalfa silage	Red clover silage		
DMI, kg/d	22.9	21.4	0.3	< 0.01
BW gain, kg/d	0.02	0.20	0.07	0.08
Milk, kg/d	32.0	31.2	0.5	0.27
3.5% FCM, kg/d	32.0	31.0	0.5	0.16
Fat, %	3.66	3.51	0.06	0.05
Fat, kg/d	1.12	1.08	0.02	0.10
Protein, %	3.03	3.00	0.02	0.29
Protein, kg/d	0.93	0.91	0.01	0.26
Lactose, %	4.78	4.81	0.02	0.27
Lactose, kg/d	1.49	1.48	0.02	0.68
SNF, %	8.53	8.56	0.05	0.59
SNF, kg/d	2.65	2.62	0.04	0.50
Milk/DMI	1.42	1.47	0.02	0.10
Milk N/N intake	0.236	0.271	0.003	< 0.01
Milk urea, mg N/dl	12.5	8.7	0.2	< 0.01
Blood urea, mg N/dl	14.2	9.3	0.3	< 0.01
Blood glucose, mg/dl	52.8	52.7	0.5	0.89
Fecal DM, kg/d	8.80	7.14	0.15	< 0.01
Apparent digestibility, %				
DM	61.5	66.6	0.7	< 0.01
OM	63.0	67.9	0.6	< 0.01
NDF	43.1	52.5	0.7	< 0.01
ADF	44.5	52.2	0.7	< 0.01
Hemicellulose	38.9	52.3	1.1	< 0.01
N	59.1	55.2	1.1	< 0.01

[1]SE = Standard error.
[2]Probability of a significant effect of forage source.

Table 3. The NEL contents of alfalfa silage and red clover silage estimated from overall mean intake and production data.[1]

Item	Silage source	
	Alfalfa	Red clover
Dietary silage, % of DM	62.7	62.4
Dietary concentrate, % of DM	37.3	37.6
NEL requirement, Mcal/d[2]		
Maintenance	9.7	9.7
BW gain	0.1	1.0
Milk output	22.6	21.7
Total requirement, Mcal/d	32.5	32.4
Total DMI, kg/d	22.9	21.4
Concentrate DMI, kg/d	8.5	8.0
Concentrate NEL,[3] Mcal/kg DM	1.88	1.88
Concentrate NEL, Mcal/d	16.0	15.1
NEL from Silage,[4] Mcal/d	16.5	17.3
Silage DMI, kg/d	14.4	13.4
Silage NEL, Mcal/kg DM	1.14	1.29
Red clover/Alfalfa, %		113

[1]Diet composition data are means from Table 1 and intake and production data are least square means from Table 2.

[2]NEL (Mcal/d) requirements computed: maintenance = 0.08 x $BW^{0.75}$ (mean BW = 601 kg), gain = 5.12 x BW gain, and milk output = Milk x (0.09464 x % fat + 0.049 x % SNF - 0.0564) (NRC, 2001).

[3]Mean NEL contents of dietary concentrate in the trials computed from NRC (2001) tables.

[4]Total NEL requirement minus concentrate NEL intake.

Effect of Replacing Dietary Starch With Sucrose on Milk Production in Lactating Dairy Cows

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Introduction

Diets based on alfalfa silage and similar hay-crop silages contain high levels of NPN and other sources of rumen-degraded protein (RDP). On such diets, ruminal carbohydrate and protein fermentation often are out of synchrony—that is, the rate of energy fermentation is too slow to allow ruminal organisms to synthesize protein from rapidly available RDP. In these circumstances, increasing the rate of carbohydrate fermentation may lead to more effective capture of RDP and improved supply of absorbable protein to the dairy cow. Sugars are more rapidly fermented in the rumen than are most sources of starch, suggesting that sugars could serve as effective supplements on alfalfa silage diets. Moreover, the Cornell model suggests that the organisms in the ruminal liquid phase that utilize soluble sugars can contribute proportionately more microbial protein than the organisms that ferment starches and other nonstructural carbohydrates. A feeding study was conducted to determine if replacing dietary starch with sucrose would improve milk production and N utilization in dairy cows fed a diet containing alfalfa silage as its major forage.

Materials and Methods

Two sets of 24 Holstein cows (mean = 41 kg/d of milk) were fed a TMR containing (DM basis) 40% alfalfa silage, 20% corn silage, 7.5% corn starch, and 32.5% other concentrate ingredients (Table 1) for a 2-wk standardization period. Production of milk and milk components during this period were used as a covariate adjustment for production during the subsequent experimental periods. After standardization, cows in each set of 24 were blocked by days in milk into six groups of four cows and randomly assigned to one of four TMR ranging from 7.5 to 0% corn starch and from 0 to 7.5% sucrose (Table 1). Cows were fed their assigned experimental diets for 8-wk. Milk yield was measured at each milking; DM intake was determined daily. Yield of milk components was determined from milk samples taken at both daily milkings one day during the covariate and every 2-wk during the experimental period. Statistical analysis was done using proc GLM in SAS. The statistical model included period (n = 2), block (n = 12), and covariate yield of each milk component. Four ruminally cannulated cows were assigned to a 4x4 Latin square trial with 4-wk periods that was superimposed over the two 8-wk experimental phases of the larger lactation trial. Ruminal metabolites were measured on the last day of each period and the results analyzed using proc GLM in SAS.

Results and Discussion

The four experimental diets ranged from 16.6 to 16.9% CP, from 29 to 30% NDF, and from 43 to 44% nonfiber carbohydrates (Table 1). Moreover, nonstructural carbohydrate analysis of the diets indicated that the target increments of sugar addition, 2.5, 5.0 and 7.5% were very nearly reached by sucrose replacement of dietary corn starch (Table 1). Generally, there was little effect of sucrose supplementation on production. Although DM intake was increased linearly when sucrose replaced dietary starch, there was no effect on body weight gain or on yield of milk, protein, lactose, and SNF (Table 2). Because milk and protein yield were not increased, despite increased intake, there were linear reductions in DM and N efficiency with sucrose addition to the diet. However, there was a linear response in both milk fat content and milk fat yield when sucrose replaced dietary corn starch. The response of milk fat content was striking in that the increase in these Holsteins was from 3.8%

on 0-sucrose to nearly 4.2% on 7.5% sucrose diet. Graded levels of sucrose were added to the diet in an attempt to identify points of maximum response to sugar supplementation. However, there were no quadratic responses ($P \geq 0.15$) in this trial and this objective could not be met.

There were small but significant effects on some ruminal metabolites with incremental replacement of dietary corn starch with sucrose (Table 3). Adding sugar to the diet increased the molar proportion of propionate in total VFA and reduced molar proportions of branched chain VFA (the VFA produced from catabolism of the branched chain AA). Reduced branched chain VFA, plus trends ($P \leq 0.17$) for lower ruminal concentrations of NH_3 and total AA, suggested that there may have been greater microbial uptake of the products from RDP with sugar addition to the diet (Table 3). Feeding sucrose has been reported to stimulate milk fat secretion by increasing butyrate production in the rumen. As mentioned earlier, milk fat secretion was increased by sucrose feeding (Table 2); however, there was no effect on molar proportions of butyrate in total VFA in this trial. It is possible that ruminal butyrate production may have altered without apparent change in butyrate concentrations. Because there are only 95% as many 6-carbon sugars per unit weight in the disaccharide sucrose as in pure starch, replacing dietary corn starch with sucrose would lead to a small dilution of dietary energy. However, this effect would have been small: we estimated that the non-fat NEL of the ration decreased from 1.63 to 1.62 Mcal/kg of DM in going from 7.5% starch and 0 sucrose to 0 starch and 7.5% sucrose.

Table 1. Composition of diets[1].

Item	Covariate	Diet A	Diet B	Diet C	Diet E
			% of DM		
Alfalfa Silage	40.0	40.0	40.0	40.0	40.0
Corn Silage	20.0	20.0	20.0	20.0	20.0
High moisture shelled corn	20.5	20.5	20.5	20.5	20.5
Solvent soybean meal	6.6	9.0	9.0	9.0	9.0
Roasted soybeans	3.0	0.0	0.0	0.0	0.0
Fat (Energy Booster®)	1.4	2.0	2.0	2.0	2.0
Sodium bicarbonate	0.4	0.4	0.4	0.4	0.4
Dicalcium phosphate	0.2	0.2	0.2	0.2	0.2
Salt	0.3	0.3	0.3	0.3	0.3
Trace minerals and vitamins[2]	0.1	0.1	0.1	0.1	0.1
Corn Starch	7.5	7.5	5.0	2.5	0.0
Sucrose	0.0	0.0	2.5	5.0	7.5
Chemical composition					
CP	16.8	16.6	16.7	16.8	16.9
NDF	30.0	30.0	29.2	29.6	29.6
ADF	19.8	20.7	20.0	20.8	20.5
NFC	42.3	42.7	43.7	42.6	42.8
NDIN	3.5	3.0	3.3	3.3	2.8
ADIN	1.6	1.7	2.0	1.8	1.7
Fat	4.6	4.0	4.0	4.0	4.0
NSC[3]	25.4	30.9	32.5	31.6	31.5
Starch[3]	22.9	28.2	27.4	24.5	21.5
Sugar[3]	2.6	2.7	5.1	7.1	10.0
Sugar increment[4]	. . .	0.0	2.4	4.4	7.3

[1]ADIN = Acid detergent insoluble N; NDIN = neutral detergent insoluble N; NFC = nonfiber carbohydrate; NSC = nonstructural carbohydrate.

[2]Provided 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E/kg of DM.

[3]Nonstructural carbohydrates determined by Tammy Miller Webster of West Virginia University.

[4]Incremental sugar addition to diets B, C, and D determined by chemical analysis.

Summary and Conclusion

Incremental replacement of dietary corn starch with sucrose resulted in a linear increase in DM intake but had no effect on the yield of milk, protein, lactose, or SNF. However, there were significant linear increases in milk fat content and yield with elevated dietary sugar. Small changes in ruminal metabolite concentrations were consistent with improved utilization of RDP when sucrose was added to a high quality lactation diet containing 40% alfalfa silage. Overall, effects of dietary sugar addition were confined to improved intake and fat production.

Table 2. Effect of replacing dietary corn starch with sucrose on intake, weight gain, and production in lactating dairy cows.

	Diets					Probability[2]	
Item	A	B	C	D	SE[1]	Linear	Quadratic
Sucrose, % of DM	0	2.5	5.0	7.5			
Starch, % of DM	7.5	5.0	2.5	0			
DM intake, kg/d	24.5	25.6	26.0	26.0	0.4	0.01	0.17
Weight gain, kg/d	0.34	0.53	0.40	0.47	0.12	0.61	0.62
Milk, kg/d	38.9	40.4	40.0	39.4	0.7	0.74	0.15
3.5% FCM, kg/d	40.5	42.2	43.9	43.2	1.3	0.11	0.38
Fat, %	3.81	3.82	4.07	4.16	0.11	0.01	0.73
Fat, kg/d	1.47	1.53	1.65	1.62	0.06	0.05	0.47
Protein, %	3.24	3.22	3.27	3.30	0.04	0.23	0.54
Protein, kg/d	1.24	1.28	1.29	1.28	0.03	0.35	0.36
Lactose, %	4.75	4.74	4.77	4.74	0.04	0.95	0.90
Lactose, kg/d	1.84	1.91	1.90	1.85	0.06	0.95	0.37
SNF, %	8.76	8.76	8.87	8.82	0.06	0.34	0.68
SNF, kg/d	3.38	3.51	3.51	3.44	0.10	0.69	0.32
Milk/DM intake	1.60	1.58	1.54	1.52	0.03	0.02	0.96
Milk N/N intake	0.312	0.291	0.291	0.285	0.01	0.01	0.26

Least square means.
[1]SE = Standard error.
[2]Probability of a significant linear or quadratic effect of sucrose level in the diet.

Table 3. Effect of replacing dietary corn starch with sucrose on ruminal pH and metabolites in lactating dairy cows.

	Diets					
Item	A	B	C	D	SE[1]	$P > F$[1]
Sucrose, % of DM	0	2.5	5.0	7.5		
Starch, % of DM	7.5	5.0	2.5	0		
pH	6.19	6.16	6.19	6.21	0.05	0.88
NH_3-N, mg/dl	6.93	6.87	6.21	5.75	0.34	0.14
Total AA, mM	4.32	3.46	3.31	3.61	0.29	0.17
Total VFA, mM	105.8	106.8	111.6	103.7	4.2	0.61
Molar proportion, mol/100 mol						
Acetate	60.9	60.8	60.1	60.4	0.7	0.87
Propionate	20.2[b]	21.1[ab]	21.4[ab]	22.0[a]	0.3	0.04
A:P ratio	3.06	2.93	2.87	2.82	0.07	0.16
Butyrate	14.3	13.1	13.5	14.0	0.6	0.63
Branched-chain VFA	3.7[a]	3.3[a]	3.1[ab]	2.4[b]	0.2	0.02
Valerate	2.0	2.4	2.5	2.0	0.2	0.26

[a,b]Least square means within the same row with different superscripts are different ($P < 0.05$).
[1]SE = Standard error.
[2]Probability of a significant effect of diet.

Effects of Feeding Dairy Cows Protein Supplements of Varying Ruminal Degradability

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Introduction

Optimizing the balance between microbial protein synthesis and degradation in the rumen will reduce ruminant N excretion and, consequently, N losses to the environment. Several newly devised systems of ruminant ration formulation require rates of ruminal protein degradation in their application. Lack of reliable information on, and methods to determine, protein degradation rates has led to the common practice of over-feeding protein to dairy cows to avoid possible amino acid shortages. Protein standards of known in vivo degradabilities are needed to develop methods for determining ruminal degradability. An omasal canal sampling technique developed by Huhtanen et al. (J. Anim. Sci. 75:1380-1392,1997) and modified by Ahvenjarvi et al. (Brit. J. Nutr. 83:67-77, 2000) has proven to be an accurate and less invasive approach for measuring nutrient flows out of the rumen. Our objectives were: 1) to quantify the effects of feeding protein supplements with differing ruminal degradabilities on milk production, and 2) to use the omasal sampling technique to quantify in vivo rates and extents of ruminal degradation of four protein supplements.

Materials and Methods

Twenty-five (ten with ruminal cannulas) lactating Holstein cows were assigned to five, 5 x 5 Latin squares and fed TMR containing rolled corn silage, alfalfa silage, rolled high moisture shelled corn, protein supplement, urea, and minerals. Diets (Table 1) differed in source of protein supplement: diet A (control; no supplement); diet B (solvent soybean meal; SSBM); diet C [expeller soybean meal (SoyPlus ® West Central Coop., Ralston, IA); ESBM], diet D (blood meal; BM); and diet E (corn gluten meal; CGM). Experimental periods lasted 21 d , consisting of 12-d for adaptation and 9-d for sample collection. Body weights were measured at the end of each period. Milk yield was recorded at all daily milkings. Milk was sampled at consecutive milkings on d-13 and d-20 of each period and analyzed for fat, protein, and SNF by infrared analysis (AgSource, Menomonie, WI). A triple marker technique using separate markers for liquid (Co-EDTA), small particle (Yb), and large particle (indigestible NDF) phases was used to quantify omasal digesta flow out of the rumen. The alternating vacuum and pressure system originally developed by Huhtanen et al. (1997) and Ahvenjarvi et al. (2000) was used to collect digesta from the omasal canal. Purines were used to distinguish between total protein AA N (TAAN) from microbial protein and rumen undegraded feed protein (RUP). Ruminal contents also were collected from all cannulated cows after Co-EDTA and Yb infusions were stopped. Samples were analyzed for marker decay (ruminal passage rates) and for VFA, ammonia and total free AA. Statistical analysis was done using proc mixed in SAS.

Results and Discussion

Cows fed control diet A consumed 1.1 to 2.1 kg/d less DM than cows fed the other diets; DMI in cows fed diet C (ESBM supplemented) was higher than that in cows fed diet D (BM) but not different from cows fed diets B (SSBM) and E (CGM) (Table 2). Although daily BW gain was not different among diets, cows fed basal diet A had the lowest yields of milk, FCM, fat, protein, and SNF, partly as a result of having the lowest DMI. Cows fed diets C (ESBM) and E (CGM) produced 1.4 and 1.9 kg/d more milk than cows fed diet B (SSBM). Milk yield of cows fed diet D (BM) was intermediate; however, of the four protein supplemented diets, DMI was numerically lowest when

BM was fed. Yields of fat, protein, and SNF were not different among diets B, C, D, and E. Milk concentrations of fat, protein, and SNF were not altered by diet. Neither ruminal pH or total free AA concentrations were affected by diet (Table 2). However, ruminal ammonia was lowest on diets A (control) and E (CGM), highest on diet B (SSBM), and intermediate on diets C (ESBM) and D (BM). Lower CP intake and higher intake of high moisture corn may have reduced ammonia concentration and enhanced microbial NPN utilization on diet A. Higher ruminal ammonia on diet B is consistent with greater ruminal degradation of SSBM. Cows fed diets B and D had higher ruminal concentrations of total VFA than cows fed diets A and E ($P < 0.05$), with cows fed diet C being intermediate (Table 2) . Ruminal VFA concentrations are the result of production and absorption and do not necessarily reflect fermentation rates. Moreover, the range of total VFA concentrations was narrow, from only 110 to 119 m*M*, and these small changes may not be biologically important. Although there were statistically significant effects of diet on ruminal acetate and propionate concentrations, differences were small and there was no effect on acetate: propionate ratio. Ruminal isobutyrate was highest on diet B (SSBM), lowest on basal diet A and diets D (BM) and E (CGM), and intermediate on diet C (ESBM). Isobutyrate partly comes from ruminal catabolism of valine and lower concentrations may be related to reduced true protein degradation. Neither ruminal OM nor NDF digestibility was altered by diet.

Estimates of extent of ruminal escape of the four protein supplements based on omasal TAAN flows are in Table 3. Ruminal escapes for SSBM, ESBM, BM, and CGM were, respectively, 35, 51, 62, and 53% based on TAAN flow. The differences in milk yields (Table 2) were consistent with these estimates of RUP. That greater omasal TAAN flow (due to greater ruminal BM escape) did not give rise to greater protein yield may be related to differences among the proteins in RUP digestibility. The NRC (2001) assigned intestinal digestibilities of 93, 93, 80, and 94% for the RUP from SSBM, ESBM, BM, and CGM, respectively. Extent of protein degradation is the resultant of the rates of passage and degradation, and both must be known to estimate the amount of protein escaping rumen degradation. The particle sizes of the protein concentrates were such that they would have been expected to pass with the small particle (Yb) phase. The NPN (fraction A) and ADIN (fraction C) contents were small and proportions of intact protein (fraction B) ranged from 98.4 to 99.5% for the four proteins (Table 3). In vivo degradation rates for these fractions B were computed using both the observed Yb passage rates (mean rate = 0.14/h) and assuming a passage rate of 0.06/h. These rates of degradation were compared to those determined using the inhibitor in vitro method (Table 3). Rates computed using the Yb passage rate ranged from 1.5 (SSBM) to about 10-times (BM and CGM) more rapid than in vitro rates. Degradation rates computed for SSBM and ESBM using the 0.06/h rate were similar to those determined in vitro. Although slower, degradation rates computed for BM and CGM using 0.06/h were still 4 and 5-times greater than in vitro. Similar milk yields with feeding of ESBM, BM, and CGM (Table 2) suggested that these proteins provided similar amounts of RUP and that in vitro degradation rates obtained for both BM and CGM were too slow. In vivo degradation rates, based on either Yb passage rates or an assumed rate of 0.06/h, will make these proteins useful standards for development of in vitro methods.

Summary and Conclusion

In vivo ruminal degradability of SSBM, ESBM, BM, and CGM was assessed in lactating dairy cows from relative production and by measuring omasal TAAN flows in ruminally cannulated animals. Responses of DMI and yield of milk, protein, and fat with supplemental protein indicated that the basal diet supplied inadequate amounts of absorbable protein. Among supplemented diets, milk yield

was greatest on ESBM and CGM, least on SSBM, and intermediate on BM. Extents of degradation for these proteins, and either passage rates observed for the small particle phase or assuming a passage rate of 0.06/h, were used to compute in vivo degradation rates. The in vivo degradation rates all were more rapid than rates determined in vitro. These four proteins will serve as standards for evaluating in vitro methods for predicting ruminal protein degradation.

TABLE 1. Composition of diets.

Item	Diet A	B	C	D	E
			(% of DM)		
Ingredients					
Corn silage	43.6	43.7	43.7	43.7	43.7
Alfalfa silage	22.2	22.2	22.2	22.2	22.2
High moisture shelled corn	30.6	21.7	20.7	25.2	23.7
Urea	2.0	2.0	2.0	2.0	2.0
Solvent soybean meal	…	8.8	…	…	…
Expeller soybean meal	…	…	9.7	…	…
Blood meal	…	…	…	5.4	…
Corn gluten meal	…	…	…	…	6.8
Sodium bicarbonate	0.5	0.5	0.5	0.5	0.5
Dicalcium phosphate	0.6	0.6	0.6	0.6	0.6
Salt	0.3	0.3	0.3	0.3	0.3
Mineral mix	0.1	0.1	0.1	0.1	0.1
Vitamin-mineral concentrate[1]	0.1	0.1	0.1	0.1	0.1
Nutrient content of diets					
CP, % of DM	15.8	19.1	19.7	20.3	19.3
NDF, % of DM	27.1	26.9	27.1	26.9	26.1
ADF, % of DM	17.5	17.3	17.3	17.0	16.7
NPN,[2] % of total N	55.3	43.9	42.0	40.0	39.0

[1]Provided (per kg of DM): Zn, 56 mg; Mn, 46 mg; Fe, 22 mg; Cu, 12 mg; I, 0.9 mg; Co, 0.4 mg; Se, 0.3 mg; vitamin A, 6440 IU; vitamin D, 2000 IU; and vitamin E, 16 IU.

[2]Proportion of total N soluble in 10% (wt/vol) trichloroacetic acid.

TABLE 2. Effect of feeding protein supplements of differing ruminal degradability on production and ruminal metabolism.[1]

Item	Diet A	B	C	D	E	SE	$P > F^2$
Protein supplement	None	SSBM	ESBM	BM	CGM		
Production							
DMI, kg/d	21.7[c]	23.5[ab]	23.8[a]	22.8[b]	23.7[ab]	0.6	<0.01
OM intake, kg/d	19.1	20.0	21.2	19.5	20.8	1.0	0.42
BW gain, kg/d	0.39	0.55	0.16	0.59	0.36	0.15	0.23
Milk Yield, kg/d	32.9[c]	36.5[b]	37.9[a]	37.6[ab]	38.4[a]	1.2	<0.01
3.5% FCM	34.8[b]	40.0[a]	38.4[a]	38.3[a]	40.5[a]	1.3	<0.01
Milk fat, %	3.77	3.87	3.57	3.64	3.73	0.11	0.09
Milk fat, kg/d	1.18[b]	1.36[a]	1.29[a]	1.28[a]	1.34[a]	0.04	<0.01
Milk protein, %	3.04	3.12	3.07	3.08	3.15	0.05	0.09
Milk protein, kg/d	0.95[b]	1.11[a]	1.12[a]	1.10[a]	1.15[a]	0.03	<0.01
SNF, %	8.51	8.66	8.62	8.66	8.71	0.08	0.10
SNF, kg/d	2.69[b]	3.07[a]	3.15[a]	3.13[a]	3.18[a]	0.1	<0.01
Ruminal metabolism							
pH	6.18	6.09	6.16	6.00	6.16	0.06	0.07
Total AA, mM	3.11	3.47	2.98	3.20	2.81	0.23	0.15
Ammonia, mM	8.01[d]	11.24[a]	10.35[ab]	9.94[bc]	9.09[cd]	0.42	<0.01
Total VFA, mM	110.2[b]	119.2[a]	114.9[ab]	119.0[a]	112.4[b]	2.7	0.03
Acetate, mM	68.8[b]	74.2[a]	72.6[a]	73.9[a]	71.3[ab]	1.4	0.02
Propionate, mM	22.6[b]	24.6[ab]	23.1[b]	25.7[a]	22.2[b]	1.3	0.03
A: P ratio	3.04	3.02	3.14	2.88	3.21	0.15	0.11
Butyrate, mM	13.7	14.7	13.7	14.2	13.1	0.5	0.24
Isobutyrate, mM	1.11[d]	1.31[a]	1.25[ab]	1.13[cd]	1.21[bc]	0.03	<0.01
Isovalerate + 2-methyl butyrate, mM	2.01	2.28	2.17	2.15	2.30	0.10	0.11
Valerate, mM	2.01	2.10	2.04	2.03	2.17	0.16	0.73
OM digestibility,[3] %	42.5	42.4	42.4	38.8	42.3	1.8	0.56
NDF digestibility,[3] %	46.9	47.9	44.8	43.6	41.5	2.9	0.50

[a,b,c]Means in rows with different superscripts differ ($P < 0.05$).

[1]BM = Blood meal; CGM = corn gluten meal; ESBM = expeller soybean meal; SE = standard error; SSBM = solvent soybean meal.

[2]Probability of a significant effect of diet.

[3]Ruminal digestibility = [(Intake – Omasal flow) / Intake] x 100.

TABLE 3. Effect of feeding protein supplements of differing ruminal degradability on intake and omasal flow of TAAN and on in vivo estimates of rate and extent of ruminal protein degradation.[1]

Item	Diet					SE	$P > F^2$
	A	B	C	D	E		
Protein supplement	None	SSBM	ESBM	BM	CGM		
TAAN intake, g/d							
from basal ingredients	238[a]	205[b]	212[ab]	238[a]	237[a]	12	0.04
from protein supplement		136[b]	138[b]	164[a]	133[b]	6	<0.01
Total TAAN omasal flow, g/d	329[c]	386[b]	408[ab]	440[a]	408[ab]	19	<0.01
Microbial[3]	214	240	235	224	223	16	0.66
RUP_{Total}[4]	115	146	173	216	185		
RUP_{Basal},[5] %	48.3						
$RUP_{Protein}$,[6] g/d		47.0	70.6	101.0	70.5		
Escape (Protein supplement),[7] %		34.5	51.1	61.6	53.0		
Fraction A (NPN), % total N		1.2	0.9	0.3	1.0		
Fraction B, % total N		98.7	98.9	99.5	98.4		
Fraction C (ADIN), % total N		0.2	0.2	0.2	0.7		
In vivo results							
Passage rate (Yb-k_{SP}),[8] /h		0.14	0.14	0.14	0.12		
Ruminal degradation rate (Yb-k_{SP}),[9] /h		0.26	0.13	0.09	0.11		
Ruminal deradation rate (k_p = 0.06/h),[9] /h		0.11	0.06	0.04	0.05		
In vitro results[10]							
Ruminal degradation rate, /h		0.17	0.04	0.01	0.01		
RUP,% of total CP		26	58	85	86		

[a,b,c]Means in rows with different superscripts differ ($P < 0.05$).

[1]BM = Blood meal; CGM = corn gluten meal; ESBM = expeller soybean meal; SE = standard error; SSBM = solvent soybean meal; TAAN = Total AA N.

[2]Probability of a significant effect of diet.

[3]Estimated from purines deterimined using the HPLC method of Makkar and Becker (2000).

[4]RUP_{Total} flow, g/d = Omasal flow, g/d – Microbial flow, g/d.

[5]RUP_{Basal}, % = [RUP_{Total}, g/d (diet A) / Intake, g/d (diet A)] x 100.

[6]$RUP_{Protein}$, g/d = RUP_{Total}, g/d – [Intake (basal ingredients), g/d x RUP_{Basal} (diet A)].

[7]Extent (protein), % = (TAAN flow from protein supplement / TAAN intake from protein supplement) x 100.

[8]In vivo ruminal Yb (small particle) passage rate.

[9]In vivo ruminal degradation rate = [(fraction B x kp) / (RUP – fraction C)] – kp, where kp was either the ruminal Yb passage rate or assumed to equal 0.06/h.

[10]Estimated using the inhibitor in vitro method, assuming a ruminal passage rate of 0.06/h.

Effects of Varying Dietary Protein and Energy Levels on the Production of Lactating Dairy Cows

G.A. Broderick

Introduction

There are complex relationships among dietary protein and energy composition and the amount of protein that can be utilized by the dairy cow. Dietary protein supplies absorbable protein by providing rumen-degraded protein to stimulate microbial protein formation in the rumen and by providing rumen-undegraded protein that can be digested directly by the cow. High energy diets stimulate microbial protein synthesis, thus increasing the supply of the major source of absorbable protein for the high producing cow. It is uneconomical to overfeed protein or energy. Overfeeding protein can result in excessive N excretion; nearly all of this N will be lost in the urine, the most environmentally labile form of excreted N. Overfeeding of concentrates will depress rumen pH, resulting in reduced fiber digestion and milk fat secretion as well as other metabolic problems for the cow. A feeding study was conducted using nine diets—three protein levels at each of three NDF (energy) levels—to help identify optimal amounts of dietary protein and energy.

Materials and Methods

Eighteen primiparous and 45 multiparous Holstein cows were blocked by lactation number and days in milk into seven groups of nine and randomly assigned to an incomplete Latin square trial with four periods. Diets were fed for 4-week periods before switching (total 16 weeks). The nine TMR were formulated from alfalfa and corn silages, high moisture corn, solvent soybean meal, plus minerals and vitamins (Table 1). Dietary forage was 60% from alfalfa silage and 40% from corn silage on all diets; NDF was varied by feeding forage at 75, 63 and 50% of dietary DM. Dietary CP was varied by adding soybean meal at the expense of high moisture corn. Milk yield and DMI were measured daily in the last 2 week of each period; yield of milk components was determined one day in each of the last 2 week of each period. Fecal and urine spot samples were collected from 36 cows in the last week of each period to estimate urinary N excretion using creatinine as a urine volume marker and fecal N excretion using indigestible ADF as an internal marker. Statistical analysis was done using proc GLM in SAS. The model included square, cow(square), period(square), CP and NDF level, and CP x NDF interactions; a conservative error term, cow(square), was used to assess statistical significance.

Results and Discussion

There were no significant interactions ($P \geq 0.21$) between dietary CP and NDF (energy) for any production or metabolism trait in the trial. Therefore, it was possible to make simple comparisons within the three levels of CP and NDF, without having confounding effects of CP on energy level or vice versa. Intake of DM and fat yield were influenced ($P \leq 0.03$) by CP content of the diet; both were lower on the lowest protein diet (15.1% mean CP) than on the two higher protein diets (Table 2). There were linear increases in milk urea and urinary N excretion, and linear decreases in N efficiency (milk N/N intake), with increasing dietary CP. Thus, the principal production effect of increasing dietary CP (by adding soybean meal) in cows averaging 34 kg/d of milk was to increase feed intake and milk fat yield. Effects of dietary energy content on milk production were more dramatic. There were linear increases in BW gain, milk yield, yield of protein, true protein, lactose and SNF, milk/DM intake, and milk N/N intake, and linear decrease in milk urea,with decreasing dietary NDF (Table 2). Component yield paralleled milk concentration except for fat, which declined with reduced dietary NDF; fat yield was higher at 32% dietary NDF than at 28% NDF. Yield of FCM was lower on the lowest energy diet. Increasing dietary energy (by reducing dietary forage and hence NDF) improved milk yield and efficiency and, except for milk fat, yield of milk components.

The effects of diet on N metabolism in this trial are shown in Table 3. As expected, there were linear increases in N intake and urinary N excretion with dietary CP, as well as a smaller influence of CP on fecal N excretion. Urinary N excretion nearly doubled, while fecal N increased a more modest 15%, with the increase from 15.1 to 18.4% dietary CP. As dietary energy content was increased by reducing mean NDF from 36 to 28%, urinary N declined linearly by about 12%. Interestingly, milk N secretion actually increased to a somewhat greater degree over this range of dietary energy, even though there was no significant effect of fecal N excretion. These trends were more clearly illustrated when N metabolism was expressed as a proportion of N intake: increasing CP from 15.1 to 18.4% reduced capture of milk N from 31 to 25% of dietary N; urinary N excretion was increased from 23 to 35% of dietary N, and fecal N declined from 45 to 41% of dietary N, over this range. Clearly, if milk production were not reduced, the greatest N efficiency would make the lowest protein diet the most economical—the one giving the least labile urinary N and the one that would be environmentally the

most sustainable. Expressed as a proportion of N intake, there was a comparable increase in milk N secretion for each percentage unit decrease in urinary N excretion, when dietary energy content was increased. A significant effect of diet on urinary purine derivative excretion, an indicator of microbial protein formation in the rumen, also was observed. When dietary energy was increased by reducing NDF from 36 to 28%, purine derivative excretion increased by about 20%. This suggested that microbial protein supply also increased by a similar amount (Table 2).

Summary and Conclusion

Three levels of protein, each at three levels of energy, were fed to lactating cows. Increasing CP from 15.1 to 18.4% by adding soybean meal to the diet had only a small positive effect on DM intake; also, milk fat yield was higher at 16.7% CP than at 15.1% CP. However, there were large increases in milk urea and NPN and in urinary N excretion, and a substantial decrease in N efficiency, over this range of dietary protein. Increasing dietary energy by reducing forage (from 36 to 28% NDF) gave rise to linear increases in BW gain, yield of milk and milk components (except for milk fat), and milk/DM intake and milk N/N intake, as well as linear decreases in milk urea and urinary N excretion. Increasing dietary energy resulted in similar degrees of increase in milk N secretion and decrease in excretion of environmentally labile urinary N.

Table 1. Composition of diets.[1]

Diet		A	B	C	D	E	F	G	H	I
	NDF		High			Medium			Low	
Ingredient	CP	Low	Medium	High	Low	Medium	High	Low	Medium	High
						(% of DM)				
Alfalfa silage		43.8	43.8	43.8	37.3	37.3	37.3	30.8	30.8	30.8
Corn silage		30.9	30.9	30.9	25.3	25.3	25.3	19.3	19.3	19.3
Rolled high moisture shelled corn		21.5	17.1	12.6	31.4	27.0	22.5	41.7	37.3	32.8
Solvent soybean meal		1.0	5.3	9.8	2.9	7.3	11.8	4.8	9.3	13.7
Roasted soybeans		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sodium bicarbonate		0.0	0.0	0.0	0.25	0.25	0.25	0.50	0.50	0.50
Salt		0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Mineral and vitamin premix[2]		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Chemical composition										
Crude protein		15.1	16.7	18.5	15.2	16.7	18.4	15.1	16.6	18.3
NDF		36.4	35.0	35.1	31.2	31.7	32.6	28.3	27.9	27.6
ADF		24.3	23.7	23.8	21.4	21.6	22.4	19.2	18.6	19.1
Ash		7.8	8.2	8.4	7.7	8.2	8.0	7.2	7.4	7.7
NDFN, % of total N		8.9	9.4	9.7	7.7	8.8	9.1	6.9	7.1	6.9
Fat		4.5	3.9	3.9	3.7	3.7	3.4	3.6	3.3	3.2
Nonfiber carbohydrate		37.5	37.8	35.9	43.4	41.3	39.3	46.8	46.0	44.5

[1]On a DM basis, diets designated as low, medium and high CP averaged, respectively, 15.1, 16.7 and 18.4% CP, and diets designated as high, medium and low NDF averaged, respectively, 35.5, 31.8 and 27.9% NDF.

[2]Provided 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E/kg of DM.

Table 2. Milk production and composition.

	CP, % of DM			NDF, % of DM				$P > F^1$	
Trait	15.1	16.7	18.4	35.5	31.7	27.9	SE	CP	NDF
Intake and yield, kg/d									
DM intake	21.3[b]	22.1[a]	22.6[a]	21.8	22.3	21.9	0.2	< 0.01	0.36
BW gain	0.45	0.61	0.51	0.39[b]	0.44[b]	0.74[a]	0.13	0.44	0.02
Milk	33.2	34.1	34.2	31.2[c]	34.0[b]	36.2[a]	0.4	0.27	< 0.01
3.5% FCM	33.2	34.5	34.3	32.9[b]	34.9[a]	34.3[a]	0.6	0.12	0.02
Fat	1.15[b]	1.22[a]	1.21[ab]	1.19[ab]	1.24[a]	1.15[b]	0.03	0.03	< 0.01
Protein	0.99	1.02	1.02	0.91[c]	1.01[b]	1.10[a]	0.02	0.20	< 0.01
True protein	0.93	0.95	0.94	0.85[c]	0.94[b]	1.02[a]	0.01	0.52	< 0.01
Lactose	1.67	1.68	1.70	1.55[c]	1.70[b]	1.81[a]	0.03	0.79	< 0.01
SNF	2.98	3.03	3.05	2.76[c]	3.04[b]	3.25[a]	0.05	0.55	< 0.01
Milk composition, %									
Fat	3.50	3.66	3.60	3.84[a]	3.69[b]	3.22[c]	0.08	0.06	< 0.01
Protein	2.98	3.03	3.02	2.95[c]	3.01[b]	3.08[a]	0.02	0.13	< 0.01
True protein	2.80	2.82	2.79	2.74[c]	2.80[b]	2.87[a]	0.02	0.29	< 0.01
Lactose	4.97	4.96	4.96	4.92[b]	4.98[a]	4.99[a]	0.01	0.74	< 0.01
SNF	8.93	8.98	8.95	8.85[c]	8.96[b]	9.06[a]	0.02	0.20	< 0.01
Efficiency, yield/intake									
Milk/DM intake	1.56	1.55	1.51	1.44[c]	1.53[b]	1.65[a]	0.02	0.13	< 0.01
Milk N/N intake	0.304[a]	0.270[b]	0.240[c]	0.248[c]	0.270[b]	0.296[a]	0.005	< 0.01	< 0.01
Milk N fractions, mg/L									
Urea N	9.2[c]	12.4[b]	16.0[a]	13.4[a]	12.7[ab]	11.5[b]	0.2	< 0.01	< 0.01
NPN	29.2[c]	32.5[b]	36.5[a]	33.0	33.1	32.3	0.3	< 0.01	0.10

[1]Probability of a significant effect of CP or NDF concentration in the diet.

[a,b,c]The three means within the same row for either CP or NDF with a different superscript are different ($P < 0.05$).

Table 3. Nitrogen metabolism.

	CP, % of DM			NDF, % of DM				$P > F^1$	
Trait	15.1	16.8	18.4	35.5	31.7	27.9	SE	CP	NDF
Nitrogen metabolism, g/d									
N intake	516.9[c]	591.1[b]	666.8[a]	587.1	597.1	590.5	9.8	< 0.01	0.71
Milk N	159.6	162.9	164.1	147.0[c]	161.5[b]	178.1[a]	3.3	0.45	< 0.01
Urinary N	120.3[c]	164.8[b]	230.1[a]	179.4[a]	177.8[a]	158.1[b]	8.5	< 0.01	0.01
Fecal N	237.0[b]	263.4[a]	272.5[a]	260.7	257.9	254.3	7.8	< 0.01	0.83
Nitrogen metabolism, % of N intake									
Milk N	31.1[a]	27.7[b]	24.7[c]	25.3[c]	27.7[b]	30.5[a]	0.6	< 0.01	< 0.01
Urinary N	23.2[c]	28.0[b]	34.5[a]	30.2[a]	29.1[a]	26.4[b]	1.3	< 0.01	0.01
Fecal N	45.7[a]	44.2[a]	40.8[b]	44.5	43.2	43.1	1.2	< 0.01	0.54
Urinary purine derivatives, mmol/d	378.2	402.2	399.9	353.4[b]	402.3[a]	424.6[a]	7.9	0.30	< 0.01

[1]Probability of a significant effect of CP or NDF concentration in the diet.

[a,b,c]The three means within the same row for either CP or NDF with a different superscript are different ($P < 0.05$).

Manure Nutrient Management

Effect of Calcium Intake on Phosphorus Excretion in Feces of Lactating Cows
Zhiguo Wu, Augustin Rius and Larry D. Satter

Introduction

It is generally considered that ruminants are quite tolerant of varying dietary Ca:P ratios as long as the ratio is between 1 and 7. Concern persists, however, amongst nutritionists and veterinarians regarding the impact of dietary Ca concentration on phosphorus uptake from the gut. The objective of this study was to determine if excretion of P in feces of lactating dairy cows is altered due to Ca intake.

Materials and methods

Eighteen multiparous Holstein cows were utilized in a crossover design. The two treatments were low or high dietary Ca content (0.70 and 1.10%, DM basis), obtained by varying the amount of dietary calcium carbonate (Table 1). Cows were paired based on similarity in milk yield and days in milk, then were randomly assigned in pairs to the dietary treatments. The experiment included two periods, each lasting 3 wk. At the beginning of the second period, cows were switched to the opposite treatment.

Cows were housed in a tie-stall barn and offered a TMR once daily at 1400 h ad libitum (5 to 10% refusal). Actual amounts of feed offered and refused by individual animals were recorded daily to obtain net intake. Milking was at 0500 and 1700 h. Milk yields were recorded, but only the last two weeks in each period were used for analysis.

Results and discussion

The DMI and all measurements of lactational performance were similar between treatments except for milk protein percentage, which was slightly lower (P=0.06) for the high Ca diet (Table 2). The similarity in cow performance suggests the cows had adequate Ca. The NRC (1989) recommends 0.58% dietary Ca for cows at a production level similar to that in this experiment.

Fecal concentration of Ca was 1.68 and 2.43%, 45% higher (P<0.01) when cows were fed 1.10% Ca than when fed 0.70% Ca (Table 2). Fecal P concentration was 0.89 and 0.94% for the two groups, and was unaffected (P=.19) by treatment (Table 2). This result is in agreement with several previous studies with ruminants.

Conclusion

Increasing the concentration of Ca from 0.70 to 1.10% of diets that contained 0.36% P increased fecal Ca concentration but did not change the concentration of P in feces. Because these amounts of Ca encompass the typical range of dietary Ca concentrations used in lactation diets, absorption of P from diets containing as little as 0.36% P does not seem to be affected by dietary Ca concentrations within the range typically fed. A reduction in dietary P for lactating cows does not require a concurrent reduction in dietary Ca concentration to maintain a Ca to P ratio of ~2:1.

Table 1. Ingredient and chemical composition of diets differing in Ca content.

Item	0.70% Ca	1.10% Ca
	% of diet DM	
Ingredient		
Alfalfa silage	20.00	20.00
Corn silage	35.00	35.00
High moisture corn	25.85	25.00
Soybean meal, 48% CP	8.00	8.00
Soybean, roasted	10.00	10.00
Dicalcium phosphate	0.15	0.15
Calcium carbonate	0.50	1.35
Salt	0.40	0.40
Mineral and vitamin mix[1]	0.10	0.10
Chemical analyses		
CP, %	18.0	17.9
NDF, %	29.5	29.4
ADF, %	19.9	19.9
Ca, %	0.70	1.10
P, %	0.36	0.36
P in diet refusal, %	0.36 (0.02)[2]	0.37 (0.01)[2]

[1]Contained 0.32 mg/g of Se, 0.43 mg/g of Co, 1.03 mg/g of I, 13.35 mg/g of Cu, 23.99 mg/g of Fe, 51.00 mg/g of Mn, 62.01 mg/g of Zn, 7,000,000 IU/kg of vitamin A, 2,222,000 IU/kg of vitamin D, and 17,630 IU/kg of vitamin E.

[2]Values in parentheses are SD.

Table 2. Lactation performance and fecal Ca and P concentrations when cows were fed diets differing in Ca content.

Item	0.70% Ca	1.10% Ca	SEM	*P*
DMI, kg/d	21.0	20.8	0.3	0.63
Milk, kg/d	29.4	28.8	0.5	0.49
3.5% FCM, kg/d	31.0	30.8	0.89	0.89
Milk fat				
%	3.83	3.93	0.12	0.57
kg/d	1.13	1.13	0.04	0.96
Milk CP				
%	3.38	3.32	0.02	0.06
kg/d	0.99	0.95	0.02	0.21
Lactose, %	4.59	4.53	0.03	0.28
SNF, %	8.95	8.84	0.05	0.14
SCC, 1000^3/ml	173	195	49	0.75
Fecal Ca, %	1.68	2.43	0.13	0.01
Fecal P, %	0.89	0.94	0.03	0.19

Phosphorus Feeding and Manure Nutrient Recycling on Wisconsin Dairy Farms

J.M. Powell, D.B. Jackson-Smith and L.D. Satter

Introduction

The dairy industry in Wisconsin continues to be land-based, that is, many farms produce most of their feed and, therefore, have sufficient land to recycle manure nutrients through crops. However, to remain economically viable, many farms are increasing herd size and importing more feed. The continuous importation of nutrients in excess of on-farm crop nutrient requirements results in excessive soil nutrient accumulation, runoff and the pollution of surface and ground waters. Newly approved federal nutrient management regulations for animal operations have targeted manure management, specifically the phosphorus (P) content of manure and the P recycling capacities of cropland, as key components to the protection of water quality. Very little information exists on how nutrient management in one dairy system component (feed) affects other system components (crops and soils) and the overall impact of livestock numbers and cropland areas on nutrient cycling under farmer conditions. The objectives of this study were to (1) assess the feeding practices of representative dairy farms in Wisconsin, (2) evaluate the relationships between feeding practices, milk production, manure P and a farm's ability to recycle manure P through crops, and (3) offer strategies that may allow dairy farms to attain P balance and conform to environmental regulations targeted at manure management.

Materials and Methods

A total of 98 dairy farms were randomly selected from the top 17 dairy counties in Wisconsin. On-farm interviews were conducted to gather information on dairy cow types, numbers and milk yield, and on feeding practices, the importance of different factors and sources of information in determining rations, etc. The types and amounts of feed being fed on the day of the interview were recorded for each feeding group. Samples were taken of each feed component and freshly deposited feces were sampled from the barn floor. Relationships between apparent dietary P concentration and milk production were determined. The amount of manure P excreted by lactating cows was calculated as the difference in apparent feed P intake and milk P output. Partial P balances (difference between annual crop P harvests and manure P production) were calculated per farm and on a per hectare basis.

Results and Discussion

Herd and cropping system characteristics

Approximately three-quarters of the surveyed farms milked between 30 to 99 cows with an average of 65 cows, 55 of which were in lactation (Table 1). Most of the farms (97%) raised all or some of their replacement heifers. Dairy heifers numbered about 80% of the cows. Of the total manure P produced on a dairy farm, an average of 75% was estimated to come from cows and 25% from heifers. Most of the cultivated area on Wisconsin dairy farms is planted to either alfalfa (56%) or corn (37%; Table 2). Of the total land planted to corn, approximately two-thirds is devoted to grain and one-third to silage. The annual amount of P harvested in crops ranged from 18 to 30 with an average of 23 kg ha^{-1}

Table 1. Herd composition, milk production, stocking rate and annual manure P excretion for 98 dairy herds surveyed in Wisconsin.

	Mean (std dev)		Range
Herd composition (numbers)			
Lactating cows	55	(40)	14 to 281
Dry cows	10	(8)	0 to 46
Heifers	53	(39)	0 to 200
Daily milk production (kg cow^{-1})	26	(8)	7.7 to 43.1
Stocking rate [(1)]			
Animal units ha^{-1}	0.71	(0.27)	0.19 to 1.68
Number of cows (cultivated ha)$^{-1}$	0.64	(0.25)	0.14 to 1.76
Annual manure P (kg)			
Lactating cows	1323	(1042)	163 to 5759
Dry cows	137	(108)	0 to 621
Heifers	400	(296)	0 to 1533

[(1)] Animal unit equals 1000 kg of animal weight (cows and heifers combined).

Phosphorus feeding

The P content of the diet ranged from 2.3 to 8.5 with an average of 4.0 g P kg^{-1}. Approximately 85% of the surveyed farms fed P in excess of current NRC recommendations. Of the total number of lactating cows (5,195) for which there was complete dietary P and associated milk production data, one half (51%) were fed P in excess of the 3.8 g P kg^{-1} DM deemed sufficient for high levels of milk production. Because many of the herds in this survey could not be considered high milk producers, excess feeding of P likely occurred to even a greater extent.

Most dairy producers (70%) reported self-sufficiency in forage (hay plus silage) and grain production. These farms had average stocking rates of of 0.70 cows and 0.54 heifers ha^{-1}. Across all stocking rates (Table 1), there were no apparent herd size differences between those farms that had low and high stocking rates (i.e. farms with the largest herd size did not have higher stocking rates than farms having smaller herd sizes).

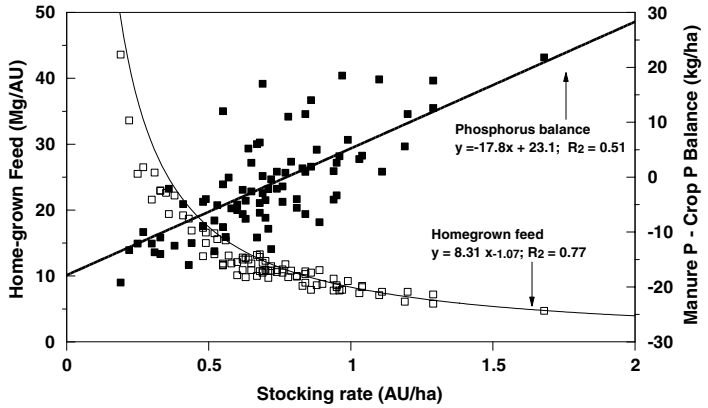
The relationship between homegrown feed (alfalfa, corn silage and grain) production and stocking rate (Figure 1) shows that most Wisconsin dairy farms have stocking rates of less than 1.1 cows ha^{-1}, the threshold value for self-sufficiency in forage (hay plus silage) and grain production. Self-sufficiency in forage and grain production generally means that a farm has adequate land to recycle its manure P through crops. Whereas a farm can attain self sufficiency in forage and grain production up to a stocking rate of approximately 1.1 cows ha^{-1}, all manure P could potentially be recycled through cropland up to a stocking rate of 1.4 cows ha^{-1}. Linking the number of animals to the area of land and cropping system available for manure utilization is critical to proper manure management.

Table 2. Cultivated areas, cropping pattern and annual crop P harvest for 98 dairy farms surveyed in Wisconsin.

	Mean (std dev)	Range
Land use (ha farm^{-1})		
Pasture	6 (5)	0 to 24
Untillable	28 (31)	1 to 172
Cultivated area	97 (68)	18 to 364
Cropping pattern (% of cultivated area)		
Corn grain	23 (13)	0 to 57
Corn silage	14 (10)	0 to 51
Hay	56 (16)	0 to 100
Small grain	3 (6)	0 to 25
Soybean	4 (7)	0 to 27
Annual crop P removal (kg farm^{-1})	2211 (1589)	381 to 8900
Annual crop P removal (kg ha^{-1})		
Corn grain	24 (2.4)	19 to 26
Corn silage	28 (2.7)	24 to 32
Hay	22 (2.2)	19 to 25
Small grain (grain plus straw)	30 (2.6)	24 to 33
Soybean	18 (2.3)	13 to 21

While animal:cropland ratios recognize that soils and their associated cropping systems have a limited capacity to recycle manure nutrients, in practice the impact of stocking rates depends on animal parameters, such as feed inputs, milk and manure outputs, and cropland characteristics that affect a field's ability to effectively recycle manure nutrients. For example, farms that feed recommended levels of dietary P produce less manure P, and therefore, can support more cows per cultivated area than farms that feed P excessively. At similar stocking rates, farms on sloping land and close to surface waters likely pose a much greater threat to water quality impairment than, for example, farms situated on parts of the landscape less susceptible to runoff. On many dairy farms, the P problem originates not so much from excessive stocking rates but rather from a combination of high dietary P levels and inadequate utilization of available cropland for manure spreading. Farms that feed adequate levels of dietary P, and utilize all of their available cropland for manure disposal can maintain higher stocking rates without increasing P losses compared to farms that feed P excessively and spread manure on only parts of their cropland.

Fig. 1. Relationships between stocking rate and sufficiency in forage and grain production and cropland P balance on Wisconsin dairy farms [1 animal unit (AU) equals 1000kg liveweight].



Many of the farms unable to grow all forage and grain are close to self-sufficiency (Figure 2). Approximately 68% of all farms would be able to produce 90% of their herds forage and grain requirements, and 80% of the farms produce 80% of their requirement. Most DM deficits are fulfilled through the purchase of corn grain, which has historically been very inexpensive. This appears to be the strategy of farmers. As stocking rates and feed deficits increase, relatively more forage than grain is produced.

Improving the phosphorus balance on dairy farms by aligning dietary P to milk production

The average diet P concentration (4.47 g/kg) fed on the 32 farms having positive field P balances is on average 25% greater than what the NRC would recommend (3.35 g/kg) for the level of milk production obtained on each farm. The adoption of NRC dietary P recommendations would reduce the number of farms having a positive P balance by 67%, and amount of land in positive P balance by 60% (Table 3). As dairy farms seek to conform to new nutrient management guidelines that restrict manure land-applications to levels that replace crop P removal, the choice of a low-phosphorus mineral and protein supplements could have a major impact on land requirement for manure application, and on-farm accumulation and loss of P.

Fig. 2. Relationship between stocking rates and home-grown feed (alfalfa, corn silage and grain) production on dairy farms not self-sufficient in home-grown feed production [1 animal unit (AU) equals 1000kg liveweight].

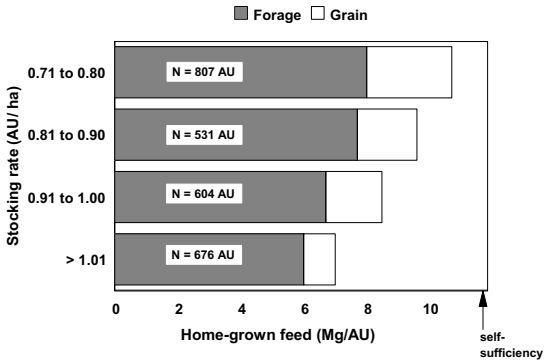


Table 3. Phosphorus balance (manure P - crop P) of dairy farms using current and NRC-recommended (NRC, 2001) feeding practices for phosphorus

Parameter	Actual feeding practice	NRC feeding practice
Number of farms with positive P balance	32	11
(% of total farms)	39	13
Crop area having positive P balance (ha)	2415	1003
(% of total crop area)	30	12
Phosphorus balance (n=82 farms)		
mean (kg farm^{-1})	271	665
range	-3945 to 6970	-1730 to 7103
mean (kg ha^{-1})	1.1	5.6
range	-39 to 19	-20 to 20

Most dairy farms in Wisconsin grow most of their feed and appear to have sufficient land for recycling manure P through crops. However, as land application of manure becomes regulated based on soil test P level and a field's risk to contribute P to surface water, farmers will need to adopt practices that reduce the amount of manure P that needs to be recycled. The manipulation of dairy diets through a more judicious selection and use of imported mineral and protein supplements will be a key practice that aligns manure P with crop P demands. For the many dairy farms that have soils already high or excessive in soil test P, feed management could very well be the most critical element of a farmer's ability to comply with nutrient management regulations Farmers and those influencing their nutrient management decisions (feed consultants, veterinarians) should seek a more holistic understanding of how nutrient management in one production component (e.g., feed) affects nutrient cycling in other production components (e.g., soils and crops) and how various components can be managed to reduce environmental risks while maintaining farm profits.

Acknowledgement

Appreciation is extended to USDA-CSREES National Research Initiative, Agricultural Systems Research Program (Grant #9703968) for partial funding of this study.

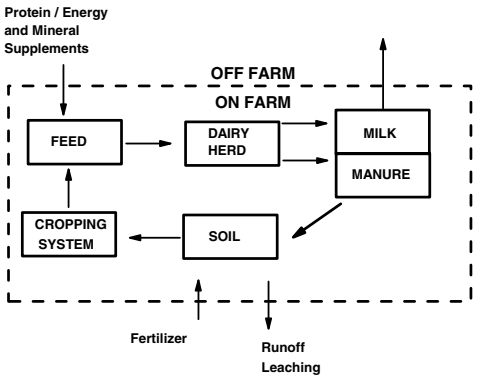
A Systems Approach to Improving Phosphorus Management on Dairy Farms

J.M. Powell, L.D. Satter; L.R. Bundy and D.B. Jackson-Smith

Introduction

The survival of many dairy farms in the U.S. will depend on farmers' ability to comply with increasingly strict environmental regulations, especially those associated with phosphorus (P) management. Many dairy farms consistently accumulate P because imports of P in the form of feed and fertilizer simply exceed exports in the form of milk, cattle, and surplus grain or hay (Fig. 1). In many areas of intensive livestock production the amount of P in manure often exceeds crop requirements. This can lead to a disposal rather than an agronomic use of manure, with a subsequent build-up of soil test P levels, much above what is needed for optimal crop yields. Newly approved nutrient management regulations for livestock operations attempt to reduce P runoff losses by controlling manure P management.

Figure 1. Phosphorus flow on dairy farms



Most efforts to improve nutrient management continue to focus on manure handling, storage, and land application. Such "rear-end" ap-

proaches neglect the effects of feeding practices on overall on-farm nutrient balances, manure nutrients and environmental impacts. For example, the newly approved USDA/EPA Unified Strategy for Animal Feeding Operations and proposed Comprehensive Nutrient Management Plan (CNMP) recognize that feed management can be an important tool for achieving a preferred balance of nutrients in manure, but the CNMP does not propose adjustments to feeding practices. Feed management is considered a planning consideration, not a technical standard. However, for farms with high levels of soil test P, feed management could very well be the most critical element of nutrient management. Separate feed, fertilizer and manure nutrient management strategies that do not consider balancing on-farm nutrient inputs and outputs can result in loss of profits through excessive nutrient use, undesirable nutrient accumulation in soil, and increased risk of negative environmental impacts from nutrient runoff, leaching and air pollution.

DFRC scientists and University of Wisconsin collaborators recently completed the first 3-year phase of a USDA-CSREES National Research Initiative-funded project entitled "A systems approach to improving phosphorus management on dairy farms". The purpose of this report is to summarize the key results obtained during the first 3 study years with an emphasis on how a more judicious use of dietary P would affect farmers' ability to comply with emerging P-based nutrient management regulations.

Materials and Methods

Strategic feeding trials were conducted to refine the estimate of what amount of dietary P is minimally needed to support high levels of milk production; manure derived from cows fed P adequate and P surplus diets were applied to cropland and runoff P was determined; a study was done to evaluate how excessive dietary P affects the land required for recycling manure P through crops, and the ability of dairy farms to recycle manure P in view of new federal guidelines that limit land application of manure based on crop P requirements; and a study of 98 dairy farms was conducted to learn about relationships between dairy feeding practices and manure P levels under farmer conditions, and between herd size, cropland area and a farm's ability to recycle manure P through crops.

Results and Discussion

Balancing P inputs and outputs through proper feed, fertilizer and manure management is the first step towards reducing soil P buildup and runoff P losses from dairy farms (Figure 1). Farms that produce manure P in excess of crop P requirements will need to amend feed and/or fertilizer practices, seek additional land for manure application, export manure, and/or reduce animal numbers on their farms if they are to achieve P balance.

Dietary P and milk production. The National Research Council recommends that the typical dairy cow diet contain between 2.7 and 4.0 g P kg^{-1}, depending on milk production (600 kg cow producing 10 to 50 kg of milk per day). A higher level of dietary P (4.8 g kg^{-1}) is recommended for the first 3 weeks of lactation. Many dairy farmers purchase and feed P in great excess of NRC recommendations. On Wisconsin dairy farms, dietary P levels range from 2.3 to 8.5 with an average of 4.0 g P $kg^{-1.}$ Approximately 85% of the surveyed dairy farms fed P in excess of NRC requirements. Over half of all cows were being fed P in excess of 3.8 g kg^{-1}, the level deemed sufficient for high levels of milk production. On these farms, the simple practice of adopting NRC's dietary P recommendations would reduce the number of farms and amount of land in positive P balance by approxi-

mately two-thirds. Perhaps the most immediate and greatest positive impact would come from reductions in the importation of unnecessary P fertilizer and diet supplements.

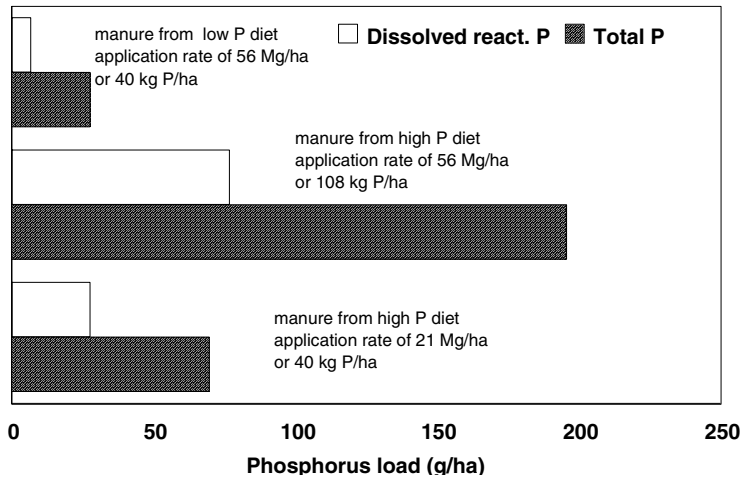
Protein supplements contain a very wide range of P concentrations (Table 1). Selecting a protein supplement with high P levels could have a profound effect on dietary, and therefore, manure P, land requirement for manure application and a farm's accumulation and loss of P. As dairy farms seek to conform to new nutrient management guidelines that restrict manure land-applications to levels that replace crop P removal, the P content of protein supplements will have to be considered.

Table 1. Protein and P concentrations in common dairy protein supplements (NRC, 2001)

Feed	Protein content g/kg	Phosphorus content g/kg	Protein:Phosphorus Ratio
Blood meal	750	3.0	317
Corn gluten meal (dried)	650	6.0	108
Soybean meal (expellers)	463	6.6	70
Soybean (roasted)	430	6.4	67
Brewer's grain (dried)	292	6.7	43
Cottonseed	230	6.0	38
Corn distiller's grain	222	8.3	27
Wheat midds	185	10.2	18
Wheat bran	173	11.8	15
Meat and bone meal	542	47.3	11

Effect of dietary P on runoff P. The type and amount of diet P supplement fed to dairy cows effect the amount and form of P in runoff from manure-amended fields. For example, when manure derived from cows fed a high (4.9 g kg^{-1}) and low (3.1 g kg^{-1}) P diet were applied at equal weights, difference in P runoff between fields amended with high diet P manure was 8 to 10 times greater than from fields amended with low diet P manure (Figure 2). When manure was applied at equivalent rates of P (40 kg P ha^{-1}), the high P manure had P runoff concentrations and loads approximately four to five times those of the low P manure. The higher soluble P in runoff from plots amended with the high P manure at the same P application rate suggests that the forms of P in the manures were different. Excessive diet P supplementation increases both total and water soluble P content of manure.

Figure 2. Soil surface runoff of P from plots amended with dairy manure derived from different dietary P levels (Ebeling et al., 2002)



Effect of dietary P on cropland needed for manure spreading. Excessive dietary P results simply in a greater excretion of manure P. If manure application to cropland becomes restricted to crop P removal, the supplementation of the dairy diet with inorganic P increases the cropland requirement for manure P recycling dramatically (Table 2). Feeding excessive P increases manure P and exacerbates the difference in nitrogen (N):P ratio between manure and crops. This means that manure from cows fed excessively high P diets, when applied to cropland in amounts to meet a crop N demand, will increase soil test P more quickly than the application of manure derived from cows fed P adequate diets. Reducing dietary P not only reduces manure P levels but also improves the N:P ratio of manure to more nearly match the N:P ratio required by plants.

Table 2. Land requirement for recycling the annual fecal P excretion by a cow fed various dietary P levels.

Dietary P level	Fecal P excretion	Cropland area to recycle fecal P	Change in land area due to diet P supplementation
g kg^{-1}	kg cow^{-1} $year^{-1}$	ha	%
3.5	19	0.63	0
3.8	21	0.70	11
4.8	30	1.00	59
5.5	35	1.17	86

Conclusion

This interdisciplinary research project showed that the elimination of inorganic P diet supplements and/or the selection of protein supplements of low P content would (1) result in less P imported and

excreted in manure, and therefore reduce the cropland area needed for manure P recycling and (2) align the N:P ratio of manure to coincide more closely with N:P ratio of crops, thereby reducing the hazard of over application of P, buildup of soil test P, and runoff from manure-amended fields. The on-farm component and subsequent group meetings with producers clearly showed that any strategy aimed at improving P use must be done in partnership with the feed and fertilizer consultants, veterinarians and manure haulers hired by farmers to make nutrient management decisions. During one of the project's workshops, Wisconsin dairy farmers said that they fully expect these hired services to incorporate any nutrient management regulation into their recommendations. While it has been shown that dairy farms can improve profitability and reduce manure P through diet P manipulation, many in the dairy industry apparently remain unconvinced that lower levels of dietary P will not adversely affect animal performance. The real and perceived risks of reduced animal performance due to diet manipulation need to be defined more clearly. Feed consultants and veterinarians need to know that their dietary P recommendations could very well be the most critical element of a farmer's ability to comply with nutrient management regulations, especially for farmers having limited cropland area upon which they can spread manure. The link between dietary practices and water quality impairment needs to be incorporated into whole-farm nutrient management planning.

Acknowledgement

Appreciation is extended to USDA-CSREES National Research Initiative, Agricultural Systems Research Program (Grant #9703968) for partial funding of this study.

Nitrogen Budget and Soil N Dynamics After Multiple Dairy Manure or Fertilizer Applications Using Unlabeled or ^{15}N-enriched Dairy Manure.

G.R. Muñoz, J.M. Powell, and K. A. Kelling

Introduction

Fertilizers, animal manure, and in some cases legumes, are the principal nitrogen (N) sources for crop production in mixed, dairy-crop production systems. Whereas fertilizer N is readily soluble in soils and becomes immediately available for crop uptake, only about half of manure N is inorganic, with the rest present in organic forms that have to be first mineralized to be used by plants or susceptible to losses. Because of its lower N availability, greater amounts of manure than fertilizer N are applied to crops. Continuous manure additions cause a steady accumulation of soil N and may negatively impact groundwater quality.

Excessive soil nutrient accumulation and pollution are pressing environmental challenges facing the dairy and other animal industries. As dairy herds expand to remain economically viable, a larger percentage of the cropland is devoted to corn silage. The effects of shifting more land to corn silage on other systems components, such as N use, buildup and loss remains to be determined. Since only a small part of applied N is ultimately taken up by corn, we wanted to track the fate of the unused portion to see whether it was lost or remained in the soil. The objective of this study was to determine total and inorganic soil N and the N balance of a continuous corn silage cropping system receiving two fertilizer or dairy manure N rates of different application frequency over three years. Unlabeled and ^{15}N-enriched dairy manure was used, and the ability of both techniques to detect

trends in soil N levels and account for applied N was compared.

Materials and Methods

A field trial was established in 1998 at the West Madison Agricultural Research Station in Madison, WI (45° 05' N, 89° 31' W) on a Plano silt loam. Initial surface (0–15 cm) soil tests were: pH 6.7 (water); organic matter, 41 g kg^{-1} (loss on ignition); Bray P1 and K levels of 50 and 146 mg kg^{-1}, respectively. Total N, NH_4^+–N and NO_3^-–N levels in the upper 30 cm of soil were 2026, 14, and 8.2 mg kg^{-1}, respectively. Trial treatments consist of two inorganic fertilizer N levels (90 or 179 kg ha^{-1}, as NH_4NO_3), two manure rates (estimated to provide approximately 90 and 180 kg available N ha^{-1} to corn the first year following application), a control receiving neither fertilizer nor manure, and three manure application intervals (every 1, 2 or 3 years). Soil samples were taken in 30cm increments to a total depth of 90 cm just prior to planting and immediately after harvest each of the three study years. Fertilizer N has been applied every year to the same plots. Estimates of soil total- and NO_3^-–N changes due to manure applications were obtained by monitoring soil ^{15}N concentrations in subplots amended with ^{15}N-labeled manure.

Results and Discussion

After three years of continuous fertilizer N or manure application, the lowest and highest NO_3^-–N levels (0 to 30 cm layer) corresponded to control and the high manure rate, respectively. There was usually no difference in soil NO_3^- levels in plots amended with the low manure rate or either fertilizer rate. Manure at the high rate significantly increased NO_3^--N over the low rate and the control. Although the fertilizer effect on topsoil NO_3^- levels was lower than that of manure, fertilizer increased NO_3^-–N concentrations in lower soil depths (30 to 90 cm) indicating that more fertilizer- than manure-N moves downward as NO_3^-–N during the growing season. This difference in behavior is probably due to the fact that NO_3^-–N applied as inorganic fertilizer is immediately solubilized in soil and therefore more susceptible to downward movement within the soil profile if there is no crop to utilize it. More than half of manure N, on the other hand, is in organic form, and virtually all of the rest is present initially as NH_4^+. Hence, manure N has to be mineralized and/or nitrified before it becomes susceptible to leaching.

There is a clear trend, which can be described by linear regression, towards increased NO_3^- contents (based on ^{14}N plus ^{15}N) with time in the topsoil for both manure rates and for the high fertilizer rate. Equations and statistics are presented in Table 1. It should be noted that the slopes for the manure treatments were generally greater than those for fertilizer, indicating that NO_3^-–N tended to accumulate to a greater degree in the manure- than the fertilizer-amended plots. This also appears to support the argument for greater short-term leaching potential from the fertilizer.

Table 1. **Regression equations used to describe soil NO_3^--N levels to 30 cm depth over time after repeated manure and fertilizer N applications in South-Central Wisconsin, 1998–2000.**

Treatment	Regression equation†	R^2	p-value
Fertilizer high‡	Y = 5.7 + 0.95 X	0.270	0.011
Fertilizer low‡	Y = 8.1	-	-
Manure high§	Y = 1.4 + 2.7 X	0.605	<0.001
Manure low§	Y = 4.4 + 1.1 X	0.407	0.001
Control	Y = 6.9	-	-

†Y is the soil NO_3^--N concentration in mg kg^{-1}, X is the sampling time (1–6 corresponding to spring, 1998 through fall, 2000).
‡Fertilizer rates were 90 and 179 kg N ha^{-1} $year^{-1}$ for the low and high level, respectively.
§Three-year average manure rate were 236 and 459 kg total N ha^{-1} $year^{-1}$ for the low and high level, respectively.

Net total soil N (^{14}N plus ^{15}N) increases in plots that received the low and high manure rates, based on regression analyses, were 2.0 and 2.9 Mg ha^{-1}, respectively, over the three year study period (Table 2). For fertilizer, these values were 1.9 and 2.0 Mg ha^{-1}. It is clear that these measurements are not sufficiently accurate, since they predict soil N increases that represent more than twice the total N applied (0.7 and 1.4 Mg ha^{-1} for manure, and 0.3 and 0.5 Mg ha^{-1} for fertilizer).

Table 2.Total N applied and recovered in harvested corn and soil to a depth of 90 cm for treatments receiving repeated manure and fertilizer applications in South-Central Wisconsin, 1998–2000.

	Initial	Applied				Crop uptake			Final	Recovery†		
Treatment	soil	1998	1999	2000	Total	1998	1999	2000	soil	Crop	Soil	Total
	kg ha^{-1}									%		
Fertilizer	13280	179	179	179	537	390	236	217	15282	46	373	419
Fertilizer	13138	90	90	90	270	301	222	210	15025	51	699	750
Manure	13499	388	501	489	1378	261	194	226	16376	6	209	215
Manure	13073	194	250	233	677	216	197	215	15024	5	288	293
Control	13528	0	0	0	0	257	172	185	13528	-	-	-

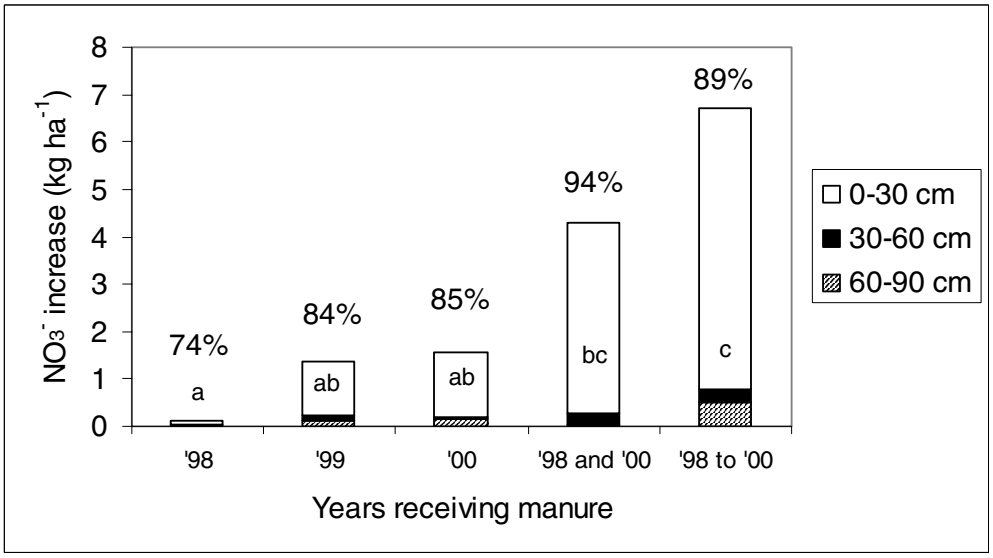
†Cumulative N recovery at the end of the third year, as a percentage of applied N.
‡Fertilizer rates were 90 and 179 kg N ha^{-1} for the low and high level, respectively.
§Three-year average manure rates were 233 and 489 kg total N ha^{-1} for the low and high level, respectively.

According to soil ^{15}N measurements, total soil N increase after three years of continuous manure applications was 289 kg ha^{-1}, or approximately 40% of the cumulative average manure N input of 743 kg ha^{-1}. Soil NO_3^-–N increases due to three years of manure application ranged from 0.1 to 6.7 kg ha^{-1} (Fig. 1). Most (74 to 94%) of this NO_3^- was found in the upper 30 cm of soil. On average, more NO_3^- was found in the 60–90 cm than in the 30–60 cm depth, but the difference was not significant and was likely due to high variability and/or the movement of N from previous year applications. The greatest amounts of NO_3^- in soils occurred in plots receiving more frequent or recent manure applications. According to ^{15}N measurements, manure increased NO_3^- levels in the subsoil only in plots that received three consecutive manure applications, and only to a small degree. These data confirm the large plot trends, that manure applied at the low rate has a low leaching potential, over this three-year period. This is not to ignore that manure could impact N leaching in the future, especially on plots continuously manured.

It might be expected that the downward movement of NO_3^-–N will eventually become a problem even for manure, especially at the high rate. Increases of 0.72 Mg NO_3^-–N ha^{-1} after 11 years of continuous manure application at a rate of 30 Mg ha^{-1} $year^{-1}$, with effects extending to the 150 cm depth. Nitrate in the 0–150 cm depth continually increased with repeated manure applications (20 years) in non-irrigated sites. After three annual manure applications, similar soil NO_3^-–N for fertilizer and manure treatments, that were usually significantly higher than controls at the 60–120 cm depth, after harvest.

Using ^{15}N measurements, from 13 to 22% of applied manure N was recovered in harvested crop during the three-year study period (Table 3). In plots receiving manure every year, 19% of applied ^{15}N was accounted for in harvested corn. The low apparent recovery of unlabeled manure N in crops using the difference method was probably due to the high N uptakes in control plots throughout the experiment. The ^{15}N method provided the best, direct estimate of manure N uptake by corn.

Fig. 1. Soil NO_3^- increase due to manure application frequencies as estimated by ^{15}N measurements in South-Central Wisconsin, 2000.



Numbers above each bar represent the percentage of recovered $^{15}NO_3^-$ present in the top 30 cm only. For NO_3^- increase in the 0-90 cm depth, bars with the same letter are not significantly different at the 0.05 level.

Table 3. ^{15}Nitrogen recovery to a soil depth of 90 cm for different manure treatments in South-Central Wisconsin, 1998–2000.

Years receiving manure	N recovery†						
	Soil depths (cm)						
	0-30	30-60	60-90	0-90	Crop	Total	Unaccounted
	%						
1998 to 2000	40	8 (2.4)	2 (0.2)	50	19	69	31 (16)
1998 and 2000	52	6 (1.6)	3 (0.7)	62	13	75	25 (24)
1998	18	4 (1.4)	1 (0.6)	24	17	41	59 (12)
1999	40	5 (0.9)	2 (0.4)	47	21	67	33 (9.7)
2000	37	7 (2.0)	4 (0.9)	48	22	70	30 (5.8)

†Cumulative recovery at the end of the third year, as a percentage of excess ^{15}N applied.
‡Standard errors are given in parentheses.

Approximately one-half to two-thirds of applied manure N was recovered in soil (0–90 cm), with one exception. Only 24% of the manure ^{15}N applied in 1998 was recovered in soil. During this study year manure remained on the soil surface for approximately 20h prior to tillage versus 4h for other study years. This likely resulted in more N loss via ammonia volatilization in 1998 than 1999 or 2000. Nitrogen recovered in the soil probably included slowly-decomposing and recalcitrant fractions of manure (undigested feed N in feces, which accounts for approximately 9% of N excreted by dairy cows), and manure N that was incorporated into new microbial biomass.

The effects of year and frequency of manure application on the amount of applied N recovered in the soil ^{15}N were not statistically significant. Depth differences in ^{15}N recovery were statistically significant ($p < 0.001$), with highest recoveries obtained from the top 0–30 cm depth (38% of applied ^{15}N). No differences in ^{15}N recovery were observed between the 30–60 cm (6%) and 60–90 cm (2%) depths. Unaccounted for ^{15}N (36% on average) was probably lost mainly through NH_3 volatilization and denitrification.

Conclusion

The use of ^{15}N-labeled manure allowed direct tracking of N in the cropping system, and provided more accurate estimates than unlabeled manure of the fate of manure N in the crop-soil continuum. Field plot soil N balances calculated using ^{15}N enrichment of total soil N were much less variable and, therefore, perhaps more reliable than soil N balances based on unlabeled N. Changes in total soil N (unlabeled) are perhaps more useful in determining long-term trends, rather than attempting to account for short-term soil N balances, such as for the three years of this study.

Comparison of Dairy Manure Nitrogen Availability to Corn Using Various Methods

G. Muñoz, K. Kelling and J.M. Powell

Introduction

Dairy manure is a valuable source of crop nutrients and also provides organic matter which improves soil physical conditions. However, when inorganic fertilizers, having a guaranteed nutrient content, readily available to the crop, became available at relatively low costs, they began to be used extensively, replacing manure, which, in turn, was considered more as a waste. Wide variation in manure composition and the difficulty of accurately predicting availability of their nutrients to crops renders manure an undependable nutrient source. Although many farmers acknowledge the beneficial effects of manure on soil quality and nutrient levels, not all of them credit these nutrients, and even less do it in an accurate fashion, although doing so would yield economic benefits through reduced fertilizer costs. The improvement of manure management is, at least, partly, dependent on more accurate, consistent, and reliable estimates of manure N availability in which farmers can have confidence. Making sound use of N resources that ensures adequate crop nutrition while avoiding environmental pollution requires an ability to predict the amount of manure N that will become available and taken up by a crop during a growing season. The objective of this research was to compare dairy manure N availability to corn using direct (^{15}N-labeled manure) and indirect (difference method and fertilizer equivalent) techniques in a field study.

Materials and Methods

A field trial was conducted from 1998 to 2000 at the West Madison Agricultural Research Station in Madison, Wisconsin (45° 05' N, 89° 31' W) on a Plano silt loam. No manure had been applied for at least four years prior to the start of the trial. Treatments consisted of five inorganic fertilizer N levels (45, 90, 135, 179 and 224 kg ha^{-1}, applied as NH_4NO_3); two manure rates (estimated to provide approximately 90 and 180 kg available N ha^{-1} to corn in the first year following application); and a control receiving neither fertilizer N nor manure. The trial was designed to test the effect of different manure application rates and intervals over a longer period of time. This summary reports first-year manure N availability to corn using only a subset of plots (those receiving manure for the first time).

Nitrogen availability and uptake calculations

Fertilizer Equivalence: The FE method compares crop yield or N uptake in the manure treatments with responses obtained from inorganic fertilizer. Each year whole-plant yields and N uptakes were regressed against fertilizer N rate. These relationships were best described by linear functions in all cases, except for whole-plant yield and N uptake in 1999 where data were best fitted to an asymptotic response model [$Y = A - B \exp(-Cx)$] where Y = crop response, A = maximum crop response attainable, B = difference between A and crop response in the unfertilized control, C = constant, x = fertilizer rate. The regression coefficients, R^2 and p-values are presented in Table 1. To solve for FE, crop response values were entered into the regression curves, and the fertilizer rate that would have produced the same response (the FE) was interpolated (Figure 1). Fertilizer equivalents for equal treatments were averaged. Percent N availability (*NA*) was calculated by dividing the FE by total applied manure N:

$$NA\ \% = \frac{FE}{Applied\ manure\ N} \times 100 \qquad [1]$$

Difference method: The difference method assumes that all crop N uptake in the amended (manure or fertilizer) plots in excess of that taken up by the control was the result of the treatment. Apparent N recovery is given by:

$$Apparent\ N\ recovery\ \% = \frac{Tmt\ N\ upt - Ctrl\ N\ upt}{Applied\ N} \times 100 \qquad [2]$$

In the above equation,
Tmt N upt and *Ctrl N upt* is the N (kg ha^{-1}) contained in the whole plant for a given treatment and control plots, respectively. Applied N is the total amount of N applied (kg ha^{-1}). Apparent recovery can also be compared to fertilizer treatments providing approximately the same amount of expected available N by creating an index of manure N availability, or "relative effectiveness":

$$Rel\ Eff\ \% = \frac{Apparent\ N\ recovery\ (manure\ tmt)}{Apparent\ N\ recovery\ (fertilizer\ tmt)} \times 100 \qquad [3]$$

The fertilizer treatments chosen were the 90 kg ha^{-1} rate for the low manure rate, and 179 kg ha^{-1} for the high manure rate, under the assumption that approximately 40% of newly-applied manure N would be available during the first growing season.

^{15}N Recovery: Apparent manurial N availability was estimated directly by measuring percentage ^{15}N recovered in above-ground corn tissue at physiological maturity:

$$^{15}N\ recov\ \% = \frac{P(c-d)}{f(a-b)} \times 100 \qquad [4]$$

In this equation, P = total crop N, f = total manure N,
a = atom % ^{15}N in the manure applied, b = atom % ^{15}N in the control manure, c = atom % ^{15}N in the treated crop, d = atom % ^{15}N in the control crop.

Results and Discussion

In general, it appeared that: 1) only in a few cases did the high manure rate result in crop responses significantly greater than the control 2) usually both manure rates resulted in similar crop responses 3) the low manure rate almost never significantly increased corn yields or N uptakes over the control 4) there were significant positive correlations between crop responses and fertilizer rates, in all cases (Table 1). The lack of significant positive crop responses to manure (in particular at the low rate) was

due to relatively high fertility at the experimental site, as indicated by the high crop responses obtained in the control plots and greater variability associated with the manure treatments. Manure applied at the lower rate appeared to provide sufficient N to the crop.

Table 1. R**egression analysis of corn responses (Y) to fertilizer N rate (X)**

Year	Response†	n‡	Equation	R^2	p-value
1998	WPNU	48	Y = 244.59 + 0.5063 X	0.366	<0.001
1999	WPNU	32	Y = 244.55 - 78.675 exp (-0.0135 X)	0.533	<0.001
2000	WPNU	24	Y = 167.71 + 0.3558 X	0.479	<0.001
1998	WPY	48	Y = 21.46 + 0.0321 X	0.392	<0.001
1999	WPY	32	Y = 21.614 - 2.843 exp (-0.0238 X)	0.246	0.017
2000	WPY	24	Y = 17.455 + 0.0155 X	0.255	0.012

† WPNU: whole-plant nitrogen uptake, WPY: whole-plant yield.
‡Number of points (single plot observations) in the regression.

Manure Nitrogen Availability

Fertilizer equivalence: Fertilizer equivalents of manure using whole-plant N uptake were solved mathematically. For example, in 2000 (Figure 1), the average whole-plant N uptake was 224 kg ha^{-1} at the low manure rate (194 kg total N ha^{-1}). Entering the regression line with this number on the Y-axis, we find that a fertilizer rate of 159 kg ha^{-1} would have resulted in the same whole-plant N uptake. This allows for the calculation of N availability according to Eq. [1]: *N availability %* = 159 kg ha^{-1}/(194 kg ha-1) x 100 = 82%. This means that manure N had approximately 82% the effect of fertilizer N in increasing whole-plant N uptake.

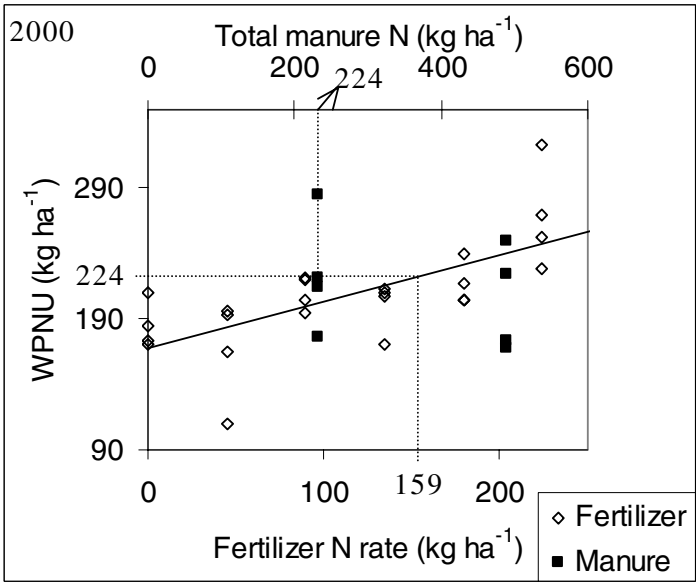
Estimates of first-year manure N availability ranged from 17 to 75%, with an overall mean (across years and crop responses) of 32%. At the high manure rate, manure N availability ranged from 2 to 76% with an overall mean of 26%. First-year dairy manure N availabilities of 27 and 26 % have been reported by Jokela (1992), based on whole-plant yield and N uptake, respectively. Our estimations are slightly higher (34 and 41%, respectively) at a similar manure N rate and are more or less comparable to the 33-60% manure N availability reported by Beauchamp (1983), the 25-100% by Xie and MacKenzie (1986) and 42% by Klausner and Guest (1981). Manure N availabilities appear to be more or less similar across a wide range of soil fertility and environmental conditions, and possibly manure characteristics.

Difference method: According to this method, the amount of N provided by manure or fertilizer was equaled to excess crop N uptake with respect to the control, and referenced to the total N applied. As discussed previously, an N availability measure (relative effectiveness) can be obtained by relating the apparent N recovery from the manure treatments to apparent recovery from a similar fertilizer rate (Eq. [3]). Both parameters for first-year manure and fertilizer treatments are presented in Table 2.

Neither apparent recovery, nor relative effectiveness of manure N were affected by year or manure rate. On average, from 15 to 18% of the total manure N applied at the low manure rate was recovered in the above-ground portion of the crop, with a weighed average of 16% across years. Relative effectiveness of manure N at the low manure rate ranged from 24 to 61% with an average of 32%.

This means that manure N was approximately 32% as effective as a similar rate of fertilizer N in increasing crop N uptake. Consistently lower estimates were obtained at the high manure rate, which ranged from 4 to 27%, with a mean of 15%, approximately half the effectiveness observed at the low manure rate. Apparent N recovery at the high manure rate ranged from 4 to 10% with a mean of 6%, substantially lower than N recovery at the low manure application rate. As discussed for FE, these results were not surprising given the general lack of crop response to the high manure rate due to high soil fertility at the experimental site during the first three study years.

Fig. 1. Corn whole-plant N uptake (WPNU) at various fertilizer and manure N rates after initial manure applications. Central Wisconsin, 2000.



Solid lines represent the relationships ($p < 0.001$) between fertilizer N rate and whole-plant N uptake. Dashed lines illustrate the fertilizer equivalence method.

^{15}N recovery: First-year recoveries of ^{15}N in whole-plant (Eq. [4]) ranged from 10 to 22%, with an average across years of 14% (Table 3). There seemed to be an increasing recovery of ^{15}N with time. An ANOVA with year and replication as main effects indicated that this trend was significant (p-value = 0.024), with recovery in 1998 (10%) being lower than in 2000 (22%).

Comparison of methods

A comparison of the apparent N recovery as calculated by the difference method and ^{15}N recovery showed that for each study-year and the across-years average, estimates were very similar (Table 3). Ranges for ^{15}N recovery are somewhat narrower than for the difference method, particularly in 1998. More importantly, several of the N recoveries, as computed by the difference method were negative (more commonly in 1998), meaning that crop N uptake in control plots exceeded than in manured plots. If during 1998 native N levels were high due to previous alfalfa, then it is reasonable that no extra N was needed. Hence, no extra N uptake was observed in many manured plots, and actually, whole-plant N uptake was not significantly different from the control.

Table 2. First-year apparent manure N recoveries (ANR) and relative effectiveness (RE) of manure according to the difference method for whole-plant N uptakes measured in Central Wisconsin, 1998–2000.

	1998		1999		2000		Mean‡	
Manure N †	ANR	RE	ANR	RE	ANR	RE	ANR	RE
kg ha^{-1}	---------- % ----------							
226	15	24	18	28	17	61	16	32
459	4	4	10	27	4	22	6	15
Mean¶	9	14	14	27	10	41	11	23

†Rate is three-year average of total N applied.
‡Across years, weighed by number of observations.
¶Across manure rates.

The difference method only compares crop responses in manured plots to those obtained in control plots. This approach has limited applicability in extreme situations, such as when the soil is either high or severely deficient in available N. The ^{15}N method does not require calibration curves or controls (the "control" is ^{15}N natural abundance) and should be, therefore, a more precise and direct estimate of crop N uptake. However, this method does not allow for N availability estimates *per se* (understanding availability as the N that can be potentially used by the crop) unless ^{15}N-enriched fertilizer treatments are included.

Table 3. Estimates of first-year manure N availability and apparent recovery using various methods, for the low manure rate in Central Wisconsin 1998–2000.

		^{15}N method		Difference Method				Fert equiv (WPNU)	
		^{15}N recovery		App recov		Rel Effec		N availability	
Year	n†	mean	range	mean	range	mean	range	mean	range
		---------- % ----------							
1998	12	10	4 to 15	15	-31 to 62	24	-51 to 100	31	-60 to 124
1999	8	17	8 to 26	18	9 to 31	28	15 to 49	43	10 to 148
2000	4	22	7 to 42	17	-4 to 43	61	-14 to 156	68	-10 to 142
mean‡		14	4 to 42	16	-31 to 62	32	-51 to 156	41	-60 to 148

†Number of observations.
‡Weighed by number of observations.

In spite of the apparent lower accuracy of the difference method, it provided virtually the same average estimate of manure N recovery estimates as the ^{15}N method. This might suggest that at least for our experimental conditions, the difference method could be the most cost-effective approach for determining manure N availability. However, considering the breadth of the N recovery ranges provided by the difference method, sometimes going from negative to more than 100%, it is somewhat surprising that it has worked out so well. Manure N recovery measurements by the ^{15}N method are invariably more consistent and reliable, although they are costly and involve much more work, from experiment setup to sample analyses.

Conclusions

This field trial was designed to evaluate the effects on N cycling of various manure management strategies, including the current, predominant practice of Wisconsin farmers: the repeated application of manure to the same field. The long-term nature of the trial (6-yr minimum) and the use of ^{15}N-labeled manure and fertilizer N should provide opportunities for comparing direct and indirect

measurements of manure nutrient dynamics under various manure management regimes, over the long-term. The ^{15}N technique appears to provide an effective tool for accurate determination of N flow in the crop/soil-environment continuum. It is expected that this information will increase our confidence in manure N credits. Ultimately, these studies may provide the basis for developing alternative, economically viable and environmentally sound manure management practices.

U.S. DAIRY FORAGE RESEARCH CENTER
ANNUAL DAIRY OPERATIONS REPORT
JANUARY 2001 (for 2000)

LEN L. STROZINSKI-HERD MANAGER

HERD STATISTICS			**CHANGE FROM PREVIOUS YEAR**
Herd Inventory			
Milking cows		320	+23
Dry cows		45	-7
average cow age	44 months		0
percent first lactation	42%		-4
percent second lactation	31%		+7
percent third lactation	14%		-1
percent greater than third	13%		-1
Herd replacements		324	-12
Total		**689**	+4
Herd Performance			
Cows calved		375	+13
Heifer calves born live		162	-2
Heifer calves born dead		20	+10
Bull calves born live		173	-12
Bull calves born dead		23	-5
Heifer calves died < 1 year old		2(1.2%)	+1
DHIA rolling herd average			
milk		22,783 lbs.	+236
protein		709 lbs.	-10
fat		829 lbs.	-6
Milk sold in 2001		7,496,467 lbs.	+80,835
Heifer calves sold		16	+2
Bull calves sold		173	+4
Cows Sold			
Cows culled for:			
Reproduction Problems		52	+27
Poor Production		16	+7
Poor Udder		10	- 9
Poor Feet and Legs		7	- 2
Mastitis		21	+8
Other		11	+4
Cattle Sales Revenue		85,234.85	+9,031.55
Herd Reproduction			
Average days open		124	+1
Average calving interval		13.02 months	+0.07
Average services per conception		3.0	+0.6
Average age at first calving		24 months	0

The herd statistics report shows little change in herd numbers and performance in 2000. Cattle numbers have stayed constant at full capacity. Much to my disappointment, milk production increased only slightly over last year's levels. The average price received for our milk in 2000 was $11.33 per hundredweight. This represents a $3.13 reduction from the 1999 price and a $5.15 reduction from 1998 prices. Since the Forage Center relies heavily on farm income to offset expenses, these are difficult times that present interesting challenges for 2001.

Dealing with labor shortage and labor turnover has been a major challenge in 2000. Although the dairy crew has a superb core of long-term employees, eight different individuals have occupied several positions in the past year. It has been a year of constant searches and training. I have personally filled in for missing members of the labor force on numerous occasions during the year. Despite long intervals of full time vacancies and no summer student help, I am proud of the fact that operations have continued and no research was jeopardized.

Several facility improvements were made in the past year. New larger water fountains were installed in the free-stall barns. New water cups and plastic manger liners were installed in the tie stall barns. A new sanitizer application unit was installed in the milking operation. In the cattle housing areas a change is being made from rubber filled mattresses in the stalls back to stalls bedded with recycled composted manure solids.

Since 1994, the Forage Center has been managing and conducting research with intensely managed grazing systems. A research technician and his summer laborers had carried out management and maintenance of the pasture fences, plots and water system. In 2000, a change in research personnel and direction placed the entire pasture program under my management with no increase in labor support. I modified the grazing system to maximize animal performance with minimal labor input.

U.S. DAIRY FORAGE RESEARCH CENTER
ANNUAL DAIRY OPERATIONS REPORT
JANUARY 2002 (for 2001)

LEN L. STROZINSKI-HERD MANAGER

HERD STATISTICS			**CHANGE FROM PREVIOUS YEAR**
Herd Inventory			
Milking cows		322	+2
Dry cows		47	+2
average cow age	44 months		0
percent first lactation	41%		-1
percent second lactation	28%		-3
percent third lactation	17%		+3
percent greater than third	12%		-1
Herd replacements		320	-4
Total		**689**	0
Rumen fistulated cows		30	
Herd Performance			
Cows calved		373	-2
Heifer calves born live		156	-6
Heifer calves born dead		20	0
Bull calves born live		170	-3
Bull calves born dead		38	+15
Heifer calves died < 1 year old		5(3.2%)	+3
DHIA rolling herd average			
milk		22,418 lbs.	-365
protein		673 lbs.	-36
fat		877 lbs.	+48
Milk sold in 2001		7,386,614 lbs.	-109,853
Average Mailbox Milk Price/cwt		$15.18	+3.85
Heifer calves sold		19	+3
Bull calves sold		170	-3
Cows Sold			
Cows culled for:			
Reproduction Problems		51	- 1
Poor Production		8	+8
Poor Udder		13	+3
Poor Feet and Legs		8	+1
Mastitis		28	+7
Other		20	+9
Cattle Sales Revenue		88,974.35	+3,739.50
Herd Reproduction			
Average days open		130	+6
Average calving interval		13.18 months	+0.16
Average services per conception		2.9	- .1
Average age at first calving		24 months	0

The herd statistics summary shows that only minor changes took place in 2001. Our goal of attaining a 23,000 pound DHIA rolling herd average continues to be elusive and remains as one of our goals in the future. Although milk production was down somewhat in 2001, the increase in the price received for our milk was a welcome and needed change. Research use of the herd increased again in 2001. Since research is our number one product, I view the year as being very successful.

2001 was a very trying year due to the economy and a set of circumstances that added to my already pressured labor situation of the previous year. It began with the resignation of one of my key Experimental Herd Assistants in December of 2000. Experimental Herd Assistants function as shift foremen in the dairy operation. After the usual announcement and recruitment delays, I was able to promote one of my laborers to that Herd Assistant position. After a short time of training, he requested a transfer to a vacant Herd Assistant position on campus. Recruitment started again and I hired a person who had good knowledge of dairy cattle but had an extremely difficult time learning and operating our computerized record system. After only one month, he resigned his position. The Herd Assistant who had transferred to campus decided to transfer back to Dairy Forage only to change his mind one more time in about a month and return to campus. While that whole episode was taking place, the wife of another key Herd Assistant in my operation was tragically killed in an automobile accident. His position was retained while he went on family leave for six months only to resign one month after returning. Recruitment action to fill that vacant position began in October. I am happy to report that after one year I have finally filled both vacant Herd Assistant positions. Both positions were filled from within my laborer pool leaving two laborer positions to fill. To date, one position remains to be filled. Both new Herd Assistants are making rapid progress in their new responsibilities. Much of my time in 2001 was spent dealing with the labor situation and personally filling in for herd assistants and laborers. Needless to say, this whole labor situation made it a very trying year and I am looking forward to the New Year working with the new leadership staff that has been put in place.

Once again, the main accomplishment that I am most proud of for this year is that I kept the ship afloat during the storm and was able to meet the needs of an increased research program.

In June of 2001 the Dairy Forage Research Center celebrated its 20th anniversary with an open house at the farm. It featured displays and discussions by our research staff as well as guest appearances by Ms. Sheri Hicken, Alice in Dairyland and Mr. Jim Harsdorf, Wisconsin Secretary of Agriculture.

The farm continues to host many visitors throughout the year however international visitors in 2001 decreased dramatically due to the Foot and Mouth disease outbreak in England.

U.S. DAIRY FORAGE RESEARCH CENTER

ANNUAL FIELD OPERATIONS REPORT
JANUARY 2002

R.P. Walgenbach, Management Agronomist & Farm Manager

The 2001 crop year began with somewhat cool temperatures but relatively dry soil conditions in April which facilitated planting of all crops (Table 2). Early and large amounts of snow in late December helped provide good insulation for alfalfa that survived the winter with few problems. Spring seeding of alfalfa went very well and resulted in excellent stands of alfalfa. Harvesting of the first crop of alfalfa (Table 3) was a very trying experience due to frequent rain. With little exception most of first crop experienced a shower or two after cutting. The rains continued in June (Table 1) but mostly ceased in July. For the last few years, moisture has not significantly limited crop yields. There is no doubt that moisture limited 2001 crop yields. The lack of soil moisture seemed to have its greatest impact on soybean growth. The soybean aphid contributed to plant stress but aphids were not as severe as they were in the 2000 crop year. White mold, which has impacted soybean production in past years, was not a significant problem this season. A few cornfields showed significant symptoms of water stress and this caused a greater variability in corn grain and silage yields than I've seen in more recent crops (Table 4). Third crop alfalfa yields also were reduced by lack of moisture.

The greatest impact on the cropping operation this season was triggered by the horrible events of September 11th. The U.S. Dairy Forage Research Center's cropland is located in the Badger Army Ammunition Plant (BAAP). After September 11, military security nation wide was put on high alert that changed the access rules to all military installations including BAAP. For a few days no access was allowed to the BAAP. When access was allowed it was only on a limited basis and only through the main entrance (Gate 1 along Highway 12). This disrupted corn silage harvest as well as other cropping activities. A high level of security is still enforced at the BAAP that is required by Army Command. The local army representatives and Olin personnel who are in charge of security have been very cooperative in helping provide access to cropland inside of the BAAP.

The K1 storage and sample drying building that was destroyed by fire was rebuilt. The facility also included plans for an automated soybean roasting operation. A 14,000-bushel soybean storage bin feeds an oil-heated roaster that operates 24 hours per day. This provides the farm a quality controlled roasted soybean for the dairy herd. In addition, the field crew built a mezzanine in this building and a new set of sample dryers were constructed and put on this mezzanine.

Harry Endres, one of the original hires at the Dairy Forage Research Center retired in May of 2000 after 20 years of excellent service to the Dairy Forage Research Center. Harry was a valued employee with many years of farming experience. Harry always had a positive attitude, was interested in research projects and was respected and well liked by fellow employees. We wish Harry the best in his retirement. Paul Weldon, our barn mechanic also retired in the winter of 2001. Paul joined the Dairy Forage Research Center in 1996 as our barn mechanic. Paul did not have an agriculture background but he was a conscientious employee who learned on the job. We also wish

Paul the best in his retirement. Scott Benson was hired in January 2001 as a farm equipment operator. Scott is originally from the Dane, Wisconsin area where his family operates a cash grain farm. Scott graduated from U.W. Platteville and had been working in Illinois for a large cash grain operator prior to joining the Dairy Forage Research Center. I am very pleased to have Scott as part of our field crew. Orie Eilertson was hired to fill the barn mechanic vacancy. Orie had been working as an independent electrician and also has had considerable experience in repairing farm equipment. I was very pleased that Orie has joined our staff. He comes with a great deal of knowledge and experience and you know he's around when you hear his happy whistle.

This past summer the Center celebrated its 20 Year anniversary with activities in Madison and the Research Farm. Jim Harsdorf, Wisconsin's Secretary of Agriculture, Trade and Consumer Protection was a keynote speaker at the research farm. Sherri Hicken the 2001 Alice in Dairyland also interacted with our guests. Many people helped to put together the successful farm celebration and I again want to thank them all.

In spite of our best efforts the transfer of crop and pasture land to USDA custody from the Department of Defense has not been finalized. The reuse committee has completed its work and a reuse plan has been proposed and accepted by the Sauk County Board. The process is continuing with the developing of a Memorandum of Understanding (MOU) by the USDA, BIA, Ho-Chunk Nation, Wisconsin Department of Natural Resources (WIDNR), Sauk County, Townships of Sumpter and Merrimac. It is hoped that this MOU would be signed by all parties. As of this date this has not occurred but work continues toward meeting the goals of the reuse plan. We are proceeding with the transfer of Badger Army Ammunition Plant land to USDA simultaneously with other discussions concerning management of this unique parcel of land. We have reached agreement with the WIDNR on modifying our original land request to accommodate a request that they will make to GSA to acquire land via National Park Services for park land adjacent to the Wisconsin River. Needless to say a lot of meetings and discussions have occurred since my past report. Progress on the transfer is slow but it is going forward. We still have work to do on the transfer and hope to have the transfer completed within the next six months.

As always I appreciate all of the work that our field and barn staff have provided this past year.

Table 1. 2001 precipitation (ppt)

Jan*	Feb	March	April	May	June	July	Aug**	Sept	Oct	Nov	Dec
ppt inches											
	2.26	0.77	2.56	4.01	4.14	1.85	8.33	5.57	1.48	2.22	1.26

*ppt not available ** 5.9 inches of rain fell on August 1

Table 2. 2001 planting and harvesting dates

		Planting		***Harvesting***	
Crop	Acres	Start	Finish	Start	Finish
Winter Wheat 01	82.0	-	-	7/16	7/27
Soybeans	284.4	5/8	5/15	10/11	10/29
Corn Grain	304.0	4/26	5/16		
Corn Silage	181.2	4/26	5/16	8/28	10/5
Alfalfa-Spring	128.0	4/18	4/28	-	-
Alfalfa-Summer	-	-	-	-	-
Winter Wheat 02	53.5	10/2	10/2	-	-

Table 3. 2001 forage cutting dates*

	Alfalfa-Established			***Alfalfa-Spring Seeded***		
Crop	Acres	Start	Finish	Acres	Start	Finish
First	301	5/27	6/11	128	7/1	7/10
Second	301	7/1	7/10	128	8/7	8/12
Third	301	8/1	8/7			

*15.5 acres of red clover were cut on 6/14 and 7/10

Table 4. 2001 crop yield data

Crop	***Acres***	***Low***	***High***	***Mean***	***Total***
	bushels per acre				
Winter Wheat	82.0	73.7	88.8	82.4	6,798
Soybeans	284.4	41.7	66.9	51.4	14,611

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