Alfalfa

Development of Value-Added Processes and Products for Profitable Alfalfa Marketing

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About this Report

Over the past three years, the Agricultural Utilization Research Institute (AURI) collaborated on a University of Minnesota-led project focused on developing a farmer-led, market-based working lands approach to increase water protection in agricultural areas through targeted expansion of alfalfa production. Funding for the project was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (LCCMR).[i] The Trust Fund is a permanent fund constitutionally established by the citizens of Minnesota to assist in the protection, conservation, preservation and enhancement of the state's air, water, land, fish, wildlife and other natural resources.

AURI Project Activities

AURI's task on this project was in support of activity 3: development of value-added processes and products for profitable alfalfa marketing. AURI's technical and value chain development team focused on three primary areas of activity:

1. Assessment and implementation of advanced processing and storage practices to reduce moisture-related spoilage and nutrient leaching of alfalfa;
2. Development and assessment of new, value-added applications for alfalfa, and
3. Development of supply chain connections and identification of market opportunities, with a focus on development of pilot projects, outreach and knowledge sharing.

AURI technical experts focused on identification, assessment and development of applications and processes with the potential to expand market opportunities for the crop and support development of high-value products from the entire alfalfa plant. As part of these activities, AURI's business development and project management teams identified, communicated and collaborated with multiple key stakeholders in Minnesota's alfalfa sector. This collaboration, coupled with guidance and support from industry experts, researchers and producers, served as the foundation for AURI's assessment of multiple potential high-value use cases.

Basis for Work: Mission, Team and Capabilities

AURI's central mission is to foster long-term economic benefit for Minnesota through value-added agricultural products. In order to pursue this mission, AURI provides a broad range of services aimed at expanding markets, developing new uses and improving processes. AURI's unique mix of facilities, professional staff and network of partners combine to provide a one-of-a-kind resource that focuses on creating more value for Minnesota's agricultural products.

As an important Minnesota crop, development of new uses and markets for alfalfa is a strong mission fit for AURI. Over the course of the project, AURI made use of several of its laboratories to pursue research in support of the project. Of particular note was work done at AURI's Coproducts Utilization Laboratory in

Waseca, where AURI technical staff pursued a wide range of work focused on processing and storage practices, along with assessment and development of new uses for the crop. AURI's technical team also pursued work aimed at development of novel, high-value uses for alfalfa at its Marshall-based Analytical Chemistry and Biobased Products Laboratories.

This combination of mission, facilities and abilities allowed AURI to pursue a wide variety of activities focused on developing new, high value uses and market opportunities for Minnesota alfalfa as part of this project. As part of our work, AURI leveraged staff knowledge and abilities tied to all its key focus areas- food, coproducts, biobased products and renewable energy. AURI also built a strong, internal project team with a wide variety of skills and areas of focus to pursue its technical, value chain development and outreach work in support of the project. AURI's project team included the following individuals:

Technical Team

- Luca Zullo, Ph.D.- Senior Director of Science and Technology
- Rod Larkins- Senior Director of Science and Technology (Retired during project)
- Michael Stutelberg, Ph.D.- Scientist, Chemistry (Principal Investigator)
- Alan Doering- Senior Scientist, Coproducts
- Riley Gordon- Principal Engineer
- Abel Tekeste- Associate Scientist, Coproducts

Supply Chain Development Team

- Jennifer Wagner-Lahr- Senior Director of Business Development and Commercialization
- Alexandra Diemer- Business Development Director of Novel Supply Chains
- Matthew Leiphon- Project Manager
- Jason Robinson- Business Development Director, Food
- Michael Sparby- Commercialization Director

AURI Connects Team (Outreach and Dissemination)

- Nan Larson- AURI Connects Manager
- Erik Evans- Director of Communications
- Lisa Martinez- Communications Coordinator
- Dan Skogen- Director of Government and Industry Relations

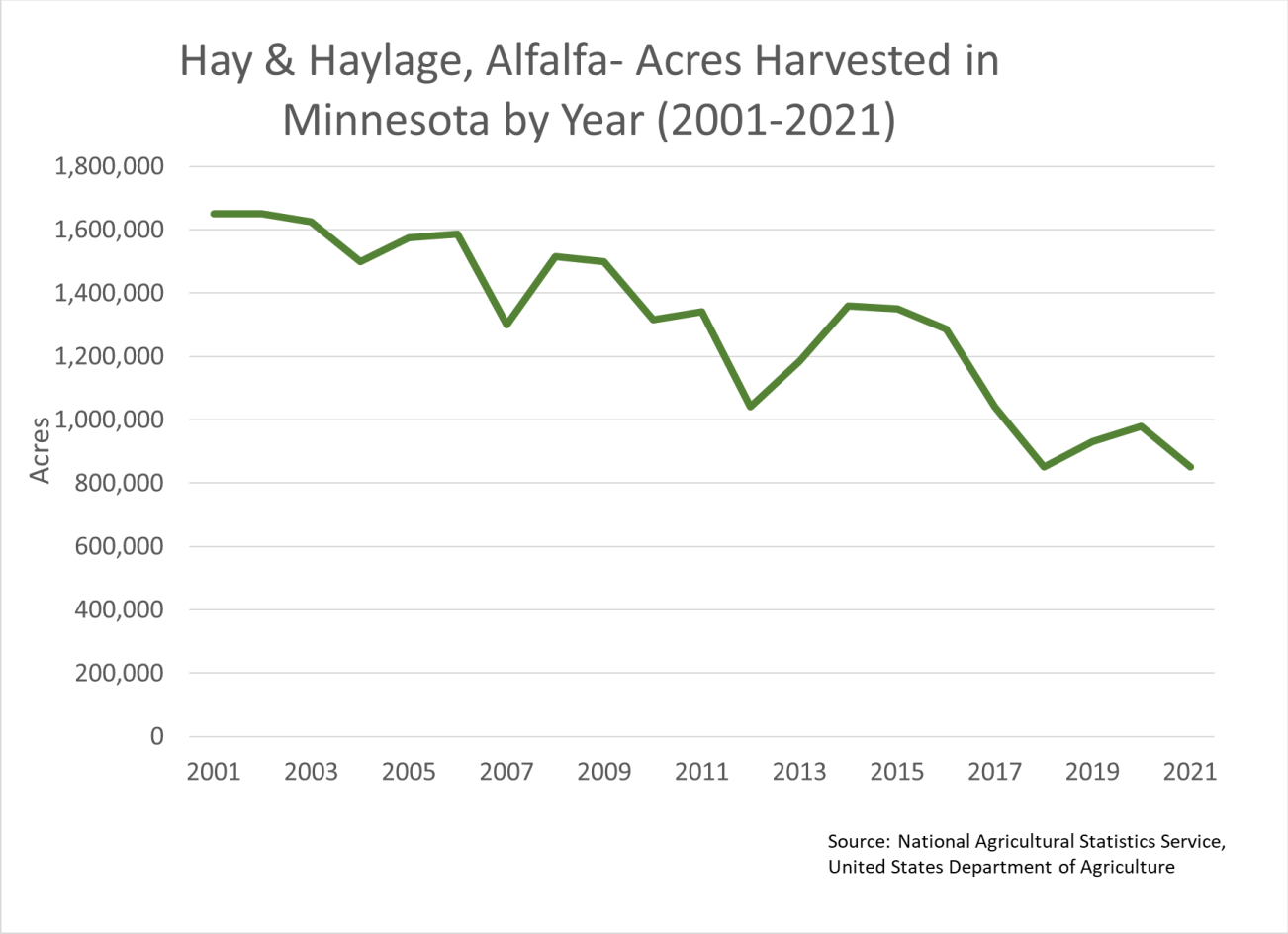
Alfalfa Industry in Minnesota - Overview

Production Trends

According to 2021 United States Department of Agriculture (USDA) data, alfalfa is Minnesota's fourth largest crop by acres, trailing only corn, soybeans and wheat. Nationally, alfalfa is one of the nation's most economically important crops, with alfalfa hay crop valued at $9.7 billion in 2021. Minnesota's alfalfa hay production in 2021 was valued at nearly $331 million.[ii]

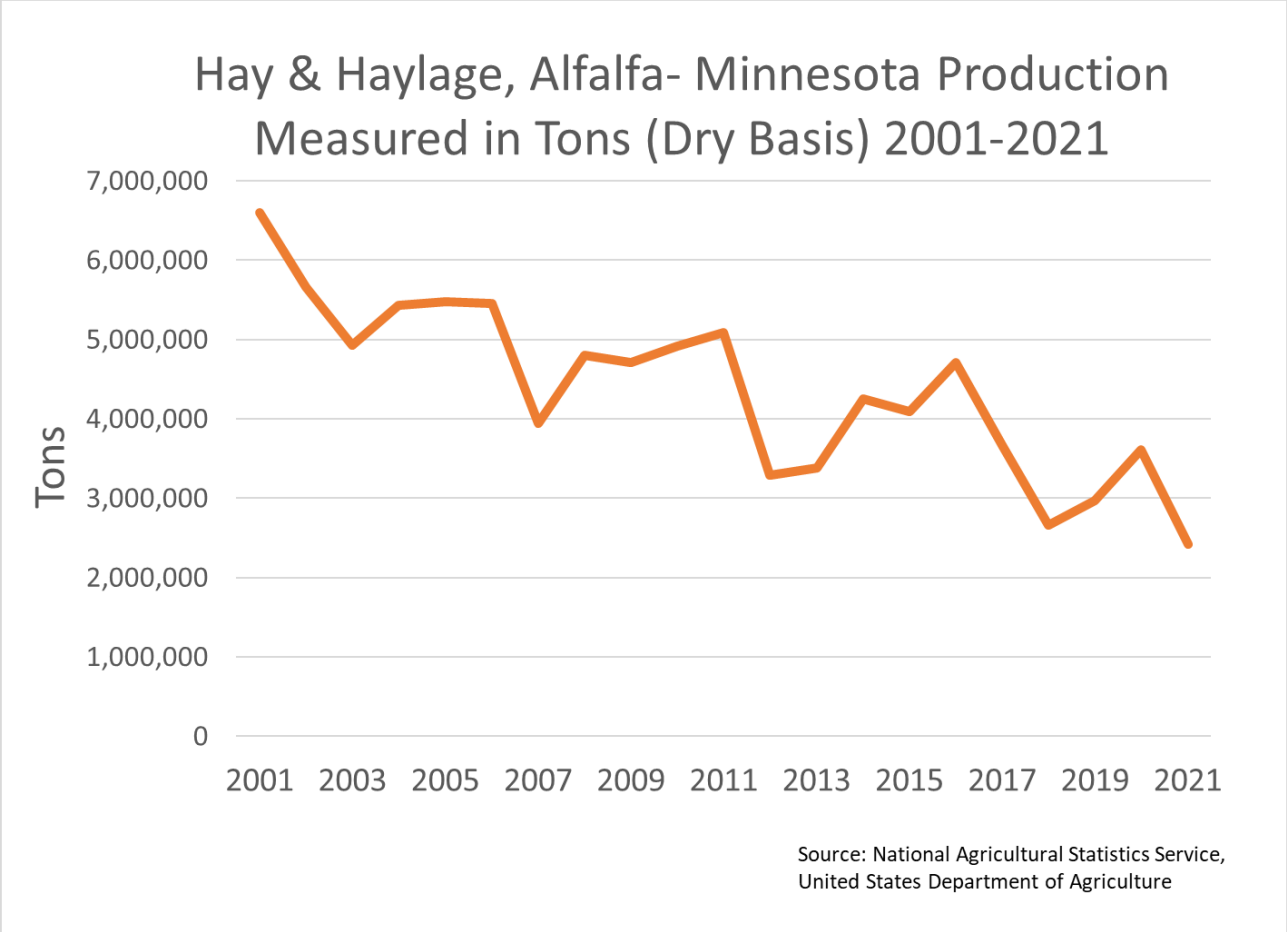
While alfalfa remains one of the largest and most important crops in the Minnesota landscape, overall production has been on a slow but steady decline over the past two decades. Between 2001 and 2021, acres of alfalfa hay and haylage harvested by Minnesota farmers dropped from 1,650,000 to 850,000- a drop of 48%. (See Figure 1)

Figure 1: Acres of Alfalfa Hay & Haylage Harvested in Minnesota (2001-2021)



As alfalfa acres have dropped in Minnesota, so have tons harvested. Over the past two decades, total production of alfalfa hay and haylage in the state dropped from 6.6 million tons in 2001 to 2.4 million tons in 2021- a decrease of 63%. (See Figure 2)

Figure 2: Tons of Alfalfa Hay & Haylage Produced in Minnesota (2001-2021)



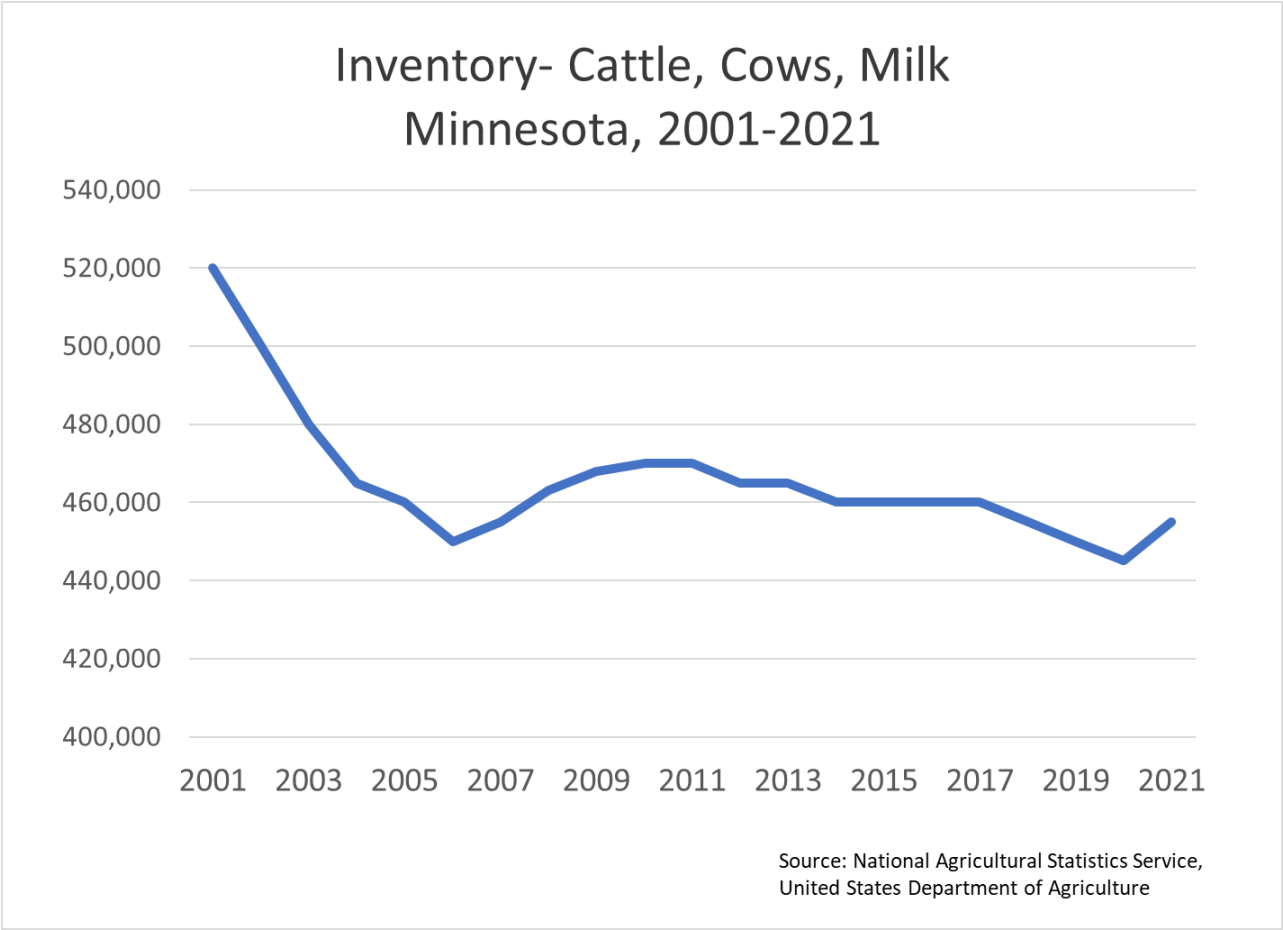
The larger decline in tonnage than acres may indicate that producers in Minnesota have chosen to shift their most productive alfalfa producing land into other crops, while maintaining alfalfa production on more marginal acres which may not be as attractive for production of alternative crops.

The shift away from alfalfa in Minnesota has been driven by multiple economic and agronomic factors. Researchers at the University of Minnesota identified several potential issues that played a role in the crop's decline in the state. They included:[iii]

- No direct government subsidies for alfalfa production- forced to compete with other, subsidized crops
- Higher cost of alfalfa has led to farmers adopting other, lower cost animal feed products, including corn silage

In addition to these challenges, overall numbers of cattle in Minnesota have dropped from a high of 2,550,000 in 2001 to 2,150,000 in 2021- a decline of 16%.[iv] Despite this wider decline in cattle numbers, Minnesota's dairy herd numbers have been nearly stable for the past 15 years, with the state reporting a total inventory of 455,000 milk cows in 2021.[v] (See Figure 3)

Figure 3: Milk Cow Inventory in Minnesota (2001-2021)



Price Trends

Over the past two decades, alfalfa hay produced in Minnesota consistently received prices notably below national average. Prices for alfalfa hay in Minnesota have on average been 21.1% lower than the national average price, with some individual years being as much as 40% below the national average. (See Table 1)

Table 1: Hay, Alfalfa- Price Received, Dollars Per Ton

Year	Minnesota	U.S.	MN Price to U.S. Price
2001	$62.50	$104.00	-39.9%
2002	$74.50	$100.00	-25.5%
2003	$71.00	$90.80	-21.8%
2004	$74.50	$98.60	-24.4%
2005	$73.00	$104.00	-29.8%
2006	$82.00	$113.00	-27.4%
2007	$117.00	$137.00	-14.6%
2008	$130.00	$165.00	-21.2%
2009	$116.00	$113.00	2.7%
2010	$110.00	$123.00	-10.6%
2011	$133.00	$196.00	-32.1%
2012	$210.00	$211.00	-0.5%
2013	$194.00	$199.00	-2.5%
2014	$136.00	$196.00	-30.6%
2015	$100.00	$158.00	-36.7%
2016	$81.00	$136.00	-40.4%
2017	$113.00	$154.00	-26.6%
2018	$147.00	$180.00	-18.3%
2019	$155.00	$179.00	-13.4%
2020	$134.00	$171.00	-21.6%
2021	$190.00	$206.00	-7.8%
Avg, 20 Years	$119.21	$149.26	-21.1%

Source: USDA National Agricultural Statistics Service, 2022

While this price data focuses on dry hay, and does not include haylage, it does offer a picture into the market challenges facing Minnesota alfalfa producers over the past two decades. Alfalfa hay prices are highly reliant on local markets, with transport costs and availability making access to wider markets a difficult and costly proposition.[vi] As noted by the University of Minnesota, local markets for alfalfa are typically tied quite closely to the dairy and cattle industries, which leads to challenges for alfalfa producers when either of these industries enters a downturn.[vii]

New Varietals, New Opportunities

Over the past decade, alfalfa breeders and researchers made multiple notable advances in shaping the crop's genetics. New varietals and genetic profiles, both conventional and GMO, were developed and released, providing alfalfa producers and end-users a wider variety of crop characteristics. While these new innovations helped reshape the industry, alfalfa tends to be seeded every several years, which means that adoption rates of new varietals occur "relatively slowly compared to other field crops, such as corn and soybeans, which are not perennials."[viii]

One of the most notable developments was the deregulation of "Roundup Ready" alfalfa varietals in 2011. This took place following a "4-year exhaustive Environmental Impact Study (EIS) by USDA-APHIS (regulatory agency responsible for genetically engineered crops), which found that this technology was safe for the environment."[ix] These herbicide tolerant (HT) varietals are aimed at producers seeking "improved flexibility in weed management, as well as overall cleaner hay and silage."[x]

In addition to developing new varietals focused on reshaping agronomic management practices, the last decade has seen the release of new varietals aimed at improving the quality profile of the harvested product. Of particular note according to experts, was the 2014 release of "HarvXtra" alfalfa varietals.[xi] This new technology makes use of "gene suppression" to "rewire the way alfalfa plants make lignin," altering the content and composition of these compounds and making the alfalfa "more digestible for cattle." These new varietals also increase yield potential by optimizing cutting intervals.[xii]

Researchers in the public and private sectors, including USDA-ARS and Minnesota-based Forage Genetics International (a subsidiary of Land O'Lakes Inc.) are continuing their efforts to optimize alfalfa genetics for agronomic and crop quality characteristics. This focus provides potential of new possibilities and markets for the crop, with the development and release of new varietals better tailored for use in monogastric animal feeds, human food products and provision of positive environmental impacts.

As advances in alfalfa genetics continue, enhancing the crop's positive environmental impacts will likely be a point of key focus. Julie Ho, an alfalfa researcher and former VP at Forage Genetics International, notes that "the most promising research for alfalfa today centers around this crop's value to farm productivity and land stewardship," and that there is "plenty of opportunity to push the envelope further through breeding, biotech and data analytics."[xiii] Alfalfa's positive environmental profile, coupled with enhanced varietals better suited for new, non-traditional uses, may open new production and market possibilities for the crop. Development of new, value-added processes and products will play a role in helping Minnesota producers take advantage of any emerging opportunities.

Barriers to Adoption and Commercialization of New Uses

Commercialization of new, value-added uses for alfalfa faces several barriers that will need to be considered in order to develop sustainable, economically feasible markets.

- Labor and Time Intensity
    - With multiple cuttings per year producing a product that requires special handling, transport and processing to preserve its quality, alfalfa is a labor and time intensive crop. This investment in time and effort has merit if the economic returns are strong but may prevent potential producers from entering crop production.
- Storage
    - Storage conditions and space available for storage of alfalfa are also a potential barrier to expanded production. In order to be marketable for high value uses, stored alfalfa will need to meet high-quality standards. Degradations to quality caused by poor storage conditions or options may prove detrimental to commercialization of new products and uses, limiting the volume of material available for production.
- Seasonality
    - With three to four annual cuttings, alfalfa presents challenges for any processor seeking to produce products year-round. While storage options exist, impacts to quality will occur, potentially creating challenges for uses that require a specific set of quality attributes to ensure a marketable product.
- Quality Issues
    - In addition to storage quality issues, the quality of product from the field is also a concern. Dry conditions are necessary during field curing to ensure a top-quality product. Minnesota's climate can prove challenging to producers, with the state's wettest months occurring during the crop's prime cutting season. While strategies and new varietals exist to address these challenges[xiv], moisture related quality issues will likely continue to be a challenge for Minnesota producers.
- Equipment- Capital Costs
    - Alfalfa production and processing requires a variety of specialized equipment. Farmers and processors may view the cost to acquire needed equipment as a disincentive to adding alfalfa to their crop rotation or product portfolio.
- Transportation Costs
    - Transporting alfalfa for processing can carry high costs, creating a barrier to selling alfalfa outside of local markets. Processors seeking to produce high-value products will likely need to secure local producers to ensure access to needed alfalfa.

AURI Research: Value-Added Processes and Products

Seeking to address existing challenges and take advantage of emerging opportunities in the alfalfa industry, AURI identified and pursued several key areas of utilization research during this project. This research included a focus on storage, processing and development of value-added opportunities for Minnesota-grown alfalfa. In addition to this work, AURI partnered with external researchers and the University of Minnesota to pursue research that complemented its work and helped further accomplishment of project deliverables.

Advanced Storage and Processing Methods

AURI's Coproduct Pilot Lab provided support focused on two main tasks: 1) developing cutting and sealing (ensiling) mechanisms to prevent humidity related spoilage or leaching due to rain; and 2) identifying and developing new applications for alfalfa. Specifically, AURI focused on processes to provide alfalfa as a nutrient source to non-ruminants while focusing on anti-nutritional content. AURI also investigated the opportunity to utilize pressed alfalfa juice as a foliar fertilizer for organic crop production.

Goal:
Identify best alfalfa processing and storage methods to efficiently enable producers to exploit value-added opportunities for expanding the adoption of alfalfa production.

Procedure:
The project team conducted alfalfa ensiling and processing trials using the same variety of alfalfa and the same alfalfa cutting period to maintain identical stages of alfalfa growth during various testing. In the case of haylage, nutrient concentrations can vary based on field and soil nutrient variability. Mineral concentrations were not a primary focus for the research conducted.

Protocol:

1. Collect fresh alfalfa sample as a control sample within 36 hours after cutting (Day 1).
2. Obtain and freeze sample of Day 1 alfalfa for nutrient analysis as the control sample. Special attention needs to be paid to **collecting moisture content** of starting material for ensiling trials.
3. Day 1 sample for liquid extraction trial and ensiling trial.
4. Press the Day 1 alfalfa sample; collect and freeze juice and solid sample for nutrient analysis and research focused on removing anti-nutritionals.
5. Primary alfalfa utilized for trials was ensiled as 'baleage.' Baleage is the practice of cutting alfalfa and baling in traditional large round or square bales within 24 hours, followed by wrapping the bales with a poly wrap to form an air-tight environment for the ensiling process to occur. Alfalfa moisture is generally 45-55 percent.
6. After 21 days of ensiling, submit a sample of ensiled Day 1 alfalfa for moisture, pH and volatile fatty acid testing specifically focusing on acetic, propionic, iso-butyric, butyric and lactic acids. This information will determine proper ensiling methods.
7. Press ensiled alfalfa haylage, (haylage which is typically around 60-70 percent moisture, is like baleage due to both practices focusing on the act of ensiling or fermentation which occurs at elevated moistures resulting in a lowered pH for forage preservation) to collect and freeze juice and solids samples for nutrient analysis and research focused on removing anti-nutritionals.
8. Submit samples of control hay, haylage, press cake and alfalfa juice for nutrient analysis and comparison.

Note: Alfalfa pressing/dewatering and liquid extraction is not successful beyond 48 hours due to the low moisture content. Extraction of alfalfa juice must utilize ensiled alfalfa within a maximum of 48 hours after cutting to obtain proper yield results.

Figure 4: Flow chart providing visual of when alfalfa samples were taken.

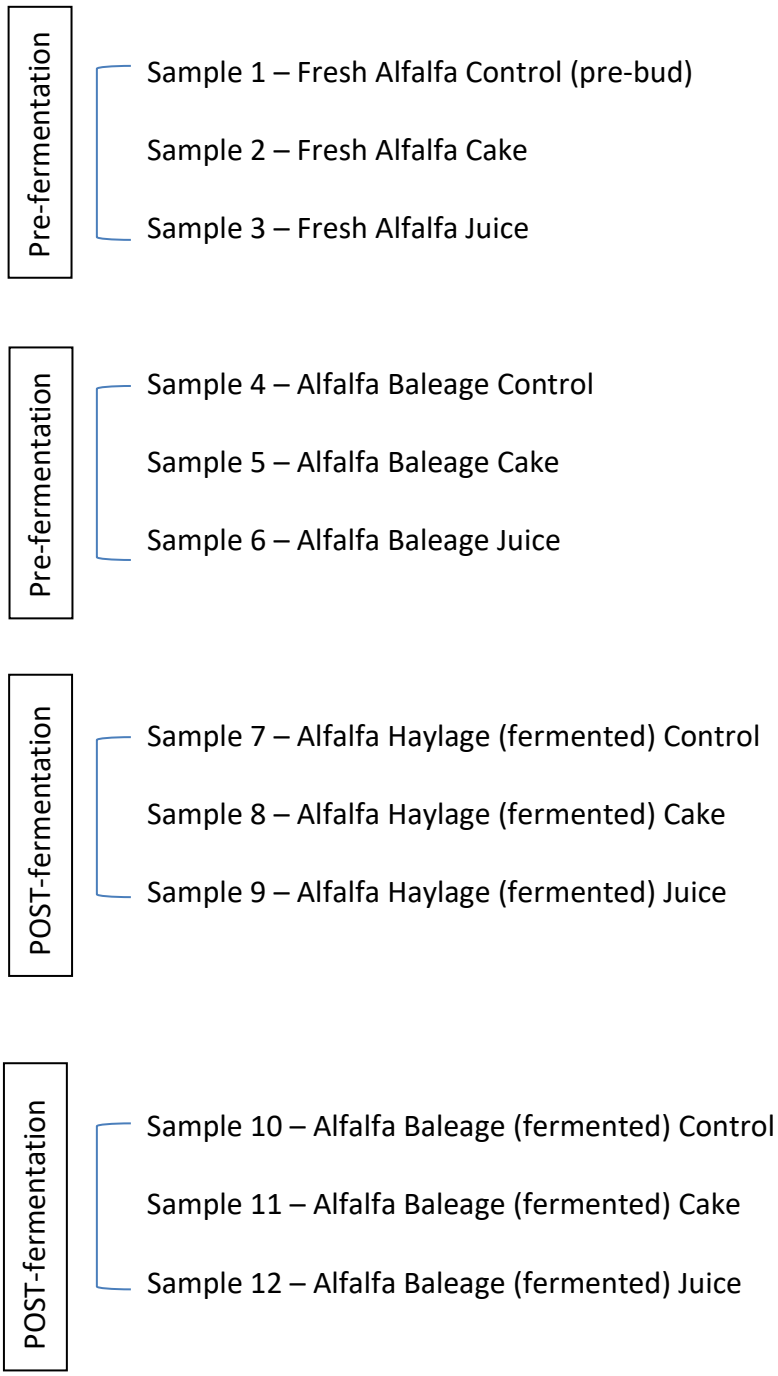


Figure 5: Alfalfa Haylage (Baleage)

Figure 6: Alfalfa Press Cake

Figure 7: Alfalfa Pressed Juice/ Solubles

Table 2: Nutritional analysis and comparison for alfalfa haylage, pressed cake, and juice.

		Alfalfa Baleage (Control)	Pressed Alfalfa Baleage Cake	Alfalfa Juice (post-fermentation)
Moisture	%	70.08	57.89	82.57
Crude Protein	% DM	24.5	20.76	36.72
Protein Solub.	% CP	65.55	60.26	
ADF	% DM	33.57	37.22	
aNDF	% DM	35.95	40.93	
Lignin (Sulf. Acid)	% DM	20.89	20.43	
Starch	% DM	0.2	1.09	
Fat (EE)	% DM	4.14	3.82	9.41
Total Fatty Acid	% DM	1.35	1.43	
Ash	% DM	15.18	13.15	
Calcium	% DM	1.5	1.3	2.24
Phosphorus	% DM	0.44	0.39	0.63
Magnesium	% DM	0.37	0.3	0.69
Potassium	% DM	3.65	3.3	9.06
Sulfur	% DM	0.31	0.24	0.57
TDN	% DM	62.75	59.91	

Due to the variability and influence of weather in the Upper Midwest, having the ability to rapidly ensile alfalfa for long-term storage, which is a widely used form of alfalfa storage by many producers, was the focus of this project to collect information related to nutritional loss due to ensiling. Secondly, by rapidly ensiling alfalfa, it provided the high moisture level required to conduct mechanical pressing to remove alfalfa solubles (in liquid form) to identify greater value opportunities for alfalfa.

Cellulosic Sugars

From pressing and ensiling the alfalfa, investigation into value-added opportunities for alfalfa juice and the available cellulosic sugars for fermentation potential and saponin was evaluated. (See Appendix A) The goal was to refine methods for extracting cellulosic sugars for conversion into high-value products.

From the pressed alfalfa, the coproducts team analyzed the alfalfa juice for available soluble sugars for fermentation applications. The team found the juice contained about 8.5% soluble sugars and utilized this in a fermentation by a third-party company, Sasya, to produce ethanol. (See Appendix B) The work performed by Sasya, indicated that the sugar content was too low, and the organic acid concentrations found inhibited fermentation production.

Researchers performed additional work on alfalfa juice to reduce saponins to improve protein quality for alfalfa by Sasya. They also performed bench top evaluations using fungal enzymes that could hydrolyze saponins. (See Appendix C) Overall, the preliminary work indicated that enzymatic hydrolysis is feasible for reducing saponin content and possibly improving protein content. However, additional work on protein isolation and purification would need to be performed after saponin hydrolysis.

Application Development

Alfalfa Liquid

Minnesota-grown alfalfa hay was ensiled for storage. After a period of fermentation, this alfalfa was pressed to extract liquid. This "juice" contains high levels of potentially useful sugars and nutrients. Juice are also pressable from fresh, non-ensiled alfalfa for use in applications requiring an unfermented ingredient for product development.

AURI also explored potential high value uses in the animal feed and fertilizer sector as a foliar feeding.

Table 3: Alfalfa Pressed Juice/Solubles

Moisture	83.35%	
Dry Matter	16.65%	
pH	5.95	
Nutrients	**Units**	**Values**
Crude Protein	%DM	28.53
Sugar (WSC)	%DM	16.16
Fat (Acid Hydro.)	%DM	24.2
Calcium	%DM	2.58
Phosphorus	%DM	0.72
Magnesium	%DM	0.54
Potassium	%DM	8.65
Sulfur	%DM	0.36
Sodium	%DM	0.06
Zinc	ppm	138
Iron	ppm	420
Manganese	ppm	42
Copper	ppm	12
Boron	ppm	42
Aluminum	ppm	282

AURI Tech Notes:

- Alfalfa haylage was ensiled and allowed to ferment for a minimum of 60 days.
- Alfalfa hay was ensiled at higher-than-normal moisture levels to increase juice volume when pressing.
- Juice was extracted using a screw press.
- Moisture and plant maturity at harvest may affect nutrient content in juice.

Spray Dried Alfalfa Juice

Minnesota-grown alfalfa hay was ensiled for storage. After a period of fermentation, this alfalfa was pressed to extract liquid as previously discussed. Due to the inconvenience, product stability and storage issues associated with a liquid, the juice (See Figure 8) was spray dried into a high-protein powder. (See Figure 9)

AURI explored the potential high-value use of spray dried alfalfa solubles in the animal nutrition sector, specifically focusing on the protein and amino acid profile as a replacement for animal-based feed ingredients. ***Note: due to the high sugar content within alfalfa juice, project team members used maltodextrin as a carrier during the spray drying process. The use of a carrier results in a reduction of nutrients.***

Figure 8: Post-Fermentation Alfalfa Juice

Figure 9: Spray Dried Alfalfa Juice/Solubles (no carrier)

Table 4: Nutrient Analysis for Spray Dried Alfalfa Juice/Solubles with Carrier

Nutrient		
Moisture	4.47%	
Dry Matter	95.53%	
Crude Protein	%DM	19.58
AD-ICP % of CP	%CP	0.77
ND-ICP w/SS	%CP	1.34
Protein Sol.	%CP	98.18
ADF	%DM	0.33
aNDF	%DM	0.75
aNDFom	%DM	0.62
Lignin	%NDFom	43.55
Sugar (WSC)	%DM	61.99
Starch	%DM	38.95
Fat (EE)	%DM	0.05
Ash	%DM	10.88
Calcium	%DM	1.15
Phosphorus	%DM	0.25
Magnesium	%DM	0.36
Potassium	%DM	3.70
Sulfur	%DM	0.46
Sodium	%DM	0.09
Zinc	ppm	30.00
Iron	ppm	247.00
Manganese	ppm	48.00
Copper	ppm	5.00
Boron	ppm	38.00
Aluminum	ppm	348.00
Calculations		
NFC	%DM	69.13
NSC	%DM	100.94
Adjusted Crude Protein	%DM	19.58
OARDC		
TDN	78.20	
Nel 3x Mcal/cwt	81.63	
Neg Mcal/cwt	59.40	
Nem Mcal/cwt	88.77	

Table 5: Amino Acid Analysis for Spray Dried Alfalfa Juice/Solubles (no carrier)

Item	Unit	Dry Basis
Crude Protein	%DM	37.95
Total Amino Acids	%DM	21.61
Total Amino Acids	%CP	56.94
Lysine	%CP	3.53
Methionine	%CP	0.76
Cysteine	%CP	0.58
Alanine	%CP	4.37
Aspartic Acid	%CP	12.94
Glutamic Acid	%CP	4.82
Glycine	%CP	2.40
Isoleucine	%CP	3.03
Leucine	%CP	4.08
Proline	%CP	5.27
Threonine	%CP	2.32
Valine	%CP	3.98
Arginine	%CP	1.00
Histidine	%CP	1.03
Phenylalanine	%CP	2.45
Tryosine	%CP	1.84
Trytophan	%CP	0.55
Serine	%CP	1.98

AURI Tech Notes:

- Alfalfa haylage was ensiled and allowed to ferment for a minimum of 40 days.
- Pressed, fermented alfalfa juice has high sugar content and may be a base for development of other high-value alfalfa applications.
- Final analytical profile of the spray dried material may vary based on varietal of alfalfa, conditions during harvest, and processing methods after cutting.

Utility of Meal for High Value Applications

After extracting liquid through pressing on high moisture haylage, the remaining product is a relatively high moisture cake that ranges between 57% and 65% moisture depending on screw press efficiency. The two options which exist to stabilize this high protein and energy feedstuffs is to re-ensile the press cake, which as previously discussed, or thermally dry the cake to prevent spoilage and capture further value-added opportunities as livestock feed in the meal or pelleted form.

Drying Trial

AURI conducted drying trials utilizing a fluid bed dryer to identify the effect drying temperatures may have on product quality. The dryer utilized by AURI's Coproduct Pilot Lab in Waseca, Minn. is a Kason Model K30/40-1FBD-SS, 30" diameter High Efficiency VIBROBED fluid bed dryer (Picture 1).

Drying trials focused on utilizing a fluid-bed dryer with a drying temperature of 140 degrees Fahrenheit (60 C) and 200 degrees Fahrenheit (93 C). Lastly, the third drying trial utilized was the use of Radio Frequency drying.

Figure 10: Haylage Press-Cake

Figure 11: Kasson Fluid Bed Dryer

Table 6 and figure 12 show the relationship between various drying time at 140°F temperature.

Table 6: Effect of Drying Time on Product Moisture Content (140 °F)

Drying Time	Moisture (%)	Temperature (°F)
H-1 Control	46.9	140
H-1 5min	30.6	140
H-1 10min	18.8	140
H-1 15mim	9.3	140
H-1 20mim	6.5	140
H-1 25mim	4.8	140

Figure 12: Moisture Vs. Time of Drying (140 °F)

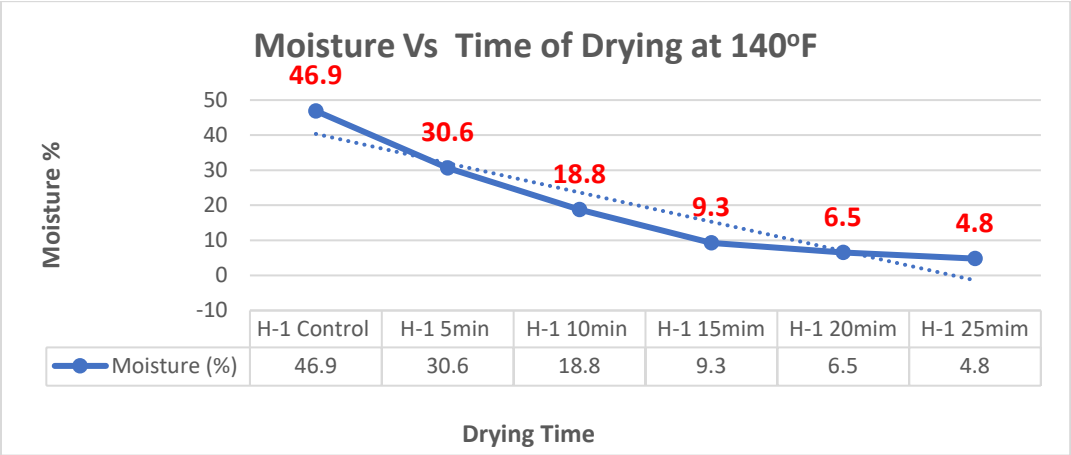


Table 7 and figure 13 show the relationship between various drying times at 200°F temperature.

Table 7: Effect of Drying Time on Product Moisture Content (200 °F)

Drying Time	Moisture (%)	Temperature (°F)
H-1 Control	50.2	200
H-1 5min	11.3	200
H-1 10min	4.2	200
H-1 15mim	2.8	200
H-1 20mim	2.6	200

Figure 13: Moisture Vs. Time of Drying (200^{O}F)

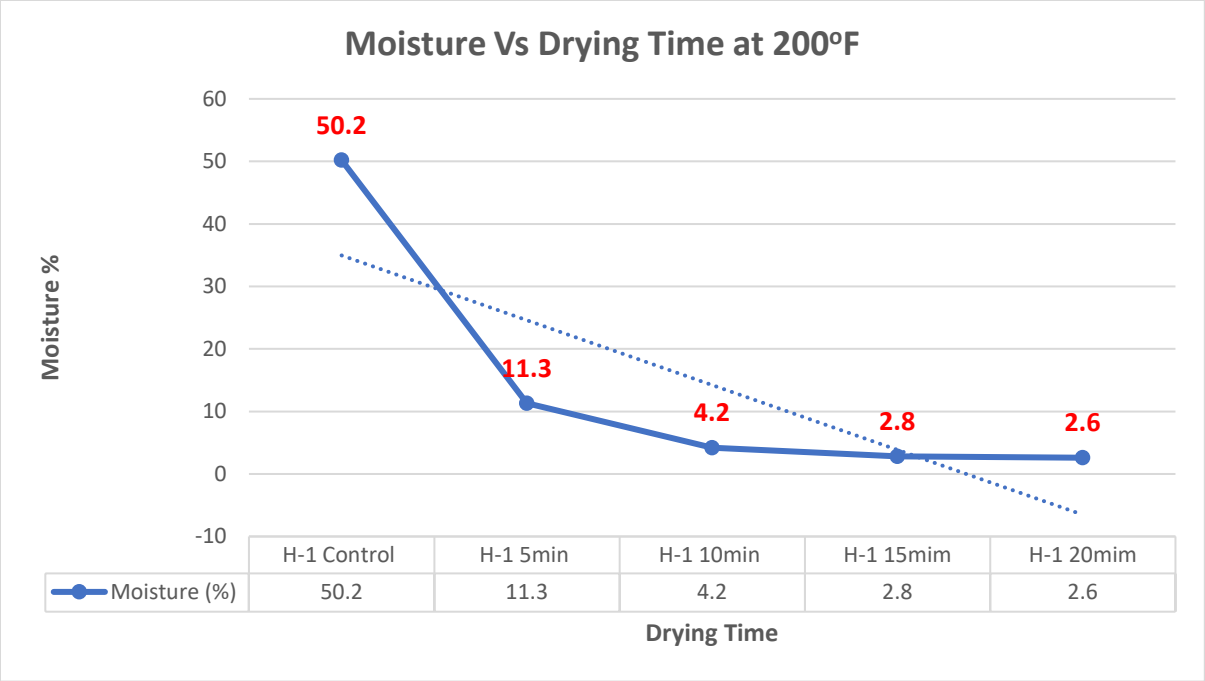


Table 8: Drying Effect on Pressed Alfalfa Cake

		Pressed Alfalfa Cake (Control)	Alfalfa Cake Dried at 140 F	Alfalfa Cake Dried at 200 F	Alfalfa Cake Dried with Radio Frequency
Moisture	%	49.0	11.5	14.4	11.8
Crude Protein	% DM	22.7	21.8	21.2	20.8
AD-Insoluble Crude Protein	% CP	6.87	7.3	6.7	6.7
Protein Solubility	% CP	52.3	54.4	54.0	55.4

AURI Tech Notes:

- Alfalfa cake was dried using fluid bed dryer at 140^{o}F and 200^{o}F.
- Direct correlation was revealed between drying time and moisture. The moisture content of the alfalfa cake declines with time of drying at 140^{o}F and 200^{o}F temperature, respectively.
- The moisture content of the alfalfa cake reduced to desired level in 15 minutes drying time at 200^{o}F temperature.
- Pressing alfalfa using de-watering press had impact on drying time.
- Drying alfalfa at 200^{o}F is more efficient than drying at 140^{o}F.
- There is no need to dry the alfalfa beyond 15 min at 200.
- Alfalfa cake drying studies conducted by AURI indicated no significant difference between the two drying temperatures evaluated. Percent insoluble crude protein and protein solubility remained similar.

Re-ensiled Meal - Assessment of Feed Value

Minnesota-grown alfalfa hay was ensiled for storage. After a period of fermentation, the alfalfa was pressed to extract liquid. This “juice” contains high levels of potentially useful sugars and nutrients.

After pressing to extract liquid, the remaining alfalfa was re-ensiled. While the pressing process extracted a portion of the alfalfa’s nutrients, the post-press material still has nutrient levels that offer potential value for animal nutrition uses.

The pH of a product is the easiest way to determine stability and if a forage has gone through the fermentation process properly; target pH levels after ensiling are 3.8 – 4.5. This target pH range indicates product stability.

Volatile fatty acids (VFAs) were tested after the completion of a 60-day test to determine if the re-ensiled pressed alfalfa with lowered moisture level would achieve optimum fermentation characteristics. Volatile fatty acids consist of lactic, butyric, iso-butyric and acidic acids to name a few. The levels of these acids in a fermented product are a good indicator of how well that product fermented due to moisture levels. Fermentation bacteria are sensitive to the amount of available water in forages. If crops are too wet, *Clostridia* bacteria will out-compete **lactic acid** (favorable acid) producing bacteria for crop sugars. *Clostridial* bacteria

produce **butyric acid**, which is much weaker than lactic acid for fermentation. *Clostridial* bacteria also convert protein into ammonia and amines. These amines are known to reduce animal feed intake.[xv] Optimum VFA parameters are listed below. (See Table 9)

Table 9: Normal Fermentation Acid Ranges

Normal Fermentation Acid Ranges	
Lactic Acid	6-8% - wet silages (>65% moisture)
	3-4% - wilted silages (<55% moisture)
	1-3% - high moisture grains
Acetic Acid	<3% - forage silages
	<1-3% - high moisture grains
Butyric Acid	< 0.1% - wet silage
	< 0.5% - legume silage
Propionic Acid	< 0.1% - wet silage
	< 0.5% - legume silage
Ammonia - CP	< 8% - wet silage (% of CP)
Lactic Acid (% of total acids)	> 70% - wet silage
	> 60% - wet legume silage

Table 10: Ensiling Variables Between Silage Bag vs. Wrapped High-moisture Bales (Baleage)

	Alfalfa Haylage #1 (Control)	Alfalfa Baleage (Control)
Moisture (%)	62.0	70.1
Crude Protein (% DM)	21.1	24.5
pH	5.75	5.69
Lactic Acid (% DM)	1.74	3.74
Acetic Acid (% DM)	0.97	4.61
Propionic Acid (% DM)	0.08	<0.01
Butyric Acid (% DM)	<0.01	<0.01

Table 11: Re-ensiled Haylage Analysis Pre- and Post- pressing.

	Haylage (Control)	Re-Ensiled Haylage
Moisture (%)	70.8	59.6
Crude Protein (% DM)	24.5	15.3
Protein Solubility (% CP)	65.6	45.3
Sugar (WSC) (% DM)	2.7	1.67
pH	5.69	4.79
Lactic Acid (% DM)	2.75	<0.01
Acetic Acid (% DM)	4.37	5.88
Propionic Acid (% DM)	0.35	0.73
Butyric Acid (% DM)	<0.01	0.39

Re-ensiling of haylage would allow producers to initially preserve alfalfa through fermentation for storage, yet allow for liquid extraction later, followed by continued storage through re-ensiling.

As indicated in the table above, re-ensiling haylage after liquid extraction did not have a dramatic effect on product stability as indicated by the volatile fatty acid profile. Researchers observed a slight increase in butyric acid production along with a reduction in lactic acid present in the sample which indicates stability of silage for preservation.

AURI Tech Notes:

- Re-ensiled haylage maintained nutrient quality during continued storage.
- Reduction in moisture and crude protein was observed due to liquid extraction.

Swine Feed Products

Spray-Dried Alfalfa Solubles in Nursery Pigs

Purpose:
Identify the metabolic and digestibility of spray dried fermented alfalfa juice in nursery swine diets.

There is currently no direct research on spray-dried alfalfa juice proteins (in some cases people have referred to 'green' protein) in swine diets. The value of identifying metabolic and digestibility of a new livestock or poultry food ingredient is to determine the actual capability of an animal to utilize the nutrients prior to conducting a performance feed trial rather than focusing on analytical values. Analytical values and actual metabolic and digestible nutrient values that the animal can utilize can be greatly different.

According to Dr. Samuel Baidoo, University of Minnesota Professor, Department of Animal Science – Swine Nutrition and Management,

> *"Protein sources derived from animal tissues or products, despite high costs, have been used extensively in feeding nursery pigs because of their increased digestibility (Gottlob et al., 2006; Emer et al., 1994), and small concentrations of antinutritional compounds (Anderson and Wolf, 1995) compared with protein sources derived from plants.*
>
> *Recent concern about animal and meat safety has heightened potential uncertainty regarding feeding animal-tissue-derived ingredients to animals. Plant-derived ingredients still have limited application in feeding newly weaned pigs because of the complexity of nutrients and anti-nutritional compounds commonly present in typical plant-derived ingredients, both of which reduce nutrient digestibility to nursery pigs."*[xvi]

Based on guidance from Dr. Baidoo, an estimated performance study trial was developed to provide the most information on product performance focusing on cost.

Swine Studies

Typically, there are three studies to conduct when evaluating the performance of a feed ingredient in swine, they include:

1) Balance study, a trial containing 24 pigs for 14 days. This study provides information on the metabolism of an ingredient within a pig's system for conducting with nursery pigs. This study includes six treatments that contain a control, 5% ingredient inclusion, 10% ingredient inclusion and 15% ingredient inclusion into the base diet. Urine, feces, blood samples and illial samples are collected from this trial to provide metabolic utilization data. Average daily feed intake (ADFI) estimates for pigs this weight and age is 500 grams per day and requireds28 pounds of spray-dried alfalfa solubles protein.

2) Illial digestibility study; consists of a trial utilizing 24 pigs for 14 days also. Digestibility of a feed ingredient is the focus of this study. This is an extensive study that tracks the feed through the digestive tract, and accounts for undigested components to identify digestibility potential of a specific species to utilize a feed ingredient. The study focused on a control treatment and 10% ingredient treatment. Illial digestibility studies require

utilizing cannulated pigs, requiring additional personnel time. Average daily feed intake at this stage is typically 800 grams per day.

3) Performance study; is the feeding trials many of us are familiar with, which focuses on evaluating the overall performance and growth rate performance over a control diet. This study is much longer for nursery pigs and generally takes 28 days.

Discussions with Dr. Baidoo led AURI to focus on the first two trials at this time, which are a required precursor for new feed ingredients prior to conducting a performance study. If a performance study is initiated prior to understanding the digestibility and metabolism capability and the animal exhibits to utilize the product, the trial will be flawed.

The University of Minnesota – Southern Research and Outreach Center conducted a Digestibility and Balance Study of spray-dried alfalfa solubles in nursery pigs to identify performance potential of this plant-derived protein and energy source as a value-added ingredient.

Initial Results

The study evaluated the efficacy of spray dried alfalfa in ileal digestibility in young pigs using inclusion rates of 0, 3, 6 and 9 percent of the diet. Results indicate the optimum inclusion rate of spray dried alfalfa is 6%. The overall ileal digestibility results of the study show inclusion of 9% spray dried alfalfa in young pig diets is the maximum level for performance advantages. Spray-dried alfalfa could be equally efficacious to spray-dried plasma, since the experimental diets formulated with no-sprayed plasma did not affect the pig's performance.

For complete information related to the feed trial, please refer to Appendix F.

AURI Tech Notes:

- Spray dried alfalfa could be equally effective in nursery swine diets as spray dried plasma.
- A nursery growth performance feed trial to compare spray dried alfalfa and spray dried plasma is required to justify the replacement of spray dried plasma with spray dried alfalfa.
- Economic feasibility and cost of producing spray-dried alfalfa solubles is required to identify market potential.
- Utilization of spray-dried alfalfa solubles does not eliminate further utilization of the remaining alfalfa fiber as haylage or dried hay as a forage for livestock.

Alfalfa Protein for Human Consumption

Traditionally, alfalfa has, and continues to be, used primarily for animal nutrition purposes. Human consumption of alfalfa is limited, typically in the form of a garnish (sprouts) added to salads or sandwiches, or a dietary supplement, as alfalfa is a rich source of calcium, potassium, phosphorus, iron and vitamins A, C, E and K. Additionally, people anecdotally use alfalfa to treat high cholesterol (due to the presence of saponins), diabetes, indigestion and other conditions, but these uses are lacking in scientific evidence.[xvii]

While scientific evidence to support the beneficial effects of alfalfa on human health is lacking, the National Institute of Health (NIH) confidently suggests that long-term use of alfalfa or alfalfa supplements might cause the immune system to become more active, exacerbating the symptoms of auto-immune diseases. Even if a person does not have an auto-immune disease, long-term use may cause reactions such as lupus. The NIH also suggests that alfalfa might cause some of the same effects as estrogen in the body, warning individuals that may have estrogen sensitivities to avoid consumption. Finally, the NIH indicates several drug and medicine interactions that are detrimental to human health.[xviii]

Despite the challenges to human nutrition, global population growth from 7.8 billion people in 2020 to almost 10 billion people by 2050[xix] will come with a corresponding increase in global protein demand, which can be met in part by developing new sources of plant protein, such as alfalfa, for human nutrition. As such, AURI partnered with the Plant Protein Innovation Center (PPIC) at the University of Minnesota to optimize extraction efficiency of, characterize and functionalize a protein isolate from the most commercially available source of alfalfa – sun dried in the field post-harvest.

The full PPIC report is available in the appendix of this report. Unfortunately, this study found “there is no efficient way to extract hydrolyzed alfalfa proteins that would have both high protein yields and isolate purity (> 60% protein)” – thus the planned characterization and functionalization work of alfalfa protein is incomplete. Literature research, however, indicates that the hydrolyzed protein found in these commercially available samples serves very little structure or functional purpose for food applications, although the nutritional profile would remain intact.

While this research did not yield a satisfactory result in terms of producing a functional alfalfa protein isolate, it did highlight an important future consideration of alfalfa protein as a benefit for human nutrition. Hydrolysis of the protein, which proved to be the primary barrier to producing a protein isolate, is linked to traditional methods of harvesting alfalfa. When commercial alfalfa is cut and left to dry in the sun, enzymes (endogenous proteases) break down the protein within 2 – 48 hours. It is thus reasonable to conclude that protein structure and function is closely related to harvesting method, and development of a functional alfalfa protein isolate for food applications is out of reach until scientists can identify a method to deactivate the enzymes (such as immediate drying to less than 13% moisture post-harvest).

Collaborative Research Activities

As part of this project, AURI collaborated and contracted with several Minnesota-based research partners in order to supplement and augment supply chain and product development activities.

- Alfalfa: Antinutrient Reduction, Protein Concentration and Sugar Extraction
    - Research Partner: Sasya LLC
    - AURI contacted with Sasya LLC, a St. Paul, Minn. based biotechnology company, to perform "proof-of-concept" research for methods of saponin reduction, protein concentration and sugar extraction for alfalfa.
    - A full report summarizing the work and findings of this research is available in Appendix A.

- Fermentation Using Alfalfa Cellulosic Sugars for High-Value Chemicals
    - Research Partner: Sasya LLC
    - AURI also worked with Sasya LLC to assess the potential uses of alfalfa-derived cellulosic sugars in high-value products.
    - A full report summarizing the work and findings of this research is available in Appendix B.

- Reduction of Saponins in Alfalfa Juice
    - Research Partner: Sasya LLC
    - AURI also contracted with Sasya LLC to perform analytical research focused on identification and quantification of saponins in alfalfa, with a focus on development of processes for reduction of these antinutrients.
    - Enzymatic hydrolysis was identified as a "far superior process for reducing saponin content and improving protein quality in alfalfa" when compared to other methods and technologies in the public domain.
    - A full report summarizing the work and findings of this research is available in Appendix C.

- Protein Isolation and Characterization
    - Research Partner: Plant Protein Innovation Center (University of Minnesota)
    - In order to assess potential high value uses for alfalfa protein in human food uses, AURI partnered with the University of Minnesota's Plant Protein Innovation Center (PPIC) to perform protein isolation and characterization of alfalfa. The objective of the project was to "determine protein extraction conditions to produce alfalfa protein isolates (APIs) of optimum yield and purity."
    - Researchers found that current methods were less than efficient but noted that harvest practices may have an impact on alfalfa "protein structure and functionality" during the extraction process. Different post-harvest handling methods for alfalfa samples may offer better results, and future research is necessary to continue development of efficient, effective protein isolation processes.
    - A full report summarizing the work and findings of this research is available in Appendix D.

- Literature Review- Alfalfa for Human Consumption
    - Research Partner: Plant Protein Innovation Center (University of Minnesota)
    - In parallel with their contracted work on protein isolation, PPIC also prepared a literature review of research focused on "opportunities to utilize the whole alfalfa plant, including grain, leaves, juice and extract in human diets." The review notes that alfalfa is a "rich source of nutrients, which could contribute to human nutrition," but that most alfalfa research is "focused on animal nutrition and its use as a forage." As a result, there is a need for further research "to characterize the functionality of alfalfa as a plant protein for nutritious food applications."[xx]
    - A copy of the literature review is available in Appendix E.

- Swine Feed Trials
    - Research Partner: Dr. Samuel Baidoo, University of Minnesota - Southern Research and Outreach Center
    - The study found that spray-dried alfalfa protein may have utility as a replacement for plasma in feed products for young pigs, reporting that the results showed "Spray-dried alfalfa could be equally efficacious to spray-dried plasma" in these products.
    - A full report summarizing the work and findings of this research is available in Appendix F.

- Supply Chain Assessment and Stakeholder Engagement
    - Research Partner: Steve Olson Consulting
    - AURI contracted with Steve Olson Consulting to support its supply chain assessment and stakeholder engagement activities, with a particular focus on the animal nutrition industry. As the former executive director of multiple poultry industry organizations, including the Minnesota Turkey Growers Association and Chicken & Egg Association of Minnesota, Mr. Olson was able to make use of his extensive connections in the animal nutrition sector as part of the assessment and development of new, high-value markets for alfalfa.
    - Olson conducted their work in two phases. Reports outlining the findings of his assessment and outreach activities are available in Appendix G (phase one) and Appendix H (phase two).

- Swine Nutrition- Feed Value of Spray Dried Alfalfa
    - Research Partner: John Goihl
    - AURI connected with John Goihl, a swine nutrition expert and consultant based in Shakopee, Minn. to review findings of the AURI/SROC Swine Feed Trials and offer additional guidance on the potential development of high-value products from alfalfa protein concentrates.
    - His review noted that more research is required, including a "performance trial comparing plasma to spray dried alfalfa juice."
    - Goihl also noted that "plasma contains immunoglobulins that the alfalfa juice does not, and is much higher in amino acids," which could create challenges related to diet formulation when using alfalfa-based products.

Outreach, Dissemination and Stakeholder Engagement

While the impacts of the COVID-19 pandemic limited outreach and engagement opportunities over the course of the project, AURI team members were able to host and take part in multiple in-person and virtual outreach events during the project period. During these events, AURI team members worked to build connections with supply chain stakeholders to raise awareness about the project, alfalfa market development opportunities and share information about potential high value uses under investigation by AURI and its collaborators.

During the project, AURI outreach and dissemination efforts in support of the project and alfalfa market development included the following activities and events:

- Roundtable Meeting- Animal Nutrition Industry Experts
    - February 3, 2020
    - Working in collaboration with Steve Olson Consulting, AURI convened a roundtable meeting of Minnesota-based animal nutrition experts in St. Paul to discuss new, value-added opportunities for alfalfa-based feed products for monogastric animals, gather and share information and identify opportunities for future research and collaboration.
- Research Meeting- USDA-ARS Plant Science Research Unit
    - February 5, 2021
    - AURI and researchers from the University of Minnesota met with alfalfa experts from the USDA Agricultural Research Service's Plant Science Research Unit in St. Paul to share information about current and future research aimed at developing new, high-value uses for alfalfa and discuss potential options for collaboration to support and build on LCCMR-funded research being carried out by AURI and UMN.
    - Led to ongoing meetings and discussions between AURI and USDA-ARS team members, with a particular focus on developing new opportunities for research to develop human food uses for alfalfa-based ingredients.
- 2021 Midwest Forage Association Symposium
    - February 2021 (Virtual)
    - AURI staff took part in the event, hosting a virtual "booth" and sharing information about the project and ongoing research.
- Midwest Forage Association "Tour de Forage"
    - Central Minnesota Forage Council, Melrose - January 26, 2022
    - Southeast Minnesota Forage Council, Rochester - January 27, 2022
    - AURI supply chain development team members attended two shows on the Midwest Forage Association's "Tour de Forage" event series for 2022. At each event, AURI hosted a booth to share information

Figure 13: 2022 Tour de Forage- Melrose, Minn.

about the project, high-value market opportunities for alfalfa and connect with growers and other key forage industry stakeholders.

- AURI Fields of Innovation Webinar
    - Growing Potential: New Markets & Opportunities for Alfalfa Production
    - June 30, 2022
    - As a capstone to AURI's outreach and dissemination activities for the project, AURI hosted a webinar focused on sharing information about the project and ongoing efforts to develop new high value uses and generate new production opportunities for alfalfa in Minnesota. Approximately 60 participants took part in the event, which included panelists from the University of Minnesota (Dr. Nick Jordan), USDA-ARS and the National Alfalfa and Forage Association, along with presentations by AURI researchers and supply chain specialists.
    - A recording of the event is available online for use in ongoing information sharing activities by AURI and project partners.[xxi]
- Informational Sheets (See Appendix I)
    - AURI technical experts developed several two-page guides with information on and assortment of the alfalfa applications and processing options being examined by AURI researchers. These informational sheets were shared with alfalfa stakeholders during meetings and events to build awareness of the project and alfalfa's potential high value uses.

In addition to these activities, AURI supply chain specialists and technical staff hosted multiple meetings over the course of project with key stakeholders in alfalfa research, production and marketing. The focus of these outreach efforts was to share information, assess market opportunities, gather input and identify opportunities for new and/or expanded collaboration. These outreach efforts helped AURI strengthen and expand its connections to Minnesota's alfalfa industry and have already led to identification and initiation of new research and product development activities that will continue following this LCCMR-funded project. AURI intends to continue these efforts and build on the knowledge and networks developed during this project, with the goal of providing an ongoing return on the LCCMR's investment in the future of alfalfa in Minnesota.

Next Steps

Over the course of this project, AURI's technical and business development team members strengthened and expanded the organization's connection to alfalfa industry stakeholders. This expanded network, coupled with the knowledge developed during AURI's process and product development research, should serve as a solid foundation for continued efforts to support the state's alfalfa industry.

As of July 2022, AURI researchers identified and initiated a new project based on the connections built during this project. This new project, supported by funding from the USDA's Rural Cooperative Development Grant Program (RCDG), is being pursued in collaboration with a Minnesota alfalfa producer, and is focused on development of new, alfalfa-based plant nutrition products.

Continued research in support of human food uses for alfalfa is also an area of potential focus. While AURI will not lead this work, the knowledge and connections developed during this project put the organization in a position to support new and ongoing research at the University of Minnesota and the St. Paul USDA-ARS. While research to develop alfalfa protein for human consumption is still some distance from being ready for commercialization, there will likely be opportunities for AURI to provide support and guidance as the work moves forward.

Support for Minnesota's existing alfalfa processors will also be an important step in preparing for future expansion in the industry. Processors such as Minnesota Valley Alfalfa Producers (MnVAP) in Kandiyohi County hold the potential to serve as key infrastructure for the commercialization of new, high-value alfalfa products. AURI worked with MnVAP on multiple occasions over the past several years and connected with them over the course of this project to share information and gather input on new opportunities. Building on these ties and forging new connections to other existing and new stakeholders seeking to build markets for Minnesota alfalfa, will be key to building on the knowledge gained during this project.

AURI also plans to continue sharing information about the project, its findings and new opportunities for value-added uses of alfalfa. AURI technical experts are slated to participate in multiple events and conferences during the second half of 2022, and plan to disseminate information about alfalfa and the technologies examined in this project when and where appropriate. The focus of this continued outreach is to identify new opportunities for collaboration and research that can build on the work done in this project. Innovation is ongoing, and the knowledge and connections built during this project will continue to play a role in that process beyond the project's end date. Moving forward, AURI hopes to identify and pursue new work that can expand the impact of this project and its findings, providing a continued return on LCCMR's investment and enhancing economic opportunities for Minnesota's agricultural producers.

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Appendices

Appendix A: Alfalfa Antinutrient Reduction, Protein Concentration and Sugar Extraction

Appendix B: Fermentation Using Alfalfa Cellulosic Sugars for High Value Chemicals

Appendix C: Reduction of Saponins in Alfalfa Juice

Appendix D: PPIC- Protein Isolation and Characterization of Alfalfa Flour

Appendix E: PPIC Literature Review- Alfalfa for Human Consumption

Appendix F: SROC Swine Feed Trials

Appendix G: Supply Chain Phase 1- Steve Olson Consulting

Appendix H: Supply Chain Phase 2- Steve Olson Consulting

Appendix I: AURI Application Information Sheets

APPENDIX A

Alfalfa Antinutrient Reduction, Protein Concentration and Sugar Extraction

Service Provider Agreement Research Deliverable

Contents

List of Figures and Tables

This document summarizes the results of the research performed by Sasya under the Service Provider Agreement for the Agricultural Utilization Research Institute (AURI). The experimental methods used are generic methods implemented in the industry and no effort was made to optimize the methods or processes to improve the efficiency for better results. Therefore, AURI must interpret the results as a "proof-of-concept" only. Although the original schedule was disrupted due to unforeseeable causes, the final deliverable was on schedule. Sasya made reasonable effort to ensure the validity and accuracy of the methods, results and procedures.

The results presented in this document are organized according to the deliverables outlined in the Service Provider Agreement (signed on Jan-10, 2020 and amended on Jun-22, 2020). The first section is on the work performed with alfalfa (1. Reducing Antinutrients from Alfalfa, 2. Protein concentrate from alfalfa and 3. Methods to extract sugars from alfalfa). This work covers the project described under FS017IN. The second section is the work on Kernza (Sugar extraction). The results are for FS016IN and FS035IN. The second section describes the methods in much greater detail and the methods developed/implemented are consistent across all the projects.

The results and data provided reflect the good faith effort employed by Sasya. The raw data is provided in the Appendix and all calculations are described in the main text. The figures are generated using the data from the Appendix using the calculations shown in the text. Some calculations are based on the analytical data provided by AURI and Sasya has used the data as received to perform the calculations. The interpretations and inferences are subject to debate.

Alfalfa (FS017IN)

1. Reducing antinutrients in alfalfa leaf extract

The main antinutrient in alfalfa is saponin. Saponins are phytochemicals, found mainly but not exclusively in plants, which exhibit foaming characteristics, and consist of polycyclic aglycones attached to one or more sugar side chains. The aglycone part, which is also called a sapogenin, is either a steroid (C27) or a triterpene (C30). Generally, most saponins tend to have short and often unbranched sugar chains containing 2–5 monosaccharide residues. The most common monosaccharide moieties found in plants are D -glucose, D -galactose, D -glucuronic acid, D -galacturonic acid, L -rhamnose, L -arabinose, D -xylose, and D -fucose.

Sasya received two samples – raw juice from alfalfa and fermented extract from AURI. According to the agreement, Sasya worked on physical and chemical methods to reduce saponin content in the raw juice from alfalfa. Only the raw juice The physical methods evaluated were (i) heat treatment, (ii) microwave treatment, (iii) membrane filtration and (iv) ion exchange. We used acid hydrolysis and water, methanol, ethanol as solvents to remove saponin.

Saponin was directly quantified using a liquid chromatography coupled with tandem mass spectrometers. Saponins from alfalfa were identified using the method (developed and optimized elsewhere). Using this method, 3-O-β-d-glucopyranosyl medicagenic acid (24%) and 3-O-β-d-glucopyranosyl-6’-malonyl-medicagenate (21%) were determined to be the majority components in alfalfa. The concentration of these two saponins will be used as representation for the total amount of saponin in each sample.

1.1. Saponin determination in extract

As received, the alfalfa raw juice had a pH of 4.46 and the fermented supernatant had a pH of 5.46. Surprisingly, the fermented supernatant had a higher specific gravity (1.04) than the raw juice (1.01). The two extracts were used as received for reducing the saponin count without any further pre-treatment. Saponin concentration in the samples was determined by aliquoting two 25 mL of representative samples of the extract and drying it in a lyophilizer. The weight of the dried samples was recorded. The dried sample was defatted by refluxing it with 80% methanol for 2 h. The solvent was evaporated under vacuum and the solids resuspended in 30% aqueous methanol added. The resulting mixture was sonicated (5 min), filtered through 0.2 µm filter syringe with nylon membrane and applied to a C18 column that was pre-conditioned with 10% aqueous methanol. Sugars and phenolics were removed in the wash step with 10% aqueous methanol. Saponins were eluted with 10 mL of dry methanol, which was evaporated under vacuum, resulting in a brown-yellow powder. The total saponin content in raw juice was determined to be 3.29% of the dry matter (as calculated by mass). Fermented extract had slightly

higher percentage of saponin – 3.62% of the dry matter. As previously evaluated, 3-O-β-d-glucopyranosyl medicagenic acid (GP) and 3-O-β-d-glucopyranosyl-6'-malonyl-medicagenate (GMP) are the largest components making up 45% of the total saponins in alfalfa.

In this report, the concentration of GP and GMP will be used as proxy for total saponin content in the samples. Furthermore, the total residual saponin content in the samples will be expressed as a percentage of the initial level of GP and GMP in the original raw juice. The processed data presented in the figures is based on the raw that is provided in Appendix.

1.2. Physical methods

1.2.1. Heat treatment

The raw juice was subjected to heat treatment at 45°C, 60°C, 75°C and 90°C. A sample of 50 mL of the sample was transferred into four 250 mL flasks and each flask was incubated at the pre-determined temperature with constant shaking for 12 h. At different timepoints, a representative sample was withdrawn and GP and GMP quantified. Saponin content reduced with increased temperature (Figure 1). The great reduction in saponin content was observed at 90°C. A majority of the saponin was hydrolyzed in the first 10 h, after which the rate of hydrolysis significantly decreased.

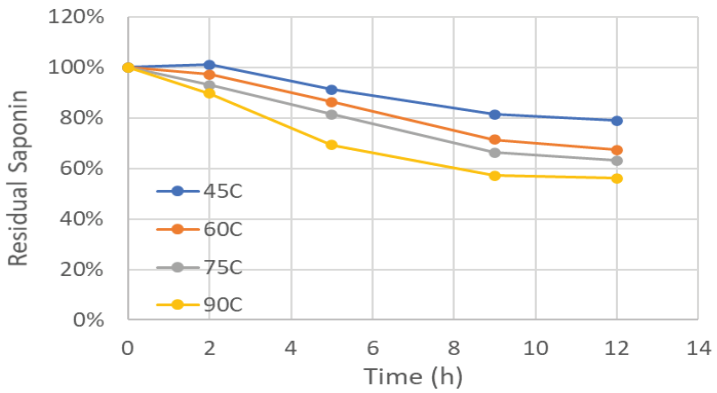


Figure 1. Residual saponin during heat treatment, relative to the initial content in the raw juice, as a function of time.

1.2.2. Microwave treatment

Many ester bonds are reversible and can be broken by providing exogenous energy. To evaluate whether the energy from a microwave can be used to breakdown the saponins, the raw juice and the fermented extract were subject to 1.2 kW of microwave energy for 30 min. This method resulted in only a 5% reduction in saponin content. It is not clear whether saponin was not hydrolyzed or the ester bonds were rejoined as a result. Interestingly, the microwave treatment resulted in a significant amount of foam which subsided subsequently. Clearly, microwave treatment is not an effective way to reduce saponins in alfalfa juice.

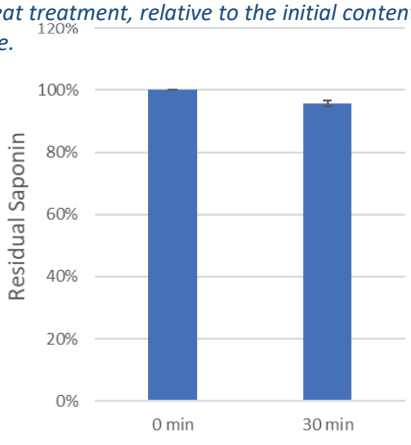


Figure 2. Residual saponin after microwave treatment

1.2.3. Membrane filtration

In this method, saponin separation was attempted on the virtue of their size. Unlike the other methods where saponin was hydrolyzed, filtration will result in intact saponins in a separate fraction, leaving the concentrated juice behind. Most saponins in alfalfa have a molecular weight between 900 and 1300. Using a 10kDa cut-off membrane, saponins were physically separated at room temperature. Membrane filtration is an ideal method to concentrate and separate components on the basis of molecular mass. The 10 kDa cut-off filter was extremely effective in removing the saponins in a small volume (~100 mL). The residual saponin content was ~19% of the initial level in the raw juice (Figure 3).

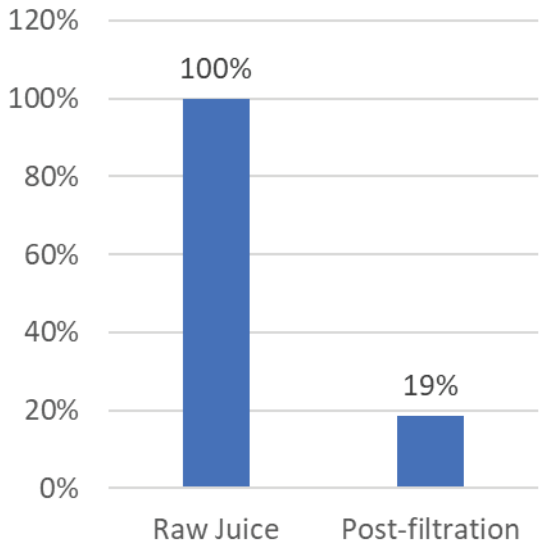


Figure 3. Residual saponin in the concentrated raw juice after membrane filtration

While membrane filtration has been extremely effective in separating saponin from the raw juice, the high cost of ultrafiltration membranes may not permit the use of this method for large-scale, low-value applications.

1.2.4. Ion exchange

An orthogonal method of separating saponins without hydrolysis is by ion exchange. Given that saponins are neutral without any charge, an anion-cation exchange chromatography may be a viable option for separating them. Three resins are considered, all with a particle diameter of 0.3 – 1.25 mm. The other properties of the resins are in Table 1.

Table 1. Properties of the three resins used to capture saponin from the raw juice

Name	Matrix	Functional group	Exchange capacity
R1	Polystyrene	$-N(CH_3)_3^+$	> 3.8 mmol/g
R2	PMMA	$-NH(CH_3)_2$	> 4.8 mmol/g
R3	PMMA	–COOH	> 10 mmol/g

The three resins were prepared by pretreating in ethanol for 48 h. They were washed with ethanol until there was no turbidity, following which a three-fold volume of water was added into the eluent. The resins were subsequently washed with DI water to completely remove any residual ethanol. Anion resin (R3) was successively washed with 1M HCl, DI water and 1 M NaOH and DI water before use. The cation resin (R1) was sequentially washed with 1M NaOH, DI water,

1 M HCl and DI water to prepare them for use. A fixed bed column (4 cm x 6 cm) was prepared and 50 g of the resin was loaded on the column. The raw juice was filtered and pumped through the columns using a peristaltic pump at 100 mL/h. The eluent was collected and analyzed for saponin content.

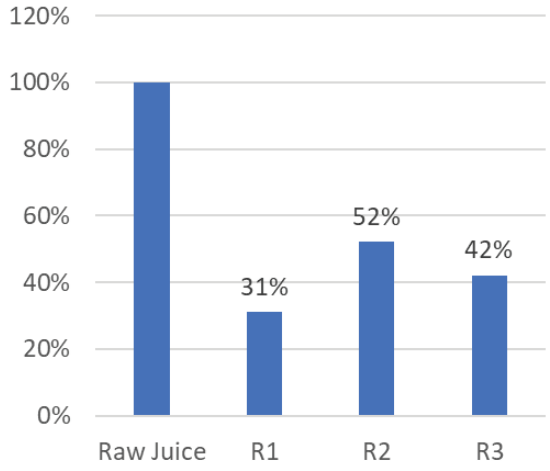


Figure 4. Residual saponin after single pass ion exchange

Overall, the three resins shown in Table 1 were able to remove saponins in the raw juice after a single pass through. Impressively, resin R1 with cationic resin reduced the saponin to 31% (Figure 4). The neutral and anionic resins were able to bind the saponins, but not as effectively. Given that the resin preparation and the separation was far from optimal, the substantial decrease in the saponin content is very encouraging, with potential scale-up possibilities. The low cost of the resin, easy availability and conditioning contribute favorable to the scale-up implications.

1.3. Chemical treatment

There is not one method that can reduce efficiently reduce saponins in alfalfa because of the structural variety arising from the different functional groups in the aglycone moiety. Hot alcohol and esters are the commonly used solvents to extract saponins. The solvents are not commonly used due to the heat labile nature of the functional groups in saponins. Since the scope of the Service Agreement is to decrease saponin content and not to extract and purify functional saponins, the method was implemented using acidified ethyl acetate, hexane, methanol, ethanol and isopropanol as the solvents for extraction.

The solvents were acidified with (1% v/v) 0.1M H_2SO_4 and added to the raw juice in 1:1 volume. They were mixed for 4 h at room temperature. At the end of the incubation, they were vacuum-distilled. The still was analyzed for saponin.

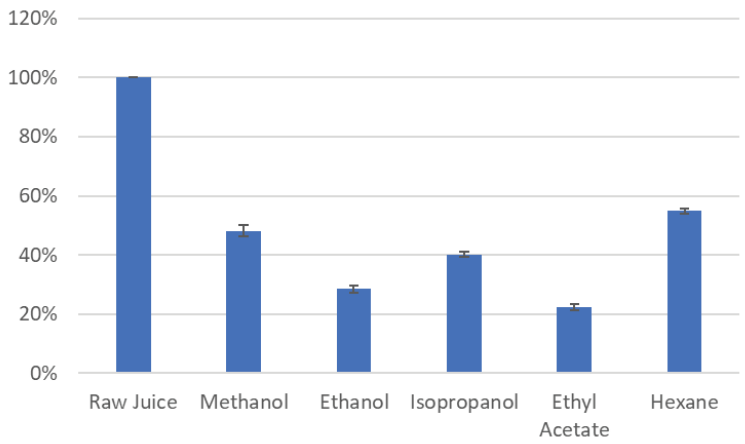


Figure 5. Residual saponin in raw juice after extraction with solvents (n = 3)

The solvents tested in the literature could efficiently remove saponins from the raw juice, as expected. Unexpectedly, ethyl acetate and hexane were also efficient in extracting saponins. Indeed, ethyl acetate treatment resulted in the greatest removal of saponins from alfalfa raw juice. This opens the possibility to explore solvent extraction as a means to separate and decrease saponin content in alfalfa juice. Clearly, the potential efficiency of other solvents, solvent ratio and extraction methods need to be optimized.

1.4. Summary – Reducing saponins in alfalfa juice

Various physical and chemical methods were demonstrated to reduce saponins in alfalfa juice. The choice of the method is largely dependent on the end use and the value of the product. For higher value, small-volume applications requiring low saponin content, membrane separation is by far the most effective process. Ion exchange using an appropriate resin is an efficient method to reduce saponin in commodity-scale applications. The diversity of saponins precludes the use of a single unit operation to completely eliminate them. For further reduction of saponin content, a combination of two or more methods may be required.

2. Protein concentrate from alfalfa

The protein, vitamins, minerals, and fiber content in alfalfa make it a highly nutritious forage for ruminants. This section of the report describes the results on extracting and recovering protein from alfalfa raw juice and fermented extract and determine the composition of the protein concentrate. Both the raw juice and fermented extract from alfalfa were used to concentrate the protein. The samples were filtered to remove the solids and the clarified broth was processed further. The generic procedure used to concentrate protein from the clarified broth from the two samples is shown below.

Figure 6. Schematic of a generic protein isolation and concentration procedure.

The samples were treated with 3 volumes of hexane and stirred for 1 h and allowed to settle. Hexane was decanted and the process repeated two more times. Any residual hexane was evaporated under vacuum overnight. The process resulted in a defatted sample that was used for concentrating the protein.

2.1. Concentrating protein in alfalfa raw juice

Protein from the samples was concentrated using ammonium sulfate precipitation. The other common method to precipitate protein (using alkali solubilization and acid precipitation) resulted in a substantial protein loss in the first attempt. While proven to be successful in other reports, this method is also likely to work for isolating alfalfa protein and will likely require substantial optimization. Therefore, for the purpose of this project, only ammonium sulfate precipitation was demonstrated.

Protein in 50 mL of the clarified sample was precipitated by slowly adding 45% $(NH_4)_2SO_4$ and stirring at 4°C overnight. The precipitate was collected and washed with PBS and freeze dried. To another 50 mL aliquot, 1N HCl was added to reduce the pH to 3.5 (where the protein had visibly crashed out of solution). The precipitate was collected and resolubilized in water after increasing the pH to 7 using 2N NaOH. The solids were discarded and the supernatant collected. An aliquot of the supernatant was stored for evaluating the size distribution of the protein. The rest of the supernatant was treated with protease and freeze dried. The protein content in the solids was also measured and found to be negligible. Therefore, a majority of the protein from the original sample was captured during the concentration process described in Figure 6. Protein determination based on nitrogen analysis is likely to be overestimated, despite using species-specific conversion factors, the protein values are also reported using Bradford method, which is an orthogonal, spectrophotometric quantification. The total protein in the samples measured using Bradford assay and shown in Table 2.

Table 2. Total protein (Bradford) in alfalfa samples

Sample	Tot prot (mg/mL)
Raw juice	0.77
Processed Raw juice (treated)	5.25
Processed Raw juice (untreated)	2.79

The processed samples refer to those from which protein is precipitated. The treatment refers to protease treatment of the protein concentrate. As determined, the protein content in post-processed samples is substantially higher than that in the raw juice (as received). Therefore, concentrating the protein using ammonium sulfate is a feasible method that can easily be scaled up as long as there is a viable process to reuse the salt.

Since the protein concentrate from the processing is typically not degraded and sufficient energy needs to be expended to convert the protein into its constituent amino acids, the protein concentrate was treated with protease to breakdown complex proteins. The samples were analyzed on a gel to evaluate the size distribution of the proteins. The gel (shown in Figure 7), in which the sample loading was normalized to have the same amount of protein, shows wide distribution of protein size (as seen by the bands) in unprocessed fermented extract (Lane 1) and raw juice (Lane 2). The processing steps shown in Figure 6 resulted in protein size distribution between 25 kDa – 45 kDa (lanes 5 and 6). There is a substantial amount of literature dedicated to process development and fine-tuning the process conditions to achieve proteins with a certain size distribution that are targeted to specific applications. When the processed protein samples were treated with protease, the protein was further broken down into amino acids, but did not alter the crude protein content.

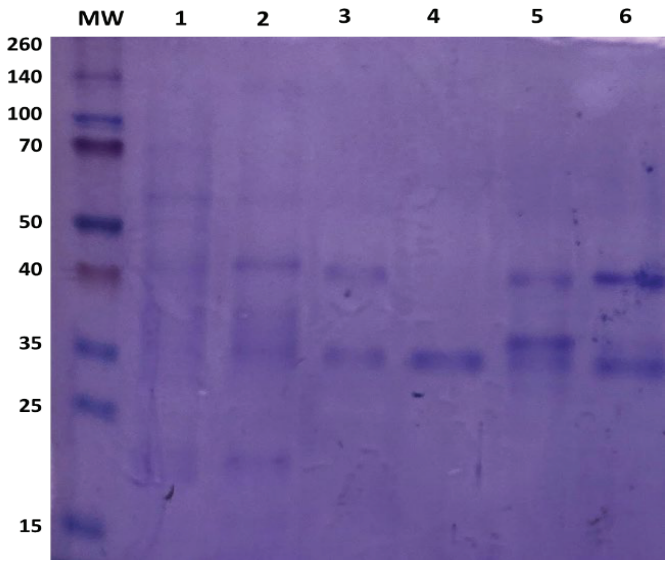


Figure 7. SDS-PAGE image that shows the distribution of the proteins in different samples. Lane 1: Fermented juice (as received), Lane 2: Raw juice (as received), Lane 3: Processed Fermented juice after protease treatment, Lane 4: Processed Raw juice after protease treatment, Lane 5: Processed Fermented juice and Lane 6: Processed Raw juice.

Table 3. Crude protein and amino acid breakdown in protein samples from alfalfa processing.

Amino acid (mol/kg)	Raw Juice	Concentrate	Concentrate (protease treated)
Crude protein (%)	29.74	78.22	80.88
Aspartic Acid	0.214	0.081	0.066
Glutamic Acid	0.173	0.052	0.069
Serine	0.098	0.049	0.052
Histidine	0.054	0.013	0.012
Glycine	0.235	0.100	0.084
Threonine	0.094	0.040	0.058
Arginine	0.098	0.019	0.023
Alanine	0.174	0.057	0.114
Tyrosine	0.055	0.022	0.029
Cystine	0.050	0.022	0.017
Valine	0.151	0.043	0.074
Methionine	0.030	0.183	0.222
Phenylalanine	0.122	0.035	0.050
Isoleucine	0.118	0.024	0.042
Leucine	0.202	0.059	0.081
Lysine	0.194	0.049	0.043

Total crude protein in the raw juice was 29.7% (as received) and the crude protein in the isolate increased to 78.2%. The crude protein and the amino acid content in the raw juice, protein concentrate and the protein concentrate treated with protease is shown in Table 3.

2.2. Summary

Protein in alfalfa raw juice was concentrated using ammonium sulfate precipitation method to ~80% and was decomposed to constituent amino acids. The amino acids profile indicates substantial enrichment of methionine. Overall, this experiment conclusively demonstrates the feasibility of using established, conventional methods to concentrate protein in alfalfa juice. With further protease treatment, the concentrate resulted in a favorable amino acid profile.

3. Extracting cellulosic sugars

Unlike other crops, alfalfa has the potential as a dual-purpose biofuel plant, with stems as the substrate for fuel and leaves for feed and other industrial products. This section of the report outlines how alfalfa solids were evaluated for application as a source of fermentable sugar. The solid samples from the raw juice were first evaluated for their sugar content by measuring the monosaccharides released from the cell-wall matrix following treatment with dilute acid (at various pH and temperature conditions) followed by enzymatic saccharification with commercial enzymes. The solids samples will be considered as our starting biomass in this section.

Carbohydrates and lignin were determined using a sequential process, according to the procedure described by NREL (https://www.nrel.gov/docs/gen/fy13/42618.pdf). Detailed procedure was outlined in the report for Task 2 (Kernza). Released sugars were analyzed using enzymatic kits using colorimetric determination. The remaining crude residue was hydrolyzed with sulfuric acid and the weight of the insoluble residue was measured and counted as lignin. The solids from the raw juice contained 47.8% cellulose, 29.6% hemicellulose and only 10% lignin. The solids from the fermented extract on the other hand contained only 17.7% cellulose, 5.1% hemicellulose and 3.9% lignin. The results are presented in Table 4. The profile for either samples is very different from raw biomass, indicating that the samples were highly processed that resulted in a partial loss of fiber and other insolubles.

Table 4. Composition of solids from raw juice and fermented extract.

Sugar	Sample	(g/kg of DB)
Cellulose	Solids from raw juice	478
	Fermented extract	177
Hemicellulose	Solids from raw juice	296
	Fermented extract	51
Lignin	Solids from raw juice	102
	Fermented extract	39

3.1. Biomass pretreatment

3.1.1. Acid Hydrolysis

Biomass pretreatment was evaluated using dilute acid method at different pH and temperature conditions. The biomass samples were evaluated at pH range of 3, 4, 5, 6 and 7 as well as using diluted sulfuric acid (5%) at three temperatures - 60°C, 75°C and 90°C. These conditions were compared with the reference used in the industry – dilute acid (10% H_2SO_4) incubation at 121°C for 3 h. The solids were separated from the reaction mixture and were subjected to enzymatic hydrolysis.

3.1.2. Enzyme hydrolysis

The acid-treated solid samples were brought to pH 5 using citrate buffer and a cocktail of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase (a gift from Novozymes) was added. The hydrolysis reaction was stopped after 6 h and the syrup separated and analyzed for hexoses (glucose and fructose) and pentoses (xylose and arabinose). After removing the solids, the clarified reaction mixture was evaluated for released sugars. The raw data is presented in Appendix Section 5.5.

3.2. Sugar extraction from alfalfa raw juice

The sugar profiles obtained were normalized to the biomass weight (dry matter) and the amount of sugar released is expressed as a percentage of the biomass weight. The results are shown in Figure 8.

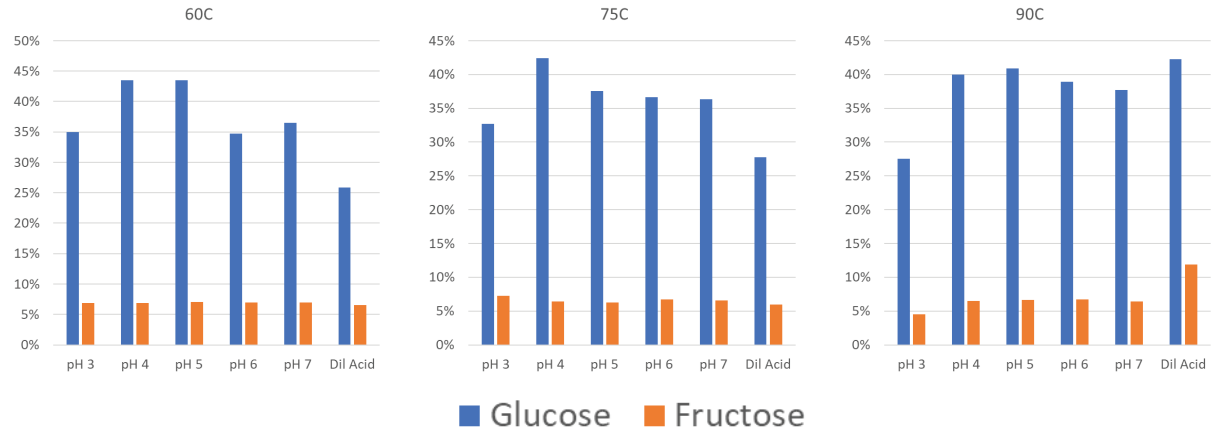


Figure 8. Hexoses from alfalfa at different pH values and temperatures. Shown in the figure are Glucose (blue) and Fructose (orange) as percentage of the biomass on a dry matter basis.

Sugars (hexoses and pentoses) produced from alfalfa raw juice had an unusual pattern and trend. Although there is a large body of literature that documents the sugar profile from alfalfa whole plant, there is no information on the sugar profile from the solids remaining after the juice was removed. Presumably, the extraction process has a significant impact on the sugar profiles. As received, the raw juice had extremely low xylose (0.68%) but higher arabinose (7.8%) content. It also contained higher amount of fructose (48.8%) than glucose (42.6%). The result is indicative of the fructose being liberating more readily during the juice extraction.

In the sugar samples, consistently, glucose was extracted in a much higher proportion than fructose. The result is not an artifact of the extraction procedure, but rather the outcome of the proportion of the two sugars present in the solids from the alfalfa raw juice. In general, the amount of glucose released increased as the pre-treatment pH decreased at all pretreatment temperatures. It reached peak 43% at pH 4 before decreasing at pH 3 (Figure 8). Interestingly,

the dilute acid pretreatment, which is the most commonly used method, resulted in liberating lower amount of glucose consistently. It appears that there is an optimal pH/temperature condition at which the cellulosic fibers are more readily disintegrated in alfalfa. Since the data is from one set of experiments, further study to validate the results is warranted.

Approximately 7% of the fructose was released under all the temperature and pH conditions studied. There was no observable trend in the amount of fructose liberated using any of the gentler conditions. However, using dilute acid at 90°C, there was a sharp increase in the amount of fructose liberated to 12% (Figure 8). Although the value is still low, the result is suggestive of a need for more stringent conditions to completely release the fructose.

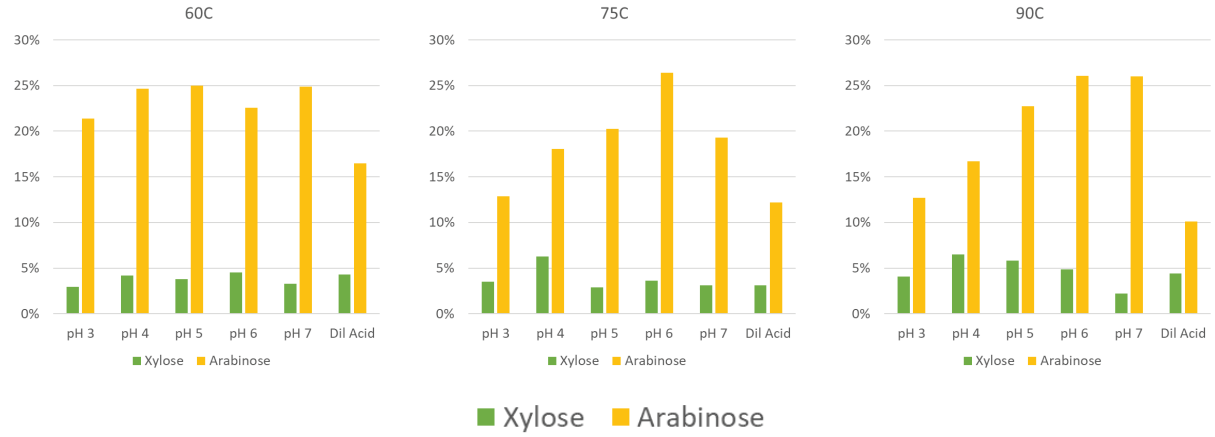


Figure 9. Pentoses released from the hydrolysis of pretreated alfalfa solids. Various pH and temperature combinations were evaluated for the pretreatment. The amount of xylose (green) and arabinose (yellow) released is indicated as percentage of biomass on a dry matter basis.

The release of pentoses was also highly unusual. Arabinose was consistently released at higher proportion than xylose. Typical biomass would have higher xylose content. At 60°C, ~20% of arabinose was released. This number decreased at 75°C and 90°C and at lower pH (Figure 8). The amount of xylose released was generally less than 5% at low temperatures (60°C and 75°C), but at 90°C and low pH xylose released increased slightly to ~7%. The pretreatment pH does not appear to have any impact on the release of xylose, but a higher pH seems to favor the release of arabinose. In general, the pretreatment temperature does not appear to have any impact on the release of pentoses from alfalfa solids.

The unusual pattern of the sugars extraction from alfalfa solids is also evident from a more holistic analysis of total C6 (glucose and fructose) and C5 (xylose and arabinose) sugars. While a higher temperature and dilute acid pretreatment conditions seem to favor the release of C6 sugars, a milder temperature (60°C) and a pH of 3.0 condition release high amount of C6 sugars. The result is shown in Figure 10, which shows the total hexoses and pentoses released as a

function of pretreatment pH and temperature. The radar graph shows that pretreatment at 90°C with dilute acid, close to 80% of the sugars released were hexoses (blue line). At these conditions, only 20% of the pentoses were released (red line). Pretreatment at 60°C and a pH of 3.0. approximately double the amount of pentoses were released while only 60% of the hexoses were released. The apparent dichotomy is suggestive of a multi-step pretreatment process that may be needed to fully release fermentable sugars from alfalfa.

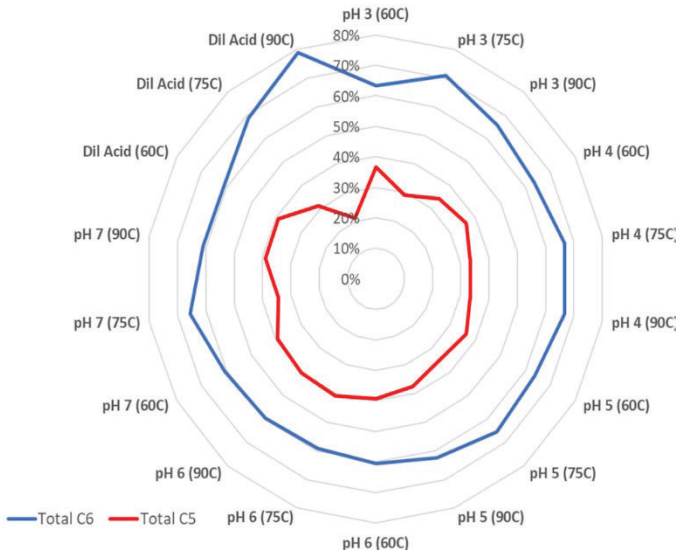


Figure 10. Holistic comparison of the pretreatment conditions as quantified by total C6 and C5 sugars released after the hydrolysis.

3.3. Summary

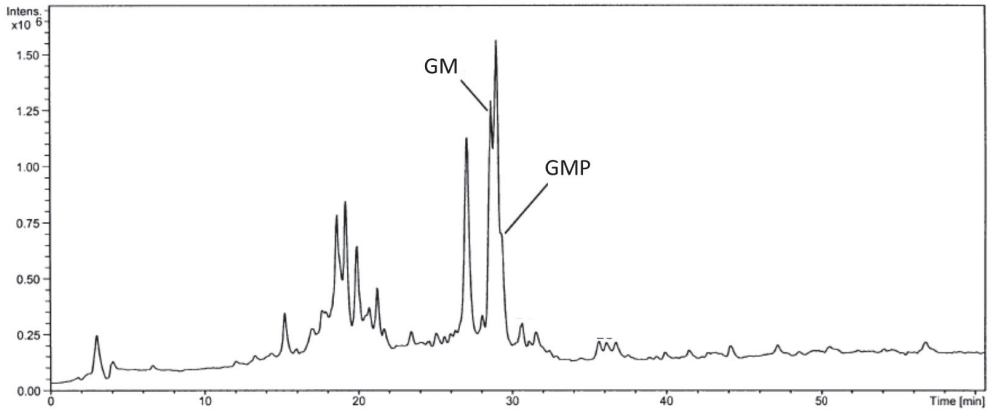
Pretreatment of alfalfa biomass (solids remaining after the juice has been removed) was used as the starting material for extracting fermentable sugars. Various combination of pH and temperature conditions were evaluated, coupled with an enzymatic hydrolysis step to determine the ideal conditions that would result in the release of sugars. The pattern of sugars extracted from the solids is highly unusual, mainly with two aspects. First, the amount of fructose released was extremely low. Second, there was substantially more arabinose released than xylose. The high amount of fructose in the juice suggests that the sugar is released readily during the juicing process and hence, is present at much lower amount in the residual solid. The dry material remaining after the sugars have been extracted still retained some crude protein (~30% of the total mass) and can be used as a supplement for added value. Overall, the experiment demonstrated the extraction of sugars using various pretreatment conditions from alfalfa.

5. Appendix

5.1. Raw data from characterizing the two samples received

	pH		Sp. Gravity	Dry weight (g)	Saponin (mg)	Fraction
Raw Juice	4.46	Replicate 1	1.01	9.46	313.28	3.31%
		Replicate 2	1.03	10.11	329.58	3.26%
Fermented extract	5.46	Replicate 1	1.04	10.46	384.28	3.67%
		Replicate 2	1.05	11.11	396.58	3.57%

5.2. Chromatogram of saponins in alfalfa



Total ion chromatograms obtained by negative-ion HPLC/MS/MS of saponins extracted from alfalfa juice. Separation was achieved using injections of 10 mg total extract, reversed-phase HPLC and gradient elution with 0.1% aqueous acetic acid and acetonitrile (20–80% acetonitrile over 60 min). The peaks for 3-O-β-d-glucopyranosyl medicagenic acid (GM) and 3-O-β-d-glucopyranosyl-6′-malonyl-medicagenate (GMP) are shown.

5.3. Raw data for Physical treatment of raw juice

5.3.1. Heat treatment

Condition	Sample ID	GM	GMP	Total
Initial	S2033-0	22831.0	78172.1	101003.1
45C	S2034-45	22457.4	79757.8	102215.2
	S2035-45	24233.9	67951.6	92185.5
	S2036-45	17209.0	65098.5	82307.4
	S2037-45	14376.5	65416.0	79792.5
60C	S2034-60	25090.0	73206.2	98296.2
	S2035-60	21127.8	66179.3	87307.1
	S2036-60	15695.8	56460.8	72156.6
	S2037-60	11644.5	56451.8	68096.3
75C	S2034-75	24150.2	69893.8	94044.0
	S2035-75	18864.1	63301.9	82166.0
	S2036-75	14123.8	52942.3	67066.1
	S2037-75	11422.2	52320.9	63743.1
90C	S2034-90	19782.2	70757.0	90539.2
	S2035-90	17389.8	52544.7	69934.6
	S2036-90	14260.7	43422.2	57682.9
	S2037-90	10232.2	46511.4	56743.6

5.3.2. Microwave treatment

Condition	Sample ID	GM	GMP	Total
Initial	S2048	23010.0	77959.8	100969.8
30 min-1	S2049	22957.2	73307.5	96264.6
30 min-2	S2050	23021.2	74562.2	97583.4
30 min-3	S2051	23104.1	72670.0	95774.1

5.3.3. Membrane filtration

Condition	Sample ID	GM	GMP	Total
Initial	S2048	23010.01	77959.78	100969.8
Post-filtration	S2061	4842.458	13953.9	18796.36

5.3.4. Ion exchange

Condition	Sample ID	GM	GMP	Total
Initial	S2048	23010.0	77959.8	100969.8
Resin 1	S2065	7720.2	23580.4	31300.6
Resin 2	S2066	12566.8	40037.0	52603.7
Resin 3	S2071	9618.3	32789.0	42407.3

5.4. Raw data for Chemical Treatment of raw juice

Replicate	Condition	Sample ID	GM	GMP	Total
	Initial	S2088	27624.1	75060.8	102684.9
Rep1	Methanol	S2091	11350.3	38746.1	50096.4
	Ethanol	S2092	6302.3	21635.6	27938.0
	Isopropanol	S2093	8736.2	31632.0	40368.3
	Ethyl Acetate	S2094	5513.2	18810.7	24323.9
	Hexane	S2095	12556.0	42770.2	55326.2
Rep2	Methanol	S2096	10383.5	36985.0	47368.5
	Ethanol	S2097	7454.3	22776.2	30230.4
	Isopropanol	S2098	10521.5	31599.9	42121.3
	Ethyl Acetate	S2099	5480.8	16678.6	22159.4
	Hexane	S2100	12836.7	43978.8	56815.5
Rep3	Methanol	S2101	13151.4	37759.8	50911.2
	Ethanol	S2102	6402.1	23314.9	29717.0
	Isopropanol	S2103	10214.2	31122.6	41336.8
	Ethyl Acetate	S2104	5779.3	16691.3	22470.5
	Hexane	S2105	13439.1	43335.4	56774.5

5.5. Raw data for the sugar analysis from alfalfa raw juice

Condition	Temp (°C)	Biomass (g)	Xylose (mg)	Arabinose (mg)	Fructose (mg)	Glucose (mg)	Total C6	Total C5
pH 3	60	9.52	283	2033	655	3332	3987	2316
pH 3	75	9.23	326	1187	668	3021	3688	1513
pH 3	90	9.26	378	1176	420	2546	2966	1555
pH 4	60	9.53	398	2350	655	4143	4798	2748
pH 4	75	9.69	608	1749	619	4112	4730	2356
pH 4	90	9.15	592	1530	596	3660	4257	2122
pH 5	60	9.63	365	2404	675	4188	4863	2769
pH 5	75	9.58	277	1942	597	3598	4195	2219
pH 5	90	9.31	544	2116	620	3810	4430	2661
pH 6	60	9.7	439	2187	672	3369	4041	2626
pH 6	75	9.85	357	2601	664	3611	4275	2958
pH 6	90	9.81	478	2556	662	3815	4477	3034
pH 7	60	9.61	316	2389	667	3508	4175	2705
pH 7	75	9.56	300	1845	629	3472	4101	2145
pH 7	90	9.69	215	2520	624	3653	4277	2735
10% H2SO4	60	9.72	416	1602	632	2510	3142	2018
10% H2SO4	75	9.79	306	1192	584	2714	3298	1498
10% H2SO4	90	7.8	345	788	926	3298	4225	1133

APPENDIX B

Fermentation Using Alfalfa Cellulosic Sugars
for High Value Chemicals

Final Report for Project No. FS017IN

Fermentation using alfalfa cellulosic sugars for high value chemicals

prepared for AURI under contract agreement FS017IN

Summary

This document summarizes the work completed for AURI by Sasya under the "Service Provider Agreement" Project No. FS017IN. AURI desired to learn the feasibility of extracting cellulosic sugars from ensiled alfalfa juice and fermenting the sugars into a higher value product. As outlined by AURI, the goal of this project was to implement the sugar extraction method optimized for alfalfa solids (see previous project report for details) for the ensiled juice and ferment the sugars into ethanol.

According to the previously optimized method for extracting sugars, dilute acid treatment followed by enzymatic hydrolysis resulted in the highest sugar yield. The ensiled alfalfa juice received from AURI was subjected to the same treatment and analyzed for sugar yield. Based on the results provided by AURI, glucose and xylose content was 0.7% each. The original sample of the ensiled juice also contained high titers of acetic (1%) and lactic (3%) acids. Despite the low sugar content in the ensiled juice, AURI instructed proceeding with the fermentation. Further, AURI desired to convert only the glucose fraction into ethanol in order to mimic a corn ethanol process. Four media conditions to maximize the fermentation of glucose extracted from alfalfa were evaluated. Using Ethanol Red® yeast, ammonia was favored over urea as the nitrogen source along with the supplementation of trace metals and vitamins to sustain metabolism.

After confirmation with AURI, the optimized condition was reproduced at 10 L scale. Even after 135 h of fermentation, only a small portion of the cellulosic glucose from alfalfa was and the final ethanol titer reached 2.5 g/L. High organic acid content in the ensiled juice was identified to be toxic to the fermentation and Sasya recommends reducing the acid content to improve the fermentation of sugars. Based on the low sugar content and prohibitively high acid content in the ensiled juice, Sasya does not recommend the use of ensiled alfalfa juice for fermentation.

Background

AURI desires to evaluate the feasibility of extracting sugars from ensiled alfalfa juice and fermenting them into products that have the potential for greater returns than current uses. Using Sasya's expertise and fermentation infrastructure, the scope of this project is to extract sugars from the feedstock provided by AURI and ferment them into ethanol as an example to demonstrate the fermentability of the alfalfa sugars. In a previous service agreement with AURI, Sasya evaluated various conditions that are conducive to extracting cellulosic sugars from alfalfa solids and identified that dilute acid deconstruction followed by enzyme hydrolysis yielded most sugars. AURI expressed a desire to implement the optimized method for obtaining cellulosic sugars from ensiled alfalfa juice as well. Furthermore, AURI also desired to "mimic" a corn ethanol process where predominantly glucose is fermented into ethanol. Since the choice of feedstock and yeast go hand-in-hand, a commercially available strain of yeast called Ethanol Red® was used in this project. This strain is capable of hyper-producing ethanol only from glucose but not other carbon sources.

Processing ensiled alfalfa juice

As received, the first aliquot of ensiled alfalfa juice was immediately processed to prevent any biological activity. Sugars from ensiled alfalfa juice were extracted by adding sulfuric acid to a final concentration of 0.1 M and incubating at 125°C for 40 min. After cooling to 30°C, an enzyme cocktail of α-amylase, β-amylase, cellulase and pectinase was added at 1% loading and the hydrolysis continued for 8 h. Solids remaining were removed and samples of the clarified hydrolysate was sent to AURI for sugar analysis.

Based on the results from AURI, the hydrolysate contained 6.79 g/L and 4.71 g/L xylose after processing. The sugar content is extremely low for use as a source of sugar. Furthermore, the acid content in the hydrolysate is extremely high for ethanol yeasts to function. The composition of the hydrolysate (as analyzed) is shown in the table below.

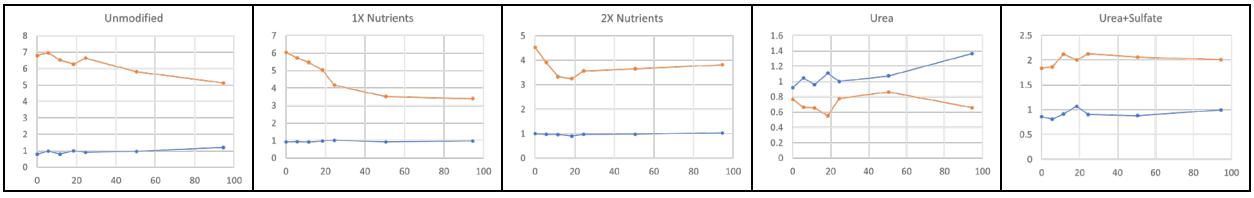
Analyte	Level (g/L)
Glycerol	0.21 ± 0.01
Ethanol	0.78 ± 0.11
Pyruvic	0.04 ±0.01
Lactic	31.46 ± 1.62
Acetic	4.98 ± 0.14
Glucose	6.79 ± 1.07
Xylose	4.71 ± 0.87

Based on this result, Sasya suggested that fermenting the hydrolysate may not yield satisfactory results due to high acid content and low sugar content. AURI confirmed continued interest in proceeding with the fermentation.

Supplemental nutrient requirements

Any requirement for supplemental nutrients for sugar fermentation was assessed by monitoring ethanol production upon adding phosphate (K_2HPO_4), nitrogen as ammonium (NH_4SO_4) or urea and trace metals. Five serum bottles were setup with 100 mL (final working volume) of hydrolysate supplemented with 1X1 of phosphate and ammonium with trace metals and vitamins, 2X the nutrient strength, 5 g/L urea and 5 g/L urea supplemented with 2 g/L sulfate (K_2SO_4). The fifth serum bottle contained unmodified hydrolysate as the control, appropriately diluted to be consistent with the other conditions.

As required by AURI's Chemistry Scientist, only the glucose part of the hydrolysate was fermented into ethanol using Ethanol Red® yeast (LeSaffre). Unlike some of the other yeasts, Ethanol Red® can ferment only glucose but not other sugars. To each of the five serum bottles, prepared in duplicate as described, 2 mL of exponentially growing culture of Ethanol Red® yeast was inoculated. The enzyme cocktail was added to each condition 2 h prior to inoculation such that hydrolysis of sugars commenced prior to fermentation. Samples were with drawn periodically to monitor glucose consumption and ethanol formation.



The figure above illustrates consumption of glucose (orange) and ethanol production (blue) in the four conditions studied along with the unmodified hydrolysate as the control (average of the duplicate conditions). The time of fermentation under microaerobic conditions is shown on the X axis and the concentration (g/L) of either residual ethanol or glucose is shown in the y axis.

Over a period of 96 h, less than 0.5 g/L of ethanol was produced. Enzymatic hydrolysis produces glucose, which is also simultaneously consumed, the residual concentration of glucose fluctuated

[1] **Salts:** 5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ 7 H_20; **Trace Metals solution (1000X):** 3 g/L $FeSO_4$-$7H_2O$, 4.5 g/L $ZnSO_4$-$7H_2O$, 4.5 g/L $CaCl_2$-$6H_2O$, 0.84 g/L $MnCl_2$-$2H_2O$, 0.3 g/L $CoCl_2$-$6H_2O$, 0.3 g/L $CuSO_4$-$5H_2O$, 0.4 g/L Na_2MoO_4-$2H_2O$, 1 g/L H_3BO_3, 0.1 g/L KI, 15 g/L Na_2EDTA; **Vitamins solution (1000X):** 50 mg/L d-Biotin, 1 g/L Ca-Pantothenate, 1 g/L Thiamin-HCl, 1 g/L Pyridoxin-HCl, 1 g/L Nicotinic Acid, 200 mg/L p-aminobenzoic acid.

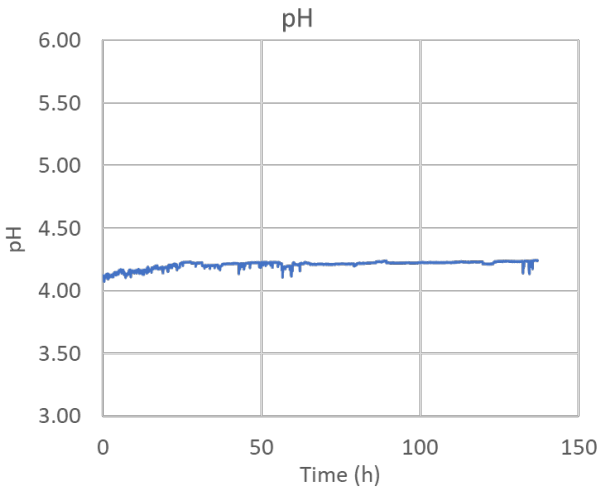
during the fermentation. Urea as a nitrogen source (with or without sulfate supplementation) did not appear to help glucose consumption and ethanol production. 1X nutrient supplementation appeared to result in the most consistent glucose consumption as well as ethanol production among the four conditions studies. After confirming with AURI of their continued interest to proceed with the fermentation, this condition was selected for the subsequent fermentation.

Fermentation of sugars

A cryovial of the yeast was thawed and the contents added to 3 mL of YPD media to activate yeast. The culture was grown overnight at 30°C, 200 rpm in a tabletop rotating incubator. After overnight growth, the activated yeast was seeded into 500 mL of the hydrolysate with 1X nutrients and conditioned to the fermentation to prepare the inoculum.

Bench-scale fermentation was conducted in a sterile 15 L glass vessel with temperature and pH controlled by Bioflo 120 console. A fresh aliquot of the ensiled alfalfa juice was received for the fermentation tests. This fresh shipment was immediately subjected to acid hydrolysis by temperature treatment in the presence of dilute sulfuric acid, as described above. After cooling to 30°C, the pH was adjusted to 4.5 and the enzyme cocktail described above was added to the deconstructed juice to initiate sugar hydrolysis for 4 h. A 10 X concentrated solution of the supplemental nutrients[1] was prepared (1 L) by sterilizing added to the vessel to bring the final volume to 10 L.

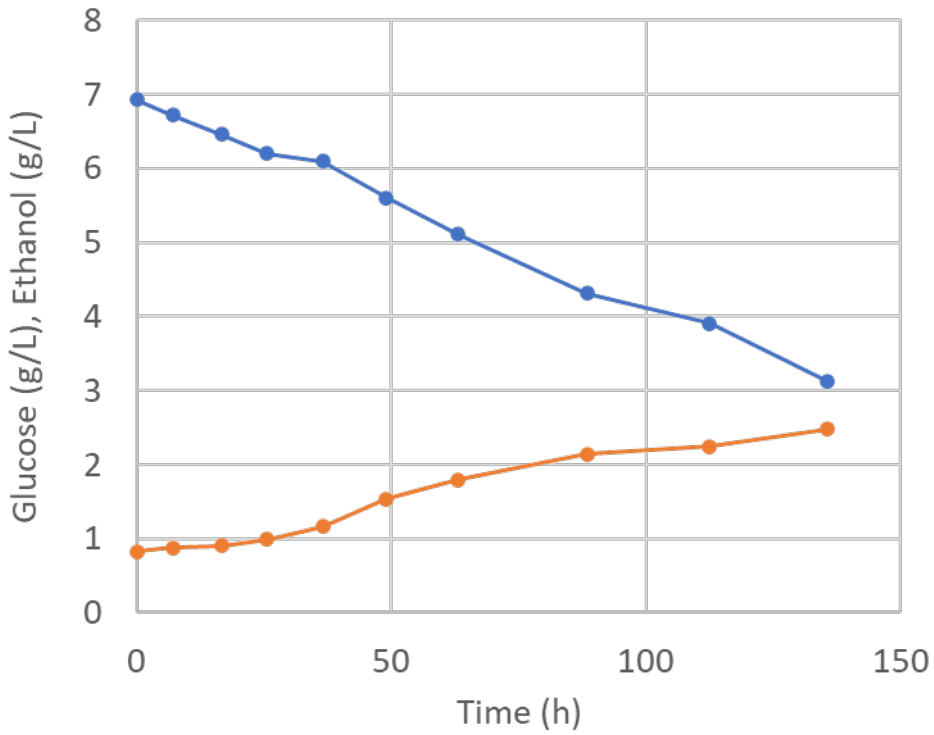
The vessel was sealed, and the motor, pH and dissolved oxygen probes, heat blanket, and the cooling water were set up. The agitation was set to 200 rpm, the temperature was set to 32°C. The inoculum was added to the vessel using a sterile funnel at t = 0 h. The fermentation progressed for five days during which real-time data on pH, temperature and agitation were collected. The pH was automatically controlled at 4.5 by the addition of 2N KOH. Periodically culture samples were collected aseptically to monitor sugar conversion and ethanol generation. These samples were centrifuged at 10,000 rpm for 5 minutes in a tabletop centrifuge to separate solids. The clarified supernatant was stored at -20°C for subsequent analysis.



Any biological activity would be indicated by changes in pH. Therefore, we monitored the

pH for any indication of metabolism. As shown in the adjacent figure, the pH of the hydrolysate was relatively steady without the need for any addition of acid/base control. Similarly, we did not observe any noticeable change to the color of the hydrolysate or its viscosity during the process. These results collectively indicate low fermentation activity. Therefore, the fermentation was discontinued after 136 h.

Samples from different time points during the fermentation were sent to AURI for quantification of ethanol and glucose. In 136 h of the fermentation, only 1.65 g/L of ethanol was produced to a final titer of 2.5 g/L. Ethanol production rate was 0.013 g/L/h during the complete fermentation process. The amount of residual glucose decreased by 3.8 g/L at a rate of 0.028 g/L/h. The figure below shows the trends of ethanol and glucose during the fermentation.



As observed previously, the hydrolysate had a very high acid content (~30 g/L lactic acid and ~10 g/L acetic acid). The level of these acids did not change noticeably during the fermentation. At the end of the fermentation, glycerol (0.7 g/L) and pyruvate (0.2 g/L) also accumulated as minor byproducts.

Conclusion

The primary conclusion is that ensiled alfalfa juice cannot be used as a source of fermentable sugars. The primary reason is the low sugar content in the feedstock, which does not permit economically viable titers for production of ethanol. Secondly, the slow progress of the fermentation likely due to high content of lactic and acetic acids, which inhibit biological activity of yeast, further impedes the use of the feedstock in a fermentation application. Indeed, high acid content could present a significant challenge for the use of ensiled alfalfa juice where low buffering capacity and low acid content are important. Through this deliverable, the Research and Deliverables according to the FS017IN agreement are satisfactorily fulfilled.

APPENDIX C

Reduction of Saponins in Alfalfa Juice

Deliverable for Contractual Services Agreement (Task 3)

Protocol development

Analytical method

Due to the absence of authentic standards, identification and quantification of saponins in alfalfa was complicated. Fortunately, detailed structural and identity information on saponins in soy is available. Method development mainly included LC-MS/MS (50:50 water:acetonitrile eluent) analyses of soy saponin (Sigma: 47036), which was used as the positive control to record retention times and characterize the mass spectra. The observed patterns were extrapolated to alfalfa saponins. The following factors were used to quantify saponins in alfalfa:

Factor	Range studied	Optimal value
Solution pH	2.0 to 9.5	3.5
Instrument polarity	-ve and +ve modes	Negative ion
Analyte concentration	1 pmol to 10 mM	-

Better chromatographic separation efficiencies were achieved in acidic mobile phases, with an optimum at pH 3.5. The abundance observed for the negative ion mode setting was lower, but the signal:noise ratio was about three-fold higher compared with that for the positive mode. Negative fragment ions corresponding to the sequential loss of sugars were observed but at lower abundances than those observed in the positive-ion mode. Increased structural information could be obtained in the negative-ion mode by increasing the capillary-skimmer voltage offset.

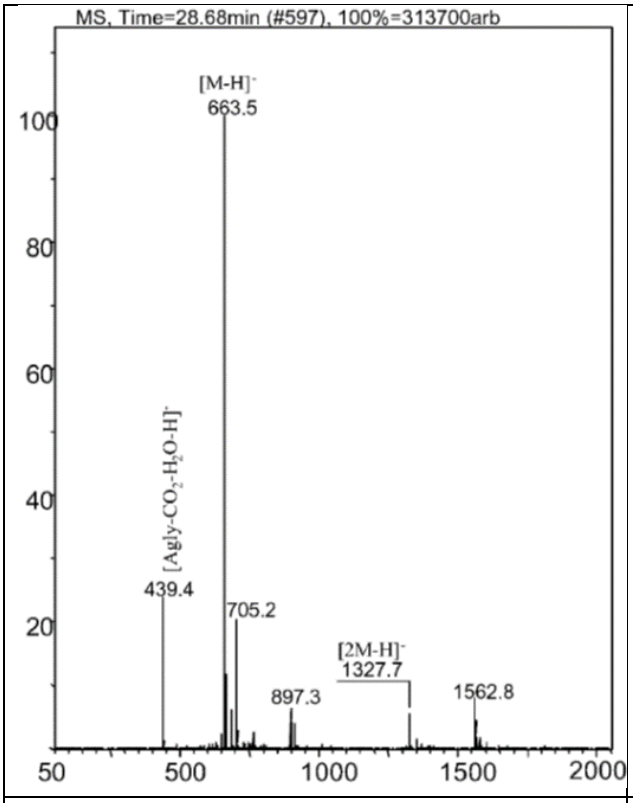
Therefore, based on higher signal:noise ratio and enhanced chromatographic separation efficiency, the optimal method for profiling complex saponin mixtures from alfalfa is negative-ion mode using a 0.1% acetic acid (pH 3.5) mobile phase. Using this method, the limit of detection was determined to be less than 1 pmol.

The developed method on the LC-MS/MS was used to identify saponins in the provided alfalfa juice. The complete chromatogram is shown in Appendix. The two major saponins were 3-O-β-d-glucopyranosyl medicagenic acid (24%) and 3-O-β-d-glucopyranosyl-6’-malonyl-medicagenate (21%), quantified as a fraction of the peak areas. The negative ion mode LC-MS/MS chromatogram of the two saponins is shown in the figure below. Soy saponin standards and the alfalfa samples were subjected to identical treatment.

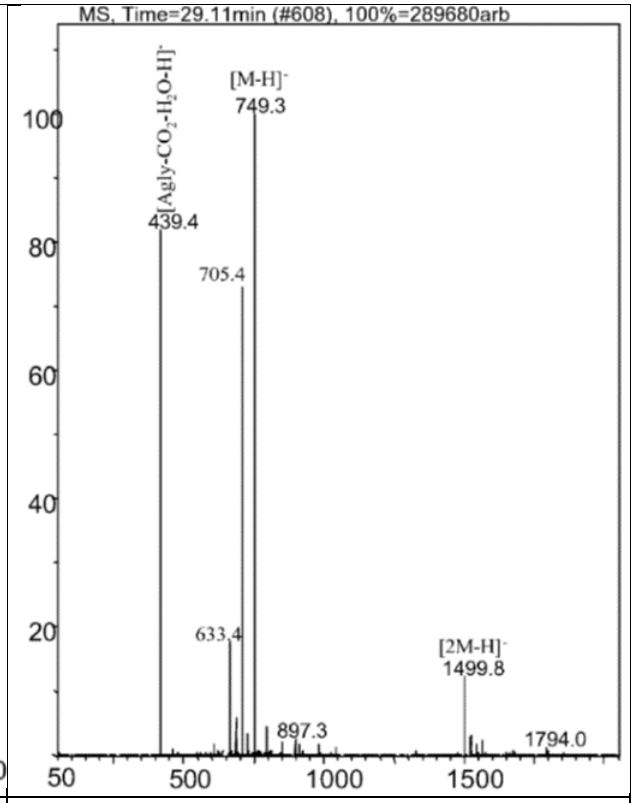
Outcome

The outcome of the work is a reliable method to detect and quantify saponins in general, and specifically in the alfalfa juice provided. The method is highly sensitive, has excellent reproducibility, simple to replicate and quick (total run time < 40 min per sample). This method will be used to quantify the saponins in this project. Since 3-O-β-d-glucopyranosyl medicagenic acid (GM) and 3-O-β-d-glucopyranosyl-6’-malonyl-medicagenate (GMP) make up for a majority of the saponin content in alfalfa, these two saponins

are quantified and are assumed to be representative of the total saponin pool in alfalfa. The underlying rationale for the assumption is that the hydrolysis is a first-order reaction and therefore, is dependent on the initial concentration.



3-O-β-d-glucopyranosyl medicagenic acid



3-O-β-d-glucopyranosyl-6'-malonyl-medicagenate

Enzyme synthesis

Three fungal saponin hydrolases (UniProt IDs: Q76BW2, Q2WGL4 and Q2WGL5) were targeted based on their reported high catalytic activity towards saponins, structural stability (as indicated by their 3D models) and promiscuity. The relevant features of these enzymes are summarized below.

NRRL ID	Enzyme ID	Seq length	Size	Source
22436	Q76BW2	638	68.4 kDa	Fusarium neocosmosporiellum
2083	Q2WGL4	618	66.6 kDa	Eupenicillium brefeldianum
447	Q2WGL5	633	68.1 kDa	Aspergillus oryzae

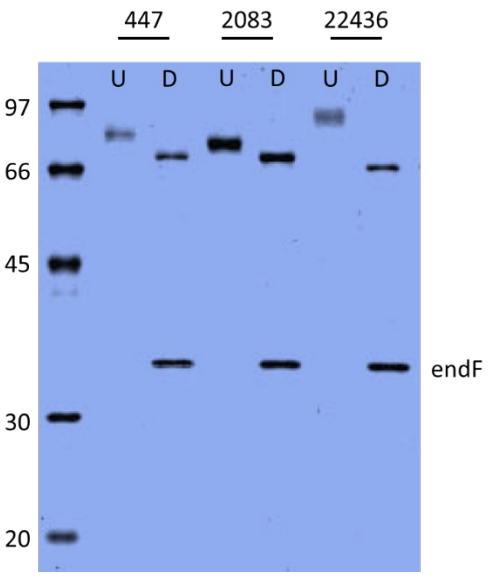
The three organisms were obtained from NRRL and were cultured in aqueous medium containing 10% V8 juice, 1% saponin and 1.5 g/L $CaCO_3$ for 3 days. The supernatant was collected by centrifugation and $(NH_4)_2SO_4$ was added to 70% saturation to precipitate the enzymes. The precipitate was dissolved in 0.1 M sodium acetate (pH 5.8) and 1M $(NH_4)_2SO_4$.

Step 1: The solution was applied to Butyl 650s column (Fisher Scientific) to bind the enzymes. The column was washed with 1M $(NH_4)_2SO_4$ and the enzymes eluted using $(NH_4)_2SO_4$ gradient (1 M to 0 M in 0.1 M sodium acetate, pH 5.8) in 10 equal fractions. Each fraction was tested for saponin hydrolase activity as described below.

Step 2: Fractions 3 and 4, which had the highest activity were applied to Resource PHE columns (Sigma Aldrich) and eluted with 1 M 1M $(NH_4)_2SO_4$ in 50 mM Tris-HCl(pH 7.5).

Step 3: In a third purification step, the partially purified enzyme was applied to Superdex 200 pg column and eluted using 50 mM phosphate buffer (pH 5.8) in the presence of .15 M sodium chloride.

Protein concentration for each enzyme was measured using Bradford assay. The concentration of Q76BW2 (from 22436) was 120 mg/mL, Q2WGL4 (from 2283) was 146 mg/mL and Q2WGL5 (from 447) was 281 mg/mL. An aliquot of the eluted enzymes was digested with glycosidase F (Roche) to ensure that post-translational modifications are intact and analyzed using SDS-PAGE (see below). The purification resulted in ~40 mL of the enzyme, which was stored at -80°C for subsequent use.



SDS-PAGE of the purified saponin hydrolases. The first lane shows MW ladder. The following lanes shows purified protein (Undigested and Digested with glycosidase F) from 447, 2083 and 22436 strains. The presence of single bands in each lane indicates >95% pure enzyme.

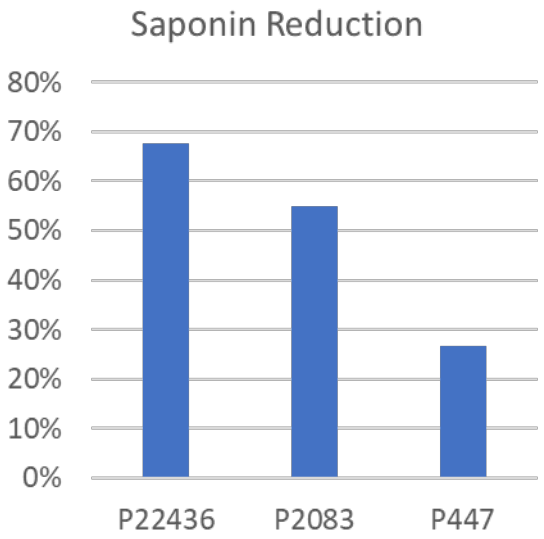
Outcome

The outcome of this task was successful purification of three fungal saponin hydrolases with their post-translational modifications intact, which promises catalytic activity.

Enzyme assay

Enzyme activity was measured by quantitative analysis of medicagenic acid produced from a reaction consisting of 1% saponin in 0.1 M sodium phosphate buffer (pH 5.8) at 37°C. The assay mixture contained 50 µg of the purified fungal hydrolase and the reaction was initiated by adding saponin. In this project, one unit of volumetric enzyme activity was defined as the amount of enzyme that reduces 1 % of the combined MS/MS signal of GM and GMP per minute from the substrate at pH 5.8 and 37°C. It is calculated as $\frac{S_0 - S_f}{S_0}$, where S_0 and S_f are the saponin concentrations at the beginning and end of the reaction and expressed as percentage. Specific activity is defined as volumetric activity normalized by the amount of protein. Triterpene glycoside saponins were quantified as described above.

The activity of the three purified hydrolases was tested on commercially purchased soy saponin. All three enzymes demonstrated significant activity on soy saponin and P22436 (*Fusarium neocosmosporiellum*) demonstrated almost twice as much activity as P447 (*Aspergillus oryzae*). P2083 (*Eupenicillium brefeldianum*) demonstrated intermediate activity. The result is unexpected not only because *Aspergillus oryzae* is widely considered to express hydrolase enzymes, but also because P22346 has higher identity with P447 (54%) than with P2083 (49%). As indicated in the adjacent figure, P22436 reduced 67% of the initial saponin while P447 reduced only 26% of the initial saponin. P2083 reduced 55% of the initial saponin under identical conditions.



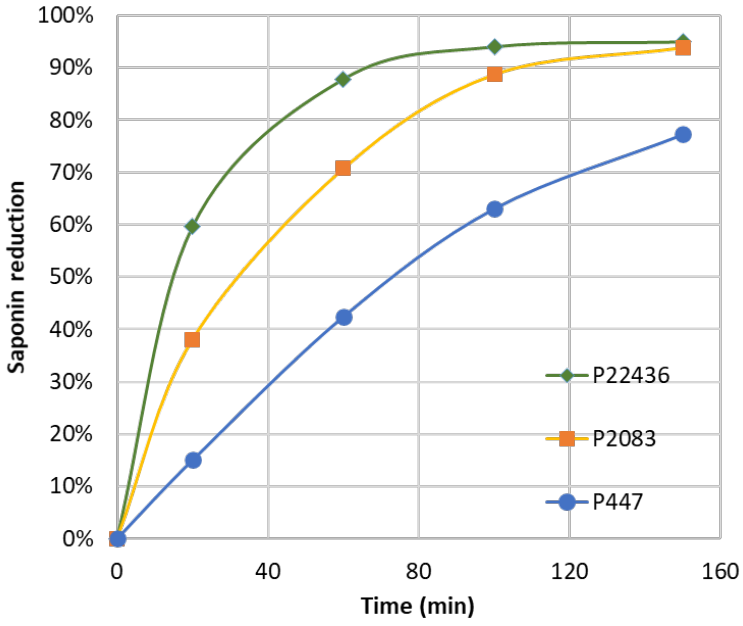
Outcome

The purified enzymes clearly demonstrated hydrolase activity. The outcome from this activity is the identification of at least three enzymes that can reduce saponin in alfalfa.

Reduction of saponins in alfalfa juice

Using purified enzyme

Based on successful demonstration of saponin hydrolase activity using soy saponin, the purified enzymes were tested with alfalfa raw juice. The assay was performed in 10 mL volume with 5 mL of 0.1 M sodium phosphate buffer (pH 5.8), 4.8 mL of alfalfa juice and 200 µL of purified enzyme. The reaction was incubated at 37°C for 3 h and 500 µL samples withdrawn periodically to monitor the saponin content using LC-MS/MS. As indicated in the figure below, saponins were hydrolyzed rapidly and efficiently by the three hydrolases. The absolute signal intensities for GM and GMP are provided in the Appendix.

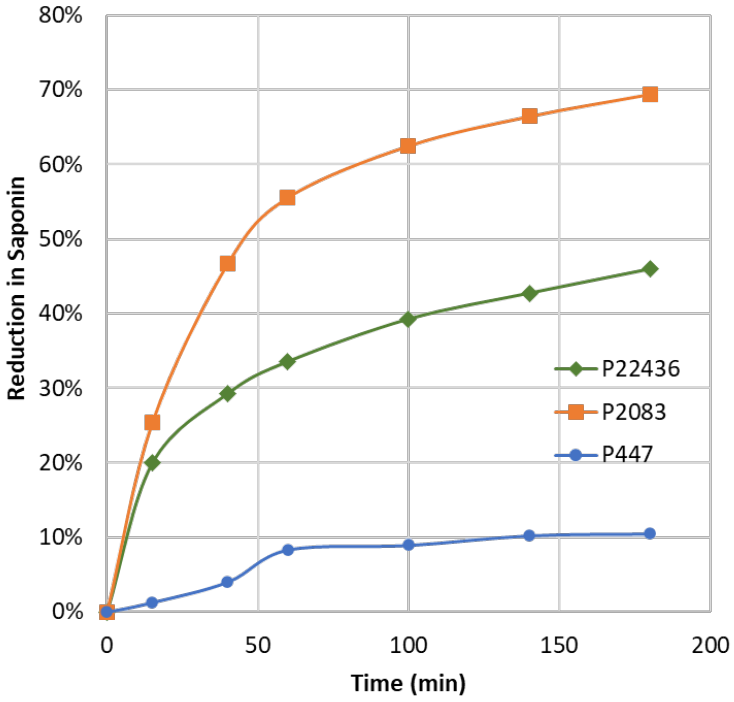


P22436 rapidly hydrolyzed the alfalfa saponins. In the first hour, the enzyme

hydrolyzed ~90% of the saponin after which the reaction rate significantly reduced. P447 exhibited the slowest reaction rate among the three, reducing alfalfa saponin by only 44% in the first hour. Consistent with the purified enzyme activity with soy saponin, P2083 demonstrated an intermediate activity by reducing alfalfa saponin by 70% in the first hour. The targeted enzymatic reduction of alfalfa saponin specifically by the hydrolases is not reported in the literature before.

Using supernatant

Alfalfa typically has low-value applications where the presence of saponins is a clear detriment. Although using purified enzymes to reduce saponin content has conclusively shown to be effective, the complexity of purification at larger scale will likely render the process economically infeasible. As an alternative, the supernatant from the fungal growth culture was tested for its efficacy in reducing saponins. Filamentous fungi have an excellent secretory signal that can transport enzymes across biological membranes. The supernatant from *Fusarium neocosmosporiellum*, *Eupenicillium brefeldianum* and *Aspergillus oryzae* were evaluated for their saponin reduction efficiency. To 4.8 mL of alfalfa juice, 0.5 mL of the 10X sodium phosphate buffer (pH 5.8) and 4.7 mL of the supernatant from the growth culture were added. The total reaction volume and the amount of raw alfalfa juice in the reaction mixture is identical to the previous setup with the only difference being in the enzyme (purified vs crude supernatant). The reaction was monitored over 3 h with periodic sampling to determine saponin content.



Saponin reduction using the supernatant was clearly not as efficient as using the purified enzyme although we did see reduction in alfalfa saponin (see above figure). Unlike with the purified enzymes, an interesting observation was that P2083 supernatant was most effective in reducing saponin. After 3 h, P447 supernatant could only reduce 10% of the saponin. Furthermore, it also exhibited a sigmoidal behavior, suggesting product inhibition. The super Surprisingly, P22436 which exhibited strongest activity as purified enzyme did not perform as well. It was able to reduce only 45% of the saponins after 3 h. The supernatant from the growth culture contains metabolites and nutrients, some of which could be inhibitory to hydrolase activity. Based on the results, P22436 enzyme appears to be more susceptible to

inhibition by components in the supernatant although in its purified state, exhibited strong activity. The inhibition of P2083 was clearly noticeable, but substantially less than that observed for P22436.

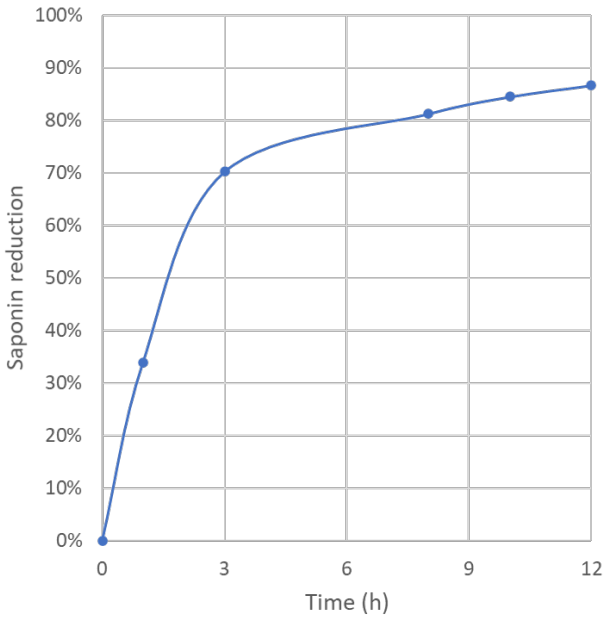
Outcome

The results conclusively demonstrate saponin reduction in alfalfa raw juice. Indeed, the rate and efficiency of reduction is substantially higher than any other physical or chemical method studied. The main outcome from this experiment is the identification of enzyme families that could reduce saponin in alfalfa.

Scale-up process

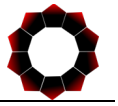
Building on the encouraging results, the saponin reduction in 2L of raw juice was evaluated using the supernatant from the culture of P2083 (selected based on process simplicity, cost and efficiency). The reaction was performed in a 3 L reactor vessel. A 1 L culture of P2083 was grown in 2 L flasks for 72 h, following which all the solids were separated by centrifugation. The clarified broth was filtered and concentrated using a 10 kDa membrane. Based on the retained volume, the supernatant was concentrated 4.2X. The concentrated supernatant was used to reduce saponin in alfalfa raw juice. The reaction was buffered with 300 mL of 1 M sodium phosphate buffer (pH 5.8) and 200 mL of the concentrated supernatant was added. The reaction mix was incubated at 37°C with constant agitation at 400 rpm for 12 h. Samples were periodically withdrawn and analyzed for residual saponin. As before, saponin reduction was calculated and expressed as a percentage.

The reaction started foaming significantly within the first 30 min and 100 µL of antifoam was added to reduce foam. There was no foam after 3 h into the reaction, which coincided with greater than 70% reduction in saponin by that time. As shown in the adjacent figure, a majority of the saponin was reduced in the first 3 – 4 h after which the reaction rate decreased. Based on the saponin determination, ~80% of the saponin was reduced in less than 6 h. The raw data are provided in the Appendix. The slowing of the reaction rate is more emphasized in the larger scale reaction and is likely due to inhibition of the hydrolysis by product(s) of the reaction.



Outcome

Using a concentrated supernatant of P2083, saponin in alfalfa could be reduced in a few hours. The direct implication of the result is its amenability for scale-up with substantially low-cost generation of the crude enzyme mix and bypassing enzyme purification.



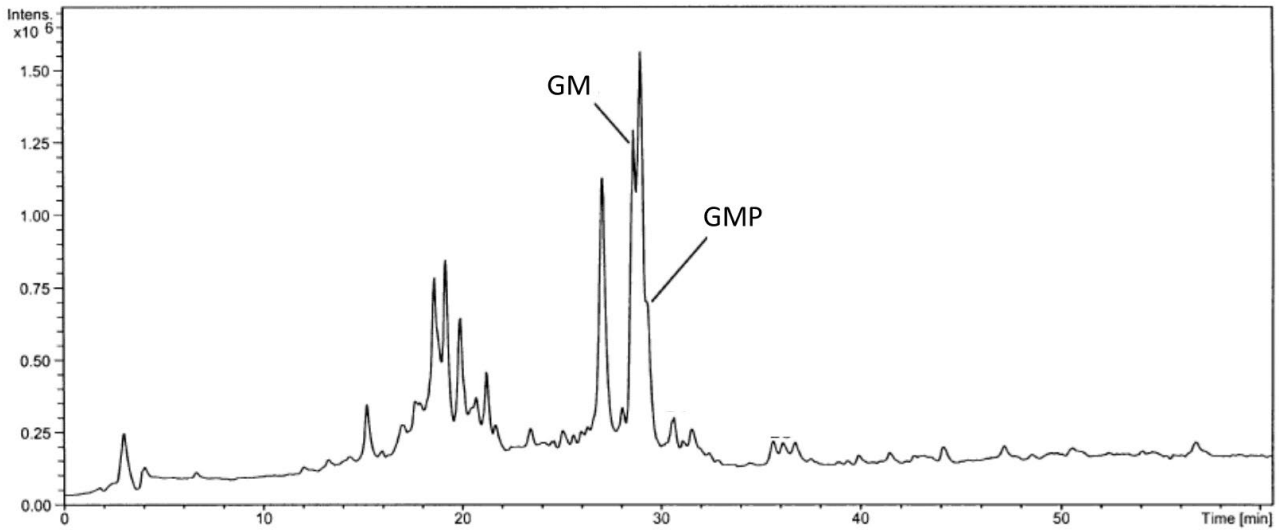
Conclusion

This report summarizes the development of analytical method to quantitatively detect alfalfa saponins. There were more than 15 significant peaks detected in the saponin analysis (see the chromatogram in Appendix), of which 3-O-β-d-glucopyranosyl medicagenic acid (GM, at 28.68 min) and 3-O-β-d-glucopyranosyl-6'-malonyl-medicagenate (GMP, at 29.11 min) were the most abundant. Given that these two saponins are representative of alfalfa saponins and the signal could be measured with least noise, the cumulative signal intensive was used as proxy for the total saponin concentration.

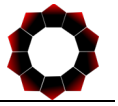
Three fungal enzymes that were postulated to have ester hydrolase activity were purified and evaluated for their ability to hydrolyze saponins. Although the purified enzyme from *Fusarium neocosmosporiellum* exhibited excellent catalytic ability, it did not perform as well in a crude preparation. However, saponin hydrolase from *Eupenicillium brefeldianum* was resistant to inhibition in the reaction with the supernatant. The feasibility of scaling the low-cost, robust process with the supernatant from this fungus was also demonstrated. The overall conclusion is that enzymatic hydrolysis is a far superior process for reducing saponin content and improving protein quality in alfalfa that any other method in the public domain. The extent of hydrolysis required will be determined by the residual amount of saponin that is acceptable for specific applications.

Appendix

Chromatogram of total saponins in alfalfa juice



Total ion chromatograms obtained by negative-ion HPLC/MS/MS of alfalfa juice. Separation was achieved using injections of 10 mg total extract, reversed-phase HPLC and gradient elution with 0.1% aqueous acetic acid and acetonitrile (20–80% acetonitrile over 60 min). The peaks for 3-O-β-d-glucopyranosyl medicagenic acid (GM) and 3-O-β-d-glucopyranosyl-6’-malonyl-medicagenate (GMP) are shown.



Raw signal data

The raw data used to calculate saponin reduction for enzyme activity is provided in this section.

Enzyme assay

Sample	Sample ID	GM	GMP	Total
Initial	S2001	57314.46	193623.7	250938.1
P22436	S2002	20408.86	60872.58	81281.43
P2083	S2003	26079.7	87256.73	113336.4
P447	S2004	45016.57	139220.7	184237.2

Saponin reduction using purified enzyme

Sample	Time (min)	Sample ID	GM	GMP	Total
	0	S2011	41644.9	126756.1	168401.0
P22436	20	S2012	16938.8	51035.2	67974.0
	60	S2013	4812.2	15653.1	20465.3
	100	S2014	2407.1	7671.7	10078.7
	150	S2015	1974.9	6501.5	8476.4
P2083	20	S2016	24259.0	80005.7	104264.7
	60	S2017	12470.2	36852.9	49323.0
	100	S2018	4719.2	14359.3	19078.5
	150	S2019	2620.0	7848.8	10468.8
P447	20	S2020	35548.9	107578.0	143126.9
	60	S2021	24182.9	72737.1	96920.0
	100	S2022	14719.6	47478.3	62197.9
	150	S2023	9255.6	29060.2	38315.8

Saponin reduction using supernatant

Sample	Time (min)	Sample ID	GM	GMP	Total
	0	S2204	39554.6	131894.9	171449.4
P22436	15	S2206	31878.4	105361.9	137240.3
	40	S2207	28321.2	92945.5	121266.7
	60	S2208	26268.4	87621.1	113889.4
	100	S2209	24274.6	79871.8	104146.5
	140	S2211	24728.1	73480.5	98208.6
	180	S2212	22164.0	70492.8	92656.8
P2083	15	S2214	32109.7	95764.3	127874.0
	40	S2215	20696.4	69144.4	91378.3
	60	S2216	17372.3	57662.1	76148.5
	100	S2217	15357.9	49947.0	64303.2
	140	S2218	13416.0	45194.8	57534.5
	180	S2219	11933.7	40672.2	52457.9
P447	15	S2220	39500.5	129718.5	169219.0
	40	S2223	39700.9	124814.4	164515.4
	60	S2224	36284.2	120842.4	157126.6
	100	S2225	40059.2	116018.4	156077.6
	140	S2226	37620.8	116240.2	153861.0
	180	S2229	36881.4	116548.9	153430.3

Scale up study

Time (h)	Sample ID	GM	GMP	Total
0	S2250	40322.3	131935.1	172257.4
1	S2251	28317.29	85452.3	113769.6
3	S2252	11777.35	39547.19	51324.55
8	S2253	8025.625	24328.98	32354.61
10	S2255	6161.055	20607.65	26768.7
12	S2260	5664.523	17365.05	23029.57

APPENDIX D

PPIC - Protein Isolation and Characterization of Alfalfa Flour

Plant Protein Innovation Center	
Project Title	Protein Isolation and Characterization of Alfalfa Flour
Project Lead	Alisa Smovzhenko
Researchers	Alisa Smovzhenko, Maddison Johnson
Start – End Date	Jan 2021 - Jan 2022

Project Executive Summary

AURI provided alfalfa flour for protein extraction optimization and protein structure and function assessment. One treatment method (twice defatted + milled) and multiple extraction methods (alkaline solubilization, isoelectric precipitation, membrane filtration, and salt solubilization) were evaluated to determine the parameters for efficient production of protein isolates.

1. Objective

The objective of Phase I of this project was to determine protein extraction conditions to produce alfalfa protein isolates (APIs) of optimum yield and purity. The prepared alfalfa (twice defatted and milled) underwent optimization of alkaline and salt extractions to try to produce APIs. The protein profiles of APIs were analyzed.

2. Materials and Methods

2.1 Sample materials

Commercial Alfalfa samples were provided by AURI.

2.1.1 Defatting

The Alfalfa flour was defatted by batch extraction with hexane in a 3:1 weight: volume ratio in three 30-minute cycles. Residual hexane was evaporated overnight under a hood. Fat content was verified to be below 3% following the Mojonnier AOAC method 922.06, a gravimetric procedure involving acid digestion followed by solvent extraction by diethyl ether and petroleum ether (AOAC International 2016).

2.1.2 Milling

Alfalfa was milled in a ball mill to decrease particle size and increase the amount of protein available. This is also essential for subsequent analysis.

2.1.3 Proximate analysis

Proximate analysis for defatted alfalfa flour (DAF), was performed in triplicate, following standard methods of analysis. Protein content was determined following the AOAC 990.03 Dumas nitrogen combustion method (AOAC International, 2016) using a Nitrogen Analyzer (LECO® TruSpecNTM, St. Joseph, MI, USA). A nitrogen conversion factor of 6.25 was used. Fat content was determined following the AOAC 922.06 Mojonnier method (AOAC International, 2016). Protein and fat content were also evaluated during different cycles of defatting.

2.1.4 Salt extraction evaluation

Optimization of salt extraction was conducted to determine the extraction conditions that would produce an isolate of the highest possible purity and yield from DAF. The main evaluated parameter was the salt concentration. In triplicate, flour was solubilized at 5% total solids (w/v) in 0.5, 0.75, or 1 M NaCl solutions for one hour while stirring. The solutions were centrifuged at 7000 rpm for 7 minutes, and the supernatant was decanted. An aliquot of the supernatant was analyzed using the Dumas method to measure protein content. The DAF solubilized in 0.75 and 1.0 had the most protein in the supernatant (**Figure 1**) yet solubility was low (under 30%) and there was no considerable difference in protein solubility as the salt concentration increased from 0.5 M to 1 M NaCl; and the risk of protein denaturation at higher salt concentrations (0.75M and 1.0M NaCl) outweighed the possibility of obtaining slightly higher protein yield, thus protein extraction using alkaline solubilization was explored.

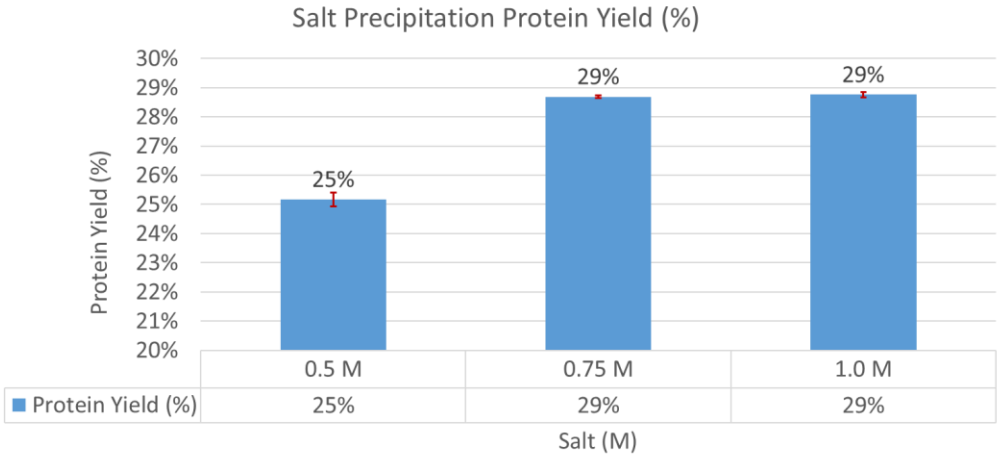
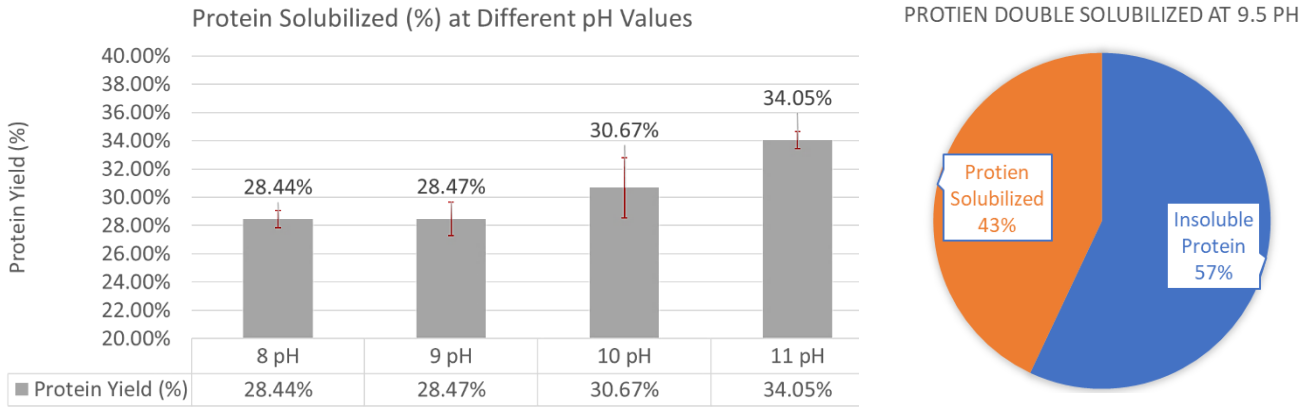


Figure 1. Protein yield percentages of defatted alfalfa flour in different NaCl concentrations.

2.1.5 Alkaline pH extraction optimization mass-balance

Optimization of alkaline pH extraction was conducted to determine the extraction conditions that would produce an isolate of the highest possible purity and yield from DAF. Parameters tested included solubilization and precipitation pH levels. Optimum solubilization pH was determined by dispersing flour at 5% total solids in DDW (w/v), in triplicate, adjusting the pH to the desired level using NaOH, and stirring the solutions for one hour. Tested pH levels for solubilization included pH 8, 9, 10 and 11. The solutions were centrifuged at 7000 rpm for 7 minutes (Thermo Electron IEC CL31 Multispeed Centrifuge, Thermo Electron Industries SAS, France), and additional floating solids were removed using a Buckman Filter. The Dumas method was used to determine the protein content of the resulting supernatants. While solubility at pH 10 was higher than that at pH, the difference was not major, thus solubilization was tested at a pH 9.5 (**Figure 2**). Solubilization at pH 9.5 was tested but with double solubilization, instead of just one round of solubilization. The pellet after the first solubilization was resubmerged in 95% DDW for an additional solubilization at 9.5 pH for one hour. Following centrifugation, the supernatants were combined, which showed adequately high protein solubilization at 43% +/- 2.41% SD.

Figure 2. Protein solubilization percentages of defatted Alfalfa flour with a single solubilization step at different pHs (A) and with double solubilization at 9.5 pH (B).



Next, protein precipitation pH level was determined by dispersing flour at 5% total solids in DDW (w/v), in triplicate, adjusting the pH to 9.5 using NaOH, and double solubilizing for one hour each time. After centrifugation at 7000 rpm for 7 minutes and removing solids with a Buckman Filter, the supernatants were combined and adjusted to either pH 3.0, 3.5, 4.0, 4.5, 6.0, 6.5, and 7.0. These pH levels were chosen as Alfalfa has an isoelectric precipitation point around 4.5 pH (Hadidi, 2019). The solutions were centrifuged under the same parameters, and only pH 3.0-4.5 showed any precipitant, while pH 6.0-7.0 did not cause any protein precipitates to form. In all trials, less than 12% of the protein had precipitated from the supernatant (**Figure 3**), indicating that pH precipitation under these conditions will not yield enough protein for analysis.

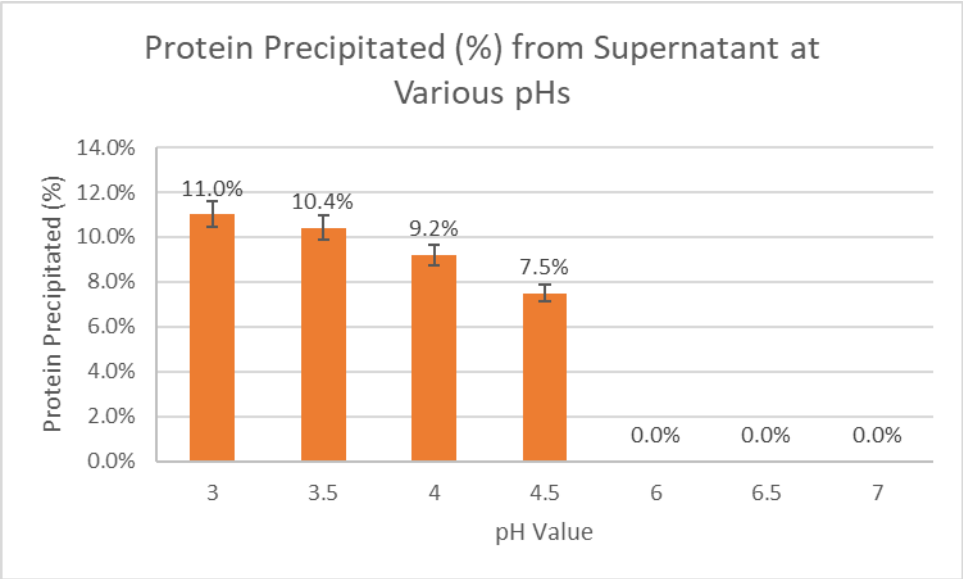


Figure 3. Protein yield percentages of defatted Alfalfa at different pH levels

2.1.4 Mass-balance extractions

Multiple mass balance experiments were done to evaluate the yield and purity of APIs produced to compare the effect of different protein concentration methods on protein extraction efficiency. These experiments included various combinations of soaking the sample overnight, ultrafiltration (UF), dialysis, precipitation overnight, and/or freeze drying to determine the protein yield, loss at different steps, and purity.

Samples soaked overnight were at 85% moisture at 4°C to mimic the moisture content of fresh alfalfa prior to further processing. All samples were then double solubilized at 9.5 pH. The supernatant containing the protein was concentrated using either ultrafiltration (UF)or precipitation overnight at 4.5 pH 4°C. For UF, the benchtop Sartorius Vivaflow® 200 system was used with two Vivaflow® membrane cassettes running in parallel to increase filtration speed. The system was assembled according to manufacturer instructions, with the protein solution in a feed reservoir and the feed tube connected to a peristaltic pump (Masterflex Easy Load Pump Head – Size 15, Masterflex Economy Drive Peristaltic Pump 230 V, Sartorius) to pump the feed solution under pressure (1.5 bars) across the membranes. Components in the solution larger than the membrane pore size (3 kDa MWCO) were retained, while smaller components passed through the permeate. Therefore, the proteins were concentrated as the small molecular weight components were filtered out of the solution. For precipitation overnight, the pH was adjusted to 4.5 using HCl, and left stirring at 4°C.

After UF or precipitation, the samples were dialyzed against deionized (DI) water at 4°C to remove any salts, following Thermo Fisher Scientific™'s specifications. Similar to UF, components in the solution larger than the

membrane pore size (3 kDa MWCO) were retained, while smaller components passed through the permeate. The samples were subsequently lyophilized. When not in use, the samples were stored at -20°C.

Experiment A included double solubilizing alfalfa flour at 9.5 pH, and then using Ultrafiltration (UF) and Dialysis to reduce impurities within the sample.

Experiment B included soaking the sample at 85% moisture overnight to replicate the moisture content fresh alfalfa. Then, the sample was double solubilized at 9.5 pH. The solubilized protein within the supernatant was precipitated overnight at 4.5 pH, followed by dialysis and freeze drying.

Experiment C included soaking the sample at 85% moisture, double solubilization at 9.5 pH, ultrafiltration, dialysis, and freeze drying.

Further trials were done to determine where protein loss is occurring and what type of protein was being extracted at various steps. This included three additional experiments outlined below:

Trial 1 aimed to determine what proteins were solubilized. Thus, the sample was soaked overnight at 85% moisture, double solubilized at 9.5 pH, and then was dialyzed to reduce impurities and lyophilized to produce an isolate.

Trial 2 aimed to determine the protein yield and profile of samples extracted from soaking the sample overnight. Thus, the sample was soaked overnight at 85% moisture, dialyzed to reduce impurities, and lyophilized to produce an isolate.

Trial 3 aimed to determine what proteins were being extracted from double solubilization without further purification; the sample was soaked at 85% moisture overnight, double solubilized at 9.5 pH, and then lyophilized to produce an isolate.

2.2 Structural Analysis

2.2.1 Protein profile by SDS-PAGE

Protein profiling of all isolates was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), following the method outlined by Laemmli (1970). Protein samples were solubilized in DDW (4 mg protein/mL) and then mixed 1:1 (v/v) with Laemmli buffer under reducing and non-reducing conditions. An

aliquot (5 µL, delivering approximately 0.05 mg protein) and 10 µL of Precision Plus MW standard were loaded onto a Criterion™ TGX™ 4-20% precast Tris-HCl gradient gel and electrophoresed at 200V. The gel was stained using Imperial™ Coomassie blue R-250 staining solution and destained with DDW. Molecular Imager Gel Doc XR system (Bio-Rad Laboratories) was used to image the gels, and bands were identified by their molecular weights.

3. Results and Discussion

3.1 Proximate analysis

The alfalfa flour was defatted by hexane extraction to reduce the flour's fat content and prevent interference with protein extraction. **Table 1** outlines the protein content of commercial Alfalfa and commercial defatted Alfalfa. Unexpectedly, the defatted flour had a slightly lower protein content relative to the defatted flour, which could be due to moisture uptake post defatting while the hexane was evaporating. The exact cause is unknown.

Table 1: Proximate analysis of alfalfa flour, and defatted Alfalfa flour (DAF)

Parameter	Alfalfa flour (% composition, w.b.)	DAF* (% composition, w.b.)
Protein	18.2[a]	16[b]
Fat	3.6[a]	1.6[b]

**Defatted alfalfa flour*
Different superscript letters in the same row indicate significant differences among the samples according to the paired t test (*P 0.0108 < 0.05).*

3.2 Protein extraction evaluation

Protein yield and purity of APIs from different extraction methods are reported in **Table 2***.* Isolates extracted from method B had the highest protein purity, yet with a protein yield of only 1.8%. There was not enough protein isolated to run further structural and functional assays; isolates from all extractions had lost more than half of the protein to UF and dialysis, indicating these proteins were smaller than the 3 kD used to purify the isolate and are likely free-floating peptides.

Table 2: Alfalfa protein isolate purity, yield, and protein loss by extraction methods

Extraction method	Protein purity (%)	Protein yield (%)	Protein lost to UF or dialysis (%)
A*	28.6[a]	8.5[b]	36[b]
B**	47.6[c]	1.8[a]	11[a]
C***	35.91[b]	9.3[b]	35.0[b]

** A: Samples were double solubilized at 9.5 pH, ultrafiltered, dialyzed, and lyophilized*
*** B: Samples were soaked overnight at 85% moisture, double solubilized at 9.5 pH, precipitated overnight at 4.5 pH, dialyzed, and lyophilized.*
**** C: Samples were soaked overnight at 85% moisture, double solubilized at 9.5 pH, ultrafiltered, dialyzed, and lyophilized.*
Different superscript letters in the same column indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (P < 0.05).

Protein yield and purity at various steps of extractions are reported in **Table 3**. While certain extraction methods had a high enough yield for analysis, such as extraction 3, the protein purity at each step was too low to be considered a suitable isolate (<60% protein purity).

Table 3: Alfalfa protein isolate purity, yield, and protein loss at different stages of extraction.

Extraction Analysis Trial	Protein purity (%)	Protein yield (%)	Protein lost to UF or dialysis (%)
1*	29.6[b]	10.21[b]	23[b]
2**	25.3[a]	2.24[a]	16
3***	23.3[a]	52.68[c]	N/A

**Extraction 1 analyses the protein isolated post soaking at 85% moisture overnight, double solubilizing at 9.5 pH, and dialysis.*
***Extraction 2 analyses protein isolated post soaking at 85% moisture overnight and dialysis.*
***Extraction 3 analyses protein isolated post soaking at 85% moisture overnight and double solubilization at 9.5 pH, without a further purification step.*
Different superscript letters in the same column indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (P < 0.05).

3.3 Protein profile by SDS-PAGE

SDS-PAGE reveals the relative distribution of protein subunits, disulfide linkages, and protein polymerization. Extracted low-protein isolates from experiments B-C and 1-3 were evaluated. under both non-reducing and

reducing conditions, and the effect of extraction methods on the protein profile of the isolates was assessed (**Figure 4 & 5**). Experiment A was not run as it had already been ruled out as an inefficient method of extraction. Among experiments B-C, similar trends are seen between both trials (**Figure 4);** there is a large amount of smearing with few distinct bands, indicating the presence of hydrolyzed peptides rather than intact protein. Additionally, there are no notable differences between reducing and non-reducing samples, indicating there is little to no protein bound by disulfide linkages. The SDS-PAGE run was stopped before fully reaching the bottom of the gel, and peptides are apparent at sizes below 1.0 kD.

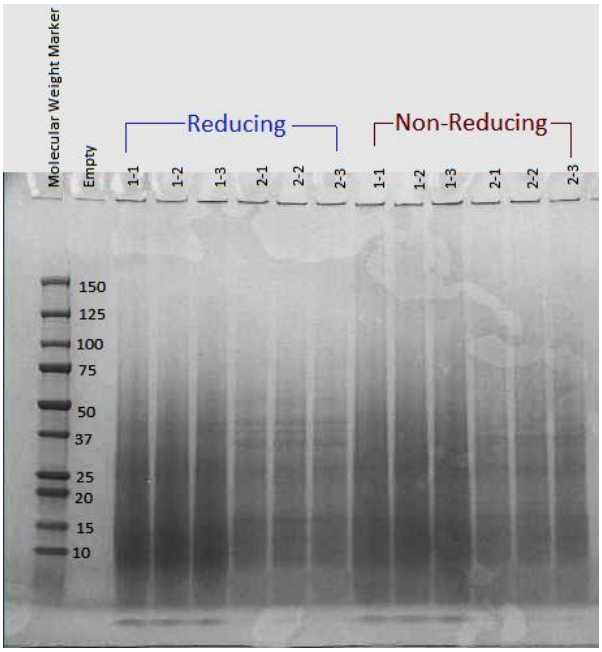


Figure 4: Protein profiling of extracted alfalfa protein under reducing and non-reducing conditions. Experiment B (1-1, 1-2, and 1-3) and Experiment C (2-1, 2-2, and 2-3) were run in triplicate.

Trial 3 (Double solubilization without dialysis) underwent less filtration/purification and showed higher amounts of hydrolyzed protein (**Figure 5**). Trial 1 (double Solubilized) and 1 (soaked overnight) show large amounts of smearing with slight band formation at 50 kD and 15 kD. This is consistent with Figure 4, where large amounts of hydrolyzed petides smear the SDS-PAGE gel.

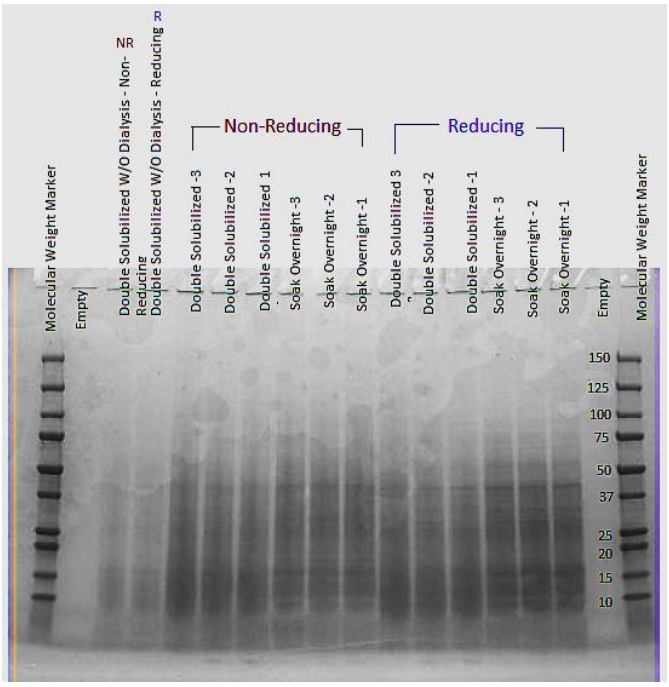


Figure 5: Protein profiling of extracted alfalfa protein under reducing and non-reducing conditions of Trial 1 (Double Solubilized), 2 (Soaked overnight), and 3 (Double Solubilized without Dialysis).

4. Conclusions and Future Work

With current methods, there is no efficient way to extract hydrolyzed alfalfa proteins that would have both high protein yields and isolate purity (> 60% protein). Additionally, hydrolyzed protein serves very little functional/structural use, although nutritional profile would remain mostly intact. Hydrolysis of alfalfa protein has previously been linked to harvesting methods of alfalfa, where endogenous proteases within alfalfa break down the protein in environments with high moisture and heat within 2-48 hours of harvesting (Hadidi, 2019). This condition is met when commercial alfalfa is harvested and left to dry in the field under the sun. This result indicates harvesting methods, specifically the deactivation of proteases soon after harvesting, are important for retaining protein structure and functionality. One proposed solution is to dry the alfalfa soon after it's harvested to limit protease activity. Other options include extracting the protein from fresh alfalfa immediately and/or adjusting the pH to limit protease activity (Hadidi 2019). Future work to determine alfalfa use in human food will require intact protein analysis.

The degradation of alfalfa protein is an important consideration for cattle digestion. While Alfalfa is relied on as a nutritional source for cattle, commercial versions are easily digested by methanogens, a group of *Archaea* microbes responsible for producing methane gas within the ruminants of livestock (Hook, 2010). They produce

large quantities of gas and slime with no healthy egress. Pressure buildup can lead to death; thus, cattle cannot be fed an alfalfa-pure diet regardless of its nutritional value. If more intact protein is fed to the cattle, it's possible that the microbes would not be able to digest it, and thus health complications (including morbidity) resulting from the buildup of gas/slime could be greatly reduced. Additionally, nearly 25% of methane production is currently produced by methanogen fermentation within cows; the activity of methanogens could be limited by feeding cattle more intact protein, reducing the amount of methane contributing to global warming. Further studies are required to confirm this (Hook, 2010). A follow up study is underway to look at the impact of post-harvest treatments of alfalfa and the impact on the protein component. Additionally, the impact of harvest time on the protein profile will be evaluated.

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Written by	Alisa Smovzhenko
Approved by	Prof. B Pam Ismail

APPENDIX E

PPIC Literature Review - Alfalfa for Human Consumption

Plant Protein Innovation Center	
Project Title	Utilization Opportunities for Alfalfa in Human Nutrition
Project Lead	Karthik Pandalaneni
Writers	Shreevats Mor and Karthik Pandalaneni
Reviewers	Abbie Krentz and Pam Ismail
Date	July 21st, 2022

Utilization Opportunities for Alfalfa in Human Nutrition

1. Introduction

The global population is expected to reach ~ 10 billion by the year 2050 (Henchion et al., 2017). This will result in an inevitable increase in the demand for food. Currently, the majority of the proteins in our diets come from animal sources such as fish, poultry, meat, and dairy. These animal sources account for 62% of the United States population's total protein intake (Górska-Warsewicz et al., 2018).

There is a general perception that animal foods have a much higher protein-to-energy conversion ratio, a better digestibility score, and a larger proportion of essential amino acids, compared to most plant protein sources. However, interest in plant proteins is constantly growing because they can be a potential solution to food security and sustainable food production for the future. In addition, plant-derived proteins are associated with lower levels of greenhouse gas emissions, lower land use requirements, and the ability maintain soil quality with smart crop rotation practices. Plant-based foods are also rich in fiber, vitamins, and minerals that promote a healthier lifestyle by providing foods with a lower calorie count and reduced levels of saturated fat, cholesterol, and sugar. These characteristics contribute to reducing the chances of chronic illness. Lastly, plant-based ingredients may be cost advantaged

relative to traditional animal sources and are accessible to a larger population, from a dietary restrictions and/or availability point of view (Henchion et al., 2017).

Among the novel plant protein sources, alfalfa is gaining interest. In 2021, the global alfalfa market was valued at US$21.6 billion, and is expected to grow at a compound annual growth rate of 7.2% to US$35.2 billion in 2028 (Fortune Business Insights, Paragraph 1). It is the fourth most widely grown crop in the United States with almost 26 million acres of land allocated for alfalfa hay (Bauchan, 2020). Alfalfa has broad economic value and is widely used as feed to improve milk and meat production for the animal industry. The entire plant, including leaves and seeds, is used as animal feed and is sometimes processed as an ingredient or dietary supplement for human consumption. Alfalfa is a rich source of nutrients, which could contribute to human nutrition. While there has been research done on the use of alfalfa, most of it has been focused on animal nutrition and its use as forage. There is a need to understand the utilization opportunities of this plant for human nutrition and its potential as a replacement for some of the traditional protein sources. Additionally, research is needed to characterize the functionality of alfalfa as a plant protein for nutritious food applications. This review is focused on current applications and will explore future opportunities to utilize the whole alfalfa plant including grain, leaves, juice, and extract in human diets. Additionally, this review will address some of the hurdles to using alfalfa in human diets, such as its anti-nutrients and sensory properties.

2. Animal feed research as it applies to human nutrition

Alfalfa is one of the most versatile crops with uses in the livestock industry, the food industry, and the medical industry. In the livestock industry, it has been used for pasture, hay, and silage because of its superior nutrient composition compared to other legumes. Alfalfa contains an excellent amino acid balance, with sufficient quantity of lysine, which is usually the first limiting amino acid in most grains (Thacker & Kirkwood, 1992).

In the past few years, research has been conducted on incorporating alfalfa into human nutrition because of its low caloric content and rich composition of minerals, vitamins, β-carotene, and the eight essential amino acids (Apostol et al., 2017). Alfalfa has been used as a medicinal herb to reduce cholesterol and blood pressure, treat arthritis and kidney stones, increase breast milk production, boost the immune system, and also as an antioxidant and a diuretic (De Leo et al., 1998; Malinow et al., 1980, 1981; Rana et al., 2010; Sadeghi et al., 2016). Unfortunately, most of these reported health benefits have only been researched in animals and further investigation is required for humans.

2.1 Factors affecting the quality of alfalfa

2.1.1. Overall composition of Alfalfa

Hay quality is assessed primarily by its percent dry matter (DM), percent crude protein (CP), acid detergent fiber (ADF) level, and neutral detergent fiber (NDF) level. Low ADF and NDF values are desirable to obtain high-quality hay (Marsalis et al., 2009). Alfalfa hay satisfies most requirements of good quality hay because of its composition. It is high in CP, which supports animal protein needs and reduces the need for supplementation unlike other types of feed. It is also high in vitamins and minerals, and contains relatively low fiber when harvested in the pre-

bud stage. Plant maturity at harvest has a major impact on the total digestible nutrients of alfalfa. The pre-bud stage at harvest is the most beneficial since it has the highest CP and lowest ADF levels. Alfalfa cultivars can differ in their yield and quality characteristics such as dry matter yield, leaf-to-stem ratio, CP content, ADF and NDF levels, and relative feed value. Relative feed value is estimated using digestible dry matter and dry matter intake (Avci et al., 2018).

Alfalfa leaves are nutrient-rich when compared to other parts of the plant. Alfalfa leaves are composed of 260-300 g/kg protein (dry basis) when compared to stems that have only 100-120 g/kg protein (dry basis) (Hojilla-Evangelista et al., 2017). A major fraction (65%) of alfalfa leaf proteins consist of ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO, E.C. 4.1.1.39) (Kobbi et al., 2017). RuBisCO is a globular protein and is the main soluble protein in alfalfa juice (Lamsal et al., 2007). Among the four forms of RuBisCO (I, II, III, IV), form I is the one usually found in plants, algae, and cyanobacteria (Di Stefano et al., 2018). This form has eight large subunits and eight small subunits, with most amino acids important for catalytic activity in the large subunit (Di Stefano et al., 2018). It can be purified from the soluble leaf concentrate fraction by heat, pH, or organic solvent precipitation, which allows for protein fractionation as well as the removal of some of the undesired green color (Tamayo Tenorio et al., 2016).

2.1.2. Alfalfa amino acid composition and protein digestibility

Alfalfa is not only high in overall CP but also is a good source of essential amino acids, namely lysine, leucine, valine, isoleucine, phenylalanine, and non-essential amino acids like proline. Whereas, methionine was reported as the only limiting amino acid in alfalfa (Taha et al., 2019).

Apart from amino acid composition, another determining factor for alfalfa protein quality is its digestibility. For ruminants, alfalfa is highly valued not just because of its nutrient content,

but also because a large proportion of its protein composition escapes rumen degradation. This minimizes the need for additional protein supplementation (Maheri-Sis et al., 2008). However, it was also suggested that the factors limiting the use of alfalfa for dairy cattle are the low digestibility of the lignified plant cell walls as well as any protein degradation that can occur during ensiling. While the protein from alfalfa can escape ruminal degradation, there are a few approaches that make the escape more efficient, so that nitrogen utilization is improved as well (Getachew et al., 2006). One approach with appropriate feed preservation methods is the optimization of rumen microbial growth for better microbial protein synthesis. Another option is the optimization of the concentration of tannins in the feed to control protein degradation. Based on these studies, the common observation for alfalfa is that as the plant maturity progresses, the plant yield increases but the nutrient composition and digestibility decrease.

A few studies have highlighted the benefit of adding alfalfa as a protein supplement in the feed due to its highly nutritious composition. A study compared the effects of replacing Bermuda grass with alfalfa hay for sheep and concluded that there were no significant effects on the digestibility of nutrients, although a small decrease in digestibility of crude protein and NDF was observed (Da Silva et al., 2017). This study also promotes the use of alfalfa as a protein supplement, since it increases feed intake and provides the animals with sufficient nutrients to promote weight gain. Similar observations were made in another study when barley was supplemented with alfalfa (Haddad, 2000). Researchers observed an improvement in the feed quality in terms of DM, organic matter, CP, and NDF. Ensiling alfalfa with other plants like corn or sorghum leads to better silage quality and improved crude protein content (Broderick, 2018).

There is a wide variety of published data comparing different feed types to alfalfa. Some studies reported alfalfa improved digestibility by supplementation in a few types of feeds, while other studies showed the opposite with deteriorated digestibility in livestock. Overall, a common observation among all these research studies can be made; alfalfa supplementation/ alfalfa hay improves feed intake and is extremely high in its nutrient composition. However, alfalfa supplementation increases NDF content which is the primary reason for lower digestibility. Therefore, while there might be a few drawbacks in terms of digestibility, alfalfa presents a high nutrient potential for animal and human diets.

2.1.2. Maturity and Leaf to stem ratio of the plant

The most important factor among plant management techniques is being able to determine the maturity of the plant at the time of harvest. As the plant matures, the leafiness of the plant decreases, and the stem-to-leaf ratio increases, leading to an overall decrease in the digestibility of the plant (Akin et al., 1977; Getachew et al., 2006). While the leaves have higher nitrogen content, the stems constitute most of the plant matter in mature plants. Plant maturity also affects the protein composition and availability in the plant. In the early stages, alfalfa contains more soluble and rapidly degradable protein fractions. As the plant matures, changes in the cell wall matrix occur making the protein harder to access and resulting in a less degradable protein (Getachew et al., 2006). The higher concentration of nutrients and intake value of alfalfa leaves compared to the stems makes it desirable to have high leaves to stems ratio at the time of harvesting (OECD, 2005; Avci et al., 2018). The relative weight of leaves to stems is an important factor to determine the quality of alfalfa during harvesting. A leaf percentage in the range of 55-65% with a leaf/stem ratio of 1.22-1.85 is considered very high-

quality alfalfa, whereas a leaf percentage of 35-45% with a leaf/stem ratio of 0.53-0.82 is considered lower quality.

Plant maturity affects the alfalfa protein digestibility in animals. Alfalfa nitrogen digestibility in horses increased with decreasing plant maturity (Woodward et al., 2011). When alfalfa is compared to other grasses, it was found that alfalfa had a higher soluble dry matter and a high extent of dry matter degradation (Elizalde et al., 1999). Dry matter is the feed material remaining post water removal. Having a higher soluble dry matter indicates better nutrient solubility for the animal.

2.1.3. Processing

Alfalfa is processed into chopped hay, cubes, or pellets to use as animal feed. Processing fresh alfalfa into silage leads to an increase in saponin concentration, which affects the palatability and availability of nutrients for poultry and animals (Kalač et al., 1996). In moist climates where field drying is a challenge, alfalfa is processed into silage for preservation. Drying alfalfa inhibits mold growth and reduces moisture levels. Therefore, dried alfalfa is less likely to undergo spontaneous heating that could result in undesirable Maillard reactions and the production of toxic metabolites (Coblentz et al., 1998). Both field and industrial drying are often practiced to dry alfalfa. Field drying can result in 30-40% of total solid losses. On the other hand, industrial drying at high temperatures can result in loss of alfalfa quality due to the enzymatic degradation of a large proportion of CP into soluble non-protein nitrogen (Siles et al., 2015). Additionally, the quality of the silage becomes significantly lower because of a decrease in water-soluble carbohydrate content, a decrease in pH with high buffering capacity, and an increase in proteolysis during ensiling. Another drawback of processing alfalfa into the dry form

is the impact this process has on the antinutrient properties of the plant, especially saponins (Szumacher-Strabel et al., 2018). In a similar study, alfalfa leaf meals were dried at 75°C or 150°C and followed by alkaline protein extractions. It was determined that the meal dried at a higher temperature was not suitable for leaf protein production (Hojilla-Evangelista et al., 2017). Therefore, alfalfa processors have to optimize the drying process and parameters to obtain high-quality alfalfa with minimum loss in yield and in functional proteins that can be used in food applications.

2.2. Benefits of alfalfa supplementation in animal feed

Alfalfa supplementation in feed improved the quality of the two most consumed animal products i.e., eggs and meat (Products, 1988). The effect of the addition of alfalfa protein concentrates in chicken feed and noticed a change in the quality of eggs (Grela et al., 2020). While most characteristics were similar in eggs from chickens fed with and without alfalfa concentrate, egg yolks from chickens receiving alfalfa concentrates had a higher polyunsaturated fat level and a darker yolk color, which was more appealing. Similar improvements in the yolk color, fat, and cholesterol content were also seen in other studies where alfalfa feed and alfalfa supplementation were compared with a soybean meal in chicken feed (Grela et al., 2014; Kocaoglu Guclu et al., 2004; Laudadio et al., 2014).

Natural alfalfa extracts also proved to have positive effects on meat quality because of their antioxidant properties. Adding alfalfa extracts to chicken feed led to increased feed intakes and better breast meat quality in broiler chickens (Dong et al., 2011). Overall improvement in the meat quality of rabbits, lamb, and chickens in terms of visible color changes in meat, reduction

in shear force, pH improvement, reduced cholesterol levels, and maintenance of the original texture profile was observed when alfalfa meal was used instead of other traditional feeds (Alhidary et al., 2016; Dabbou et al., 2018; Dal Bosco et al., 2014; Ouyang et al., 2016; Ponte et al., 2004). One common factor in all these studies was that the increase in the flavonoid level led to an improvement in the meat quality. These results also suggest that flavonoids are not only beneficial but also essential for feed protein conversion when used as feed for livestock.

While alfalfa is recognized as a premium forage for ruminants and animals like horses and rabbits, it is not the best option for monogastric animals (including humans), due to low protein digestibility, low digestible energy, high fiber, presence of saponins and phenolics, and low palatability (OECD, 2005). Further research is needed to overcome these challenges to make it a desirable protein ingredient and supplement for human consumption.

3. Challenges in using alfalfa as a protein ingredient for human consumption

Alfalfa has been emerging as a promising source of high quality protein, minerals, and vitamins. However, there are a few hurdles that restrict the use of alfalfa as a protein ingredient suitable for human consumption. The food industry needs to address these challenges when using alfalfa, while considering post-harvest treatments, upstream and downstream processing and product development.

3.1. Antinutritional factors

Antinutritional factors are divided into four categories – factors affecting protein utilization and lower digestibility (protease inhibitors, tannins, saponins, lectins, etc.); metal ion scavengers (oxalate, phytate, gossypol pigments, glucosinolates, etc.); antivitamins (antivitamin

A, antivitamin E, antivitamin D), and other factors (mycotoxins, nitrates, alkaloids, isoflavones, etc.) (Makkar, 1993). Each of these has a different effect on the overall nutrient composition of alfalfa, which is discussed in the sections below.

3.1.1. Saponins

Saponins are the major anti-nutrients present in alfalfa that hinder the digestibility of protein. The function of saponins in plants is to protect them from insect predation due to their toxicity (Sen et al., 1998). Saponin content in the alfalfa plant is influenced by several factors such as the variety, maturity of the plant, and the number of leaves on the plant (Sen et al., 1998). Several studies have reported that seeds and leaves have higher saponin content than stems and flowers. Additionally, immature plants contain more saponin than mature plants. Fenwick & Oakenfull (1983) estimated that alfalfa contains up to 56 g of saponins per kg of dry material. Whereas alfalfa sprouts contain 87 g saponins per kg of dry material, and prepared food after cooking with sprouts contains only 12 g per kg.

Alfalfa saponins exist as a diverse group of compounds. They are a complex mixture of triterpenic pentacyclic glycosides with the most abundant ones being medicagenic acid, hederagenin, zanhic acid, and soyasapogenol (Szumacher-Strabel et al., 2018). Depending on the structure, length, and composition of side chains, saponins present different biological activities such as hemolytic and antimicrobial activities, fungicidal activity, enzyme inhibition, and nutrient absorption (Cheeke, 1971). Alfalfa saponins also affect cell membrane permeability and nutrient absorption in the gut. Consequently, this affects the growth of livestock. In addition, alfalfa saponins have hypocholesterolemic and other biological effects that can cause toxicity to animals (Price et al., 1987). Saponins bind to nutrients, causing a

reduced utilization and absorption of these nutrients. Saponins also inhibit digestive enzymes such as amylases and proteases (Thompson, 1993). Hegsted & Linkswiler (1980) compared the protein quality of alfalfa protein concentrates (APC) with different levels of saponins. According to this study, APC with low levels of saponin had a higher CP percent and higher protein digestibility. Saponins are also responsible for reducing microbial protein synthesis. As a result, saponins consequently can cause bloating in ruminants, growth retardation, and blood plasma cholesterol reduction in non-ruminants (Sen et al., 1998). Apart from their effect on nutrient absorption, saponins also impart a bitter taste, which leads to reduced feed intake and decreased weight gain in animals (Thompson, 1993).

On the positive side, saponins do have some health benefits. Saponins have anticarcinogenic characteristics as they bind to cholesterol and bile acids, thus preventing bacterial reactions and tumor formation (Thompson, 1993). When alfalfa saponins were fed to chickens, monkeys, and rats, the cholesterol levels decreased, indicating a potential to reduce the risk of heart disease. However, more research is needed to understand the impact of saponins present in alfalfa on humans.

3.1.2. Condensed Tannins

Tannins are phenolic compounds that form complexes with proteins and enzymes. The ability of tannins to bind to proteins and enzymes (which are also proteins) depends on the plant species and the plant maturity. Tannins are found in various parts of the plant, most commonly in the leaves, roots, and seeds (Hoard et al., 1998). Since tannins are extremely diverse compounds, their effects are highly dependent on their concentration. Similar to saponins, tannins have both negative and positive effects in ruminants. Studies suggest that tannins can

cause significant negative effects such as a reduction in the uptake of proteins, carbohydrates, amino acids, minerals, and vitamins (Makkar, 1993). These studies have also suggested that as the concentration of tannins in the feed increases, feed intake by animals decreases. This observation is due to the insolubility and decreased digestibility of complexes formed, the palatability of the feed, and the presence of other compounds secreted during mastication. Other negative effects include decreased fiber digestion and nitrogen fraction uptake by the animals (McLeod, 1974). Tannins also lead to the inhibition of necessary digestive enzymes when present at concentrations higher than the beneficial levels (Getachew et al., 2006). Some studies have suggested that a tannin dietary concentration higher than 50 g per kg leads to detrimental effects on protein uptake (OECD, 2005). In addition, tannins are also linked to increased risks of mouth and esophagus cancer (Thompson, 1993).

Not all tannins, however, have the same effects. A limited number of studies have suggested that tannins can produce beneficial effects in ruminants such as better protein utilization, higher milk yield, and better fertility (Mueller-Harvey, 2006). Another review reported that condensed tannins reduce pasture bloating in animals and improve the conversion efficiency of plant protein to animal protein due to their metal chelator and antioxidant properties (OECD, 2005). A low to moderate tannin concentration of 4-5% is desirable and improves protein utilization (Hoard et al., 1998). At moderate concentration, tannins are also helpful in lowering starch digestion rates and therefore blood glucose levels (Thompson, 1993). The function of condensed tannins in inhibiting protein degradation is also important for ruminants. This is necessary as forages must be able to meet protein requirements by providing both degraded crude protein for microbial synthesis and protein

that escapes ruminal degradation (Broderick, 1995). Rapid alfalfa protein degradation in the rumen can also cause significant bloating (Getachew et al., 2006). Hence, the ability of tannins to inhibit protein breakdown is beneficial by allowing minimal degradation in the rumen and preventing bloating. However, more research is required to confirm that these compounds can be used therapeutically.

Depending on the type of tannins, different processing techniques such as the addition of tannin binding polymers, alkaline treatments, and processing in silages can be opted to mitigate the negative effects of tannins (Mueller-Harvey, 2006).

3.1.3. Phytoestrogens

In alfalfa, the major fraction of phytoestrogens consists of coumestans in concentrations ranging from 2.99 to 104.37 ppm (OECD, 2005). Similar to many other antinutrients, the concentration of phytoestrogens is determined by plant maturity, with relatively lower concentrations present during the early stages of the plant (Seguin et al., 2004). Harvesting before complete maturity is the stage at which alfalfa plants have to be harvested for animal forage to make them desirable as animal feed. The concentration of some phytoestrogens is also dependent on the part of the plant in which they are present. Flowers contain higher concentrations than the leaf and stem (Seguin et al., 2004). However, coumestans are found to be present in similar concentrations in the remaining parts of the plant, including the roots and seeds. Phytoestrogens are believed to have infertility effects on ruminants, especially sheep, and could also be carcinogenic at high concentrations (Adams, 1995; Thompson, 1993). Similar detrimental effects were also observed in the developmental stages of rats (Casanova et al., 1999).

Alfalfa does contain several other types of health-promoting phytoestrogens. Just like saponins, phytoestrogens are also linked to reducing blood lipid levels and therefore decreasing the chances of coronary heart disease in animals (Thompson, 1993). However, more research is required to confirm the negative and positive effects of alfalfa phytoestrogens on ruminants and potentially humans.

3.1.4. Other secondary metabolites

In addition to the aforementioned antinutrients, alfalfa contains a secondary metabolite that is also a potentially toxic compound called canavanine (OECD, 2005). Canavanine is also widely present in most legumes. L-canavanine is usually found in the seeds, cotyledons, and emerging shoots of alfalfa (OECD, 2015). Currently, there is limited information available on the effect of canavanine in the feed when compared to other secondary metabolites in alfalfa.

3.2. Effect of alfalfa on sensory properties of food

Key sensory properties such as color, taste, flavor, and mouthfeel are evaluated to predict consumer acceptance of an ingredient in food applications. One of the major drawbacks of using alfalfa for human consumption is its impact on the sensory characteristics of food. Opportunities to use alfalfa in food applications have been explored by few researchers. Alfalfa extracts in tofu resulted in an increase in protein content, but a decrease in moisture, pH, and tofu yield (Kim et al., 2012). In general, sensory properties such as color, hardness, and taste of the tofu with alfalfa extracts were perceived negatively. There were also studies regarding the sensory properties of gluten-free grain-based cookies prepared with alfalfa seed flour as a nutritional additive (Giuberti et al., 2018; Ullah et al., 2016). The nutritional properties of the

final products were improved, but sensory properties were inferior. The gluten-free cookies had less acceptable flavors and a reddish/ yellowish color, which is not typical of baked products. Additionally, the cookies had high hardness values due to the higher protein and fiber content. These results suggest that the sensory properties of products with alfalfa need to be improved to gain consumer acceptability.

Research targeting the mitigation of off-flavors was done primarily in applications with soy protein. These studies have concluded that off-flavors are limited by maximizing the removal of residual lipids during the extraction process of soy protein (Wu et al., 2011). This off-flavor mitigation approach has mostly focused on soy protein ingredients and products, thus research is required to adopt this and other approaches to improve the sensory properties of alfalfa protein ingredients and products.

4. Whole alfalfa plant applications as human food

While the whole alfalfa plant has a long history of use as animal feed, only a few parts of the plant have proven to be edible by humans. This section explores the application opportunities of the alfalfa plant and its derived ingredients for human consumption.

4.1. Sprouts and Seeds

In human nutrition, alfalfa is popularly used in the form of sprouts. Alfalfa sprouts and are high in Vitamin B, C, and K, and saponins, which help in lowering cholesterol levels. During sprouting, the naturally occurring anti-nutrients and enzyme inhibitors breakdown, and the concentration of minerals, vitamins, antioxidants, and lectins increases (Chavan & Kadam, 1989; Fahey et al., 1997; Gupta, 1994; Li & Zhang, 2013; Sreenivasan & Wandrekar, 1950; Tang et al., 2014;

Vadivel & Biesalski, 2012). The nutritional profile of alfalfa sprouts and seeds is further improved by a drying process called "*détente instantanée contrôlée*" (DIC) process or "controlled instantaneous pressure release" (Plaza et al. 2003). During the DIC process, the product is heat-treated to temperatures below 200 °C for less than a minute under pressure. This is followed by releasing the pressure abruptly causing quick cooling and massive evaporation. Because of the short duration of the heat treatment, thermally sensitive biological compounds are preserved in the alfalfa seeds and sprouts. This results in significantly higher Vitamin A and C content.

4.2. Alfalfa Protein Concentrates

Alfalfa protein concentrates (APCs) contains 45 - 60% protein along with low levels of fat, polysaccharides, minerals, and vitamins (Bresson et al., 2009). However, APCs contain many antinutritional factors like saponins, phytates, and secondary metabolites. However, their composition varied during different phases of processing such as chopping, grinding, and heating. Bresson et al. (2009) concluded that APC consumption for humans is completely safe at the recommended intake of 10g per day.

4.3. Leaf Protein Concentrates

Alfalfa leaf protein concentrates (LPCs) have a high potential for human consumption due to their high RuBisCO content and associated amino acid composition (approximately 65%) (Apostol et al., 2017; Li & Zhang, 2013; Livingston et al., 1984; Zhang et al., 2017). LPCs contain certain anti-nutrients such as phenolic compounds and flavonoids, yet these do have beneficial attributes such as antioxidative and anti-inflammatory properties when consumed in moderate amounts (Karimi et al., 2013). In addition, alfalfa LPCs have been shown to cure symptoms of

protein deficiency diseases such as kwashiorkor (Knuckles & Kohler, 1982). Alfalfa leaves are widely used as supplements for low protein/low lysine diets in children and have biological and digestibility values very close to other commonly used meals such as soybean and sunflower (Ghaly & Alkoaik, 2010). Alfalfa leaf protein extraction can occur via a variety of different methods, including mechanical tissue disruption, pH, heat, or salt-induced protein precipitation, and ultrafiltration (Tamayo Tenorio et al., 2016; Zhang et al., 2017). A few research studies have attempted to extract RuBisCO from the alfalfa leaves. One particular study investigated RuBisCO extraction from the juice of alfalfa leaves (Kobbi et al., 2017). RuBisCO was successfully extracted with a protein yield of 69% and a protein purity close to 90%. This study highlights a novel, simple and sustainable method of RuBisCO protein recovery and purification from alfalfa leaf green juice. The functional properties, such as solubility and emulsification properties, of alfalfa leaf RuBisCO are highly influenced by the extraction process used (Di Stefano et al., 2018). In general,alfalfa LPCs exhibited better emulsifying properties than soy protein concentrates (Wang & Kinsella, 1976). Additionally, LPCs had high solubility following alkali solubilization, with a progressive increase in solubility with an increase in extraction pH from pH 4.5-9, along with high emulsification properties (Di Stefano et al., 2018). Alfalfa LPCs, therefore, show a promising use in potential food applications due to their high protein content, nutritional quality, and desirable functional properties. However, to be suitable for human consumption, LPCs must be free of chlorophyll and off-flavor (Hernández et al., 1997). Additionally, the concentration of antinutrients such as saponins needs to be minimized to improve the nutritional profile. Further research into the nutritional benefits, functional

properties, and consumer acceptability of LPCs is required to promote them as an ingredient in various food applications.

5. Roadmap to increase the application of alfalfa in foods

In addition to addressing the knowledge gaps outlined in the earlier sections, increasing the use of alfalfa for human applications will also require continued research into processing technologies to optimize the extracted protein. Additionally, clinical studies need to be conducted to fully understand the physiological implications of alfalfa in human foods.

5.1. Advancements in protein extraction technology

Recent research advancements in processing techniques have increased opportunities to use alfalfa for human consumption. Determining the optimal protein extraction process is crucial to obtaining a high purity protein isolate with a high yield. The selection of the protein extraction process to follow is dependent on the composition of the starting material and the structural properties of the protein. The main goal of optimizing a protein extraction process is to recover protein with good quality, yield, and functional properties. The use of conventional protein isolation and purification steps to isolate alfalfa leaf protein results in the degradation of protein and a brown color leading to poor quality and recovery. To improve the quality of the LPCs, alfalfa leaves can be blanched immediately after harvest to inactivate polyphenol oxidases (PPO), polyphenol peroxidase (POD), and plant proteases. Hadidi et al. (2019) found that steaming for 4.36 min of 23mm leaves without cutting, at a maximum time of 2 hours from harvesting to blanching resulted in an LPC with reduced PPO and POD activity, and proteins with higher molecular weights. LPCs obtained from blanched samples exhibited lower browning

and protein degradation rates when compared to the control samples after 60 days of ensiling (Hadidi et al., 2019).

In addition to traditional protein extraction methods, membrane filtration was also researched as a method to isolate alfalfa protein to obtain LPCs of good quality. Membrane technology was suggested as an alternative to the thermo-coagulation process to preserve the alfalfa leaf protein structure and thereby improve its functional properties (Zhang et al., 2017). High-temperature protein extractions above the denaturation temperature of alfalfa leaf protein (57 °C) will result in protein denaturation, thus negatively impacting the protein quality. Hadidi et al. (2020) also explored a new ultrasound-ultrafiltration- assisted alkaline solubilization coupled with isoelectric precipitation (UUAAIP) process as an alternative to thermo-coagulation and alkaline isoelectric precipitation extraction of protein. This study resulted in an alfalfa LPC with improved average molecular weight protein, color, and improved solubility, water-holding, and oil-binding capacities. This ultrasound-assisted protein extraction was also efficient in removing saponins and phenolic compounds. However, emulsifying and foaming properties of the extracted LPC were inferior to the LPCs obtained using thermo-coagulation and alkaline isoelectric precipitation methods. Alfalfa extracts have been seen to have the highest solubility at pH less than 3 or greater than 7, depending on the extraction method, with increasing solubility when subjected to alkaline pH shift methods. These extracts also have better foam stability than egg whites and soy, around their isoelectric point (pH 4.5), as a result of less electrostatic repulsions. The extracts also had excellent gelation potential at neutral pH due to the low gelation temperature of RRuBisCO (Nissen et al., 2021). Further research is required to optimize protein extraction from alfalfa without intense heat and alkali

treatments, to avoid browning and off-flavors. Obtaining APC with enhanced functional and sensory properties improves its opportunity to be used as an ingredient in food applications.

5.2. Clinical trials

Historically, alfalfa has been used in treating many health issues in humans (Bora & Sharma, 2011). These issues include kidney pain, cough, diabetes, and memory loss. Alfalfa has also historically been used in Ayurvedic and homeopathic medicine. Leaf extract, specifically, has been used in the treatment of neuro vegetative menopausal symptoms in women, while seed extract has been used in the reduction of total cholesterol. Some studies have suggested that alfalfa may have digestive and bone health benefits (Mikaili & Shayegh, 2011).

Diabetes mellitus is one of the most common diseases among humans and the primary treatment to date involves the use of hypoglycemic drugs. However, in many parts of the world, alfalfa, specifically the leaves, are used as an herbal drug due to its protein, calcium, vitamin content, and lesser side effects compared to other drugs. Alfalfa leaves also have the ability to stimulate insulin secretion in rats, possibly because of the high levels of manganese (Amraie et al., 2015). Alfalfa saponins fed to rats inhibited cholesterol esterase, acetyl coenzyme, and carboxylase enzymes, resulting in the inhibition of fatty acid synthesis and an increase in the ratio of high-density lipoprotein cholesterol to low-density lipoprotein cholesterol. However, not enough evidence has been provided in humans.

Apart from this, there is ongoing research on the potential use of alfalfa genes in creating plant-based vaccines for both humans and animals, along with clinical trials in humans to cure diseases like rabies (Aguirreburualde et al., 2013; Takeyama et al., 2015). The use of alfalfa in

antibody production was also investigated. It was shown that alfalfa can rapidly produce clonal transgenic populations, making it an ideal choice for molecular farming (Busse et al., 2002).

Clinical trials conducted thus far show that not only is alfalfa safe for human consumption when consumed in moderation, but it also has multiple health benefits. With further human clinical trials, alfalfa use can be expanded in food applications and in medicinal products.

6. Conclusion

As the demand for protein increases, there will be a need for a wider variety of plant-derived protein sources. From this review, it is evident that alfalfa is a very promising emerging plant protein source for human consumption, with its high protein, vitamin, and mineral concentration and good functional properties. Currently, the applications of alfalfa in the industry are mostly limited to cattle feed. The challenges involved in increasing their utilization in various food applications include the presence of anti-nutrient factors like saponins and tannins along with color and flavor challenges. Additionally, post-harvest handling and processing need to be further explored to enhance the feasibility of producing functional and nutritional APC ingredient.

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APPENDIX F

SROC Swine Feed Trials

FINAL REPORT

Project No.

Contract No.

Study Title: Dietary evaluation of spray dried fermented alfalfa juice on nutrient balance, ileal digestibility, and performance of weaned pigs.

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Date: December 2021

Introduction:

Protein sources derived from animal tissues or products, despite high costs, have been used extensively in feeding nursery pigs because of their increased digestibility (Gottlob et al., 2006; Emer et al., 1994), and small concentrations of antinutritional compounds (Anderson and Wolf, 1995) compared with protein sources derived from plants. Recent concern about animal and meat safety has heightened potential uncertainty regarding feeding animal-tissue-derived ingredients to animals. Plant- derived ingredients still have limited application in feeding newly weaned pigs because of the complexity of nutrients and anti-nutritional compounds commonly present in typical plant-derived ingredients, both of which reduce nutrient digestibility to nursery pigs.

Objectives:

1.To determine the effect of titrated level of spray-dried fermented alfalfa juice on nutrient balance of nursery pigs.

2.To evaluate the effect of titrated level of spray-dried fermented alfalfa juice on ileal nutrient digestibility for nursery pigs

.

Materials and Methods:

Thirty-six ileal canulated pigs, average initial BW 20 kg were fitted with a simple T cannula at the distal ileum and fed four diets:

1. 0% Spray-Dried Alfalfa Juice (SDAJ)
2. 3% SDAJ.
3. 6% SDAJ.
4. 9% SDAJ

The ileal and the balance study was performed in a swine metabolic unit based on the 4 dietary treatments stated above. After surgery, pigs were covered with warm towels to provide thermal support. Pigs were given (IM) Carprofen at 3 mg/kg every 12-24 hours for 72 hours after the surgery as analgesic agent. The monitoring plan was daily for 10

days at which time all pigs are expected to return to normalcy. Additionally, all pigs were monitored daily for any adverse health issues. The pigs were allowed 10 days to recover after the surgery. There were two periods for collection of digesta samples. Each period was for 7 d with 5 d adaption and 2 d for collection periods. A wash-out period of 7 days was observed to prevent any potential gastric upset (Florence et al., 2018). The experimental diets contained 0.4% Celite (a source of AIA) as indigestible marker (Kim et al., 2017). Equal meals were provided at 0800 and 2000 h daily. Ileal digesta samples were collected from 0800 to 1630 h on each day during collection periods. Collection of digesta was 0800, 0830,1000, 1030, 1200, 1230, 1400, 1430, 1600 and 1630 h. There was no collection between 1700 to 0730 h. The cannulated pigs for ileal and nitrogen balance studies were housed in individual pens equipped with a feeder and a nipple drinker in a swine metabolic facility. The ileal and the balance study was terminated on d 32 and pigs did not enter the food chain. The SOP for the pigs in the metabolic unit was IACUC #1710B11921.

Table 1. Composition[1] of spray dried alfalfa

Dry matter (%)	89.93
Crude Protein (%)	37.95
Acid Detergent Fiber (%)	0.48
Neutral Detergent Fiber	0.88
Sugar (WSC) (%)	8.31
Starch (%)	0.58
Fat (EE) (%)	2.31
Ash (%)	19.75
Lysine (%CP)	3.53
Methionine (%CP)	0.76
Threonine (%CP)	2.32
Tryptophan (%CP)	0.55
Valine (%CP)	3.98
Calcium (%)	2.13
Phosphorus (%)	0.55
Magnesium (%)	0.71
Potassium (%)	7.32
Sodium (%)	0.12
Zinc (ppm)	71.00
Iron (ppm)	252.00
Manganese (ppm)	63.00
Copper (ppm)	16.00

[1]Laboratory analyses by DairyLand Laboratories Inc., Arcadia. WI

Table 2: Dietary composition and analyses of spray dried alfalfa diets					
Dietary Treatments	**1**	**2**	**3**	**4**	
Level of Spray Dried Alfalfa (%)	**0**	**3**	**6**	**9**	
Corn	63.27	59.42	55.40	51.37	**NRC 2012**
SBM	26	26	26	26	
Fish Meal	2	2	2	2	
Whey Powder	3	3	3	3	
Spray Dried Alfalfa	**0**	**3**	**6**	**9**	
Blended Animal Fat	0.5	1.62	2.74	3.86	
Monocalcium Phosphate	1.21	1.11	1.11	1.11	
Limestone	1.1	1.1	1	0.92	
Salt	0.3	0.3	0.3	0.3	
Vitamin- Mineral Premix	0.5	0.5	0.5	0.5	
Lysine	0.63	0.62	0.62	0.61	
Methionine	0.14	0.14	0.14	0.14	
Threonine	0.19	0.19	0.19	0.19	
Total	**100**	**100**	**100**	**100**	
Nutrient Analyses					
Net Energy, kcal/kg	2464	2428	2393	2357	**2412**
Crude protein (%)	21.4	21.56	21.72	21.88	
Calcium (%)	0.8	0.82	0.85	0.87	**0.8**
Total Phosphorus (%)	0.69	0.69	0.69	0.69	**0.65**
STTP (Phosphorus) (%)	0.44	0.44	0.44	0.44	**0.4**
SID Lysine (%)	1.37	1.38	1.38	1.38	**1.38**
SID Methionine (%)	0.39	0.39	0.39	0.39	**0.39**
SID Threonine (%)	0.84	0.85	0.85	0.85	**0.79**

Table 3: Digestible and Metabolizable Energy of Sprayed Dried Alfalfa Diets

	Inclusion of spray dried alfalfa, %				CV, %	SEM	P-value
	0	3	6	9			
Digestible energy, kcal/kg	2590.87	2525.45	2532.01	2457.26	34.76	34.76	0.643
Metabolizable energy, %	83.5 a	85.1 a	81.2 ab	75.8 b	0.01	0.01	**0.002**
Metabolizable energy, kcal/kg	2442.63	2386.89	2397.06	2313.95	33.36	33.36	0.625
Nitrogen intake, g/day	39.54	40.51	39.45	41.17	0.48	0.48	0.582
N in urine, g/day	2.20	2.34	2.26	1.76	0.15	0.15	0.537
Dry matter intake, kg/day	1.25	1.28	1.27	1.26	2.46	0.01	0.337
Apparent total tract dm digestibility, %	87.79	85.81	86.39	84.65	5.91	1.11	0.807

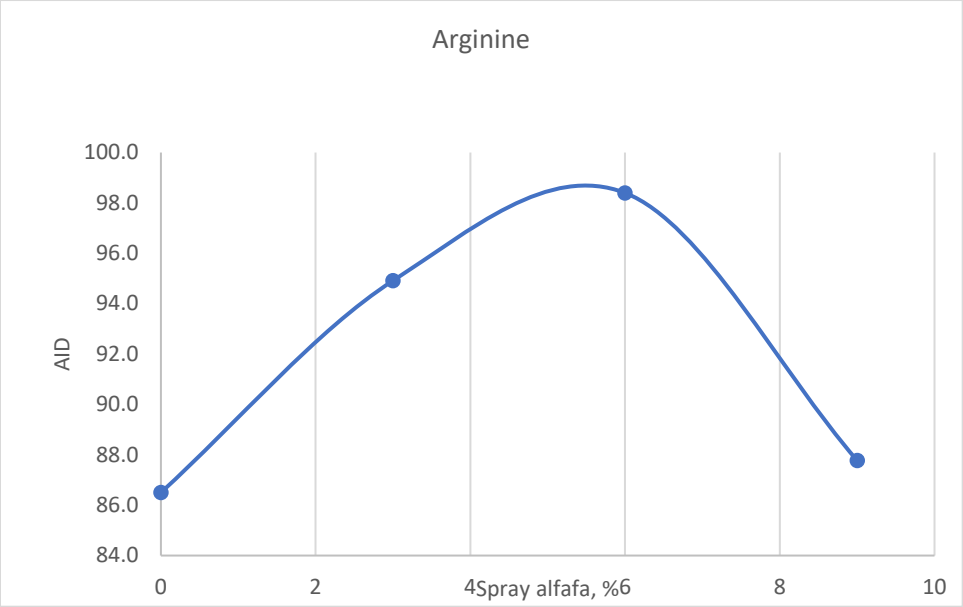
Increasing dietary level of spray dried alfalfa in the diet did not influence digestible energy. Metabolizable energy was significantly ($P<0.002$) reduced with 9% inclusion of spray dried alfalfa in the diet. Apparent total tract dry matter digestibility, nitrogen intake, nitrogen in urine were not influenced ($P>0.05$) by inclusion of spray dried alfalfa in the diet.

Table 4: Amino Acid Composition of Sprayed Dried Experimental Diets:

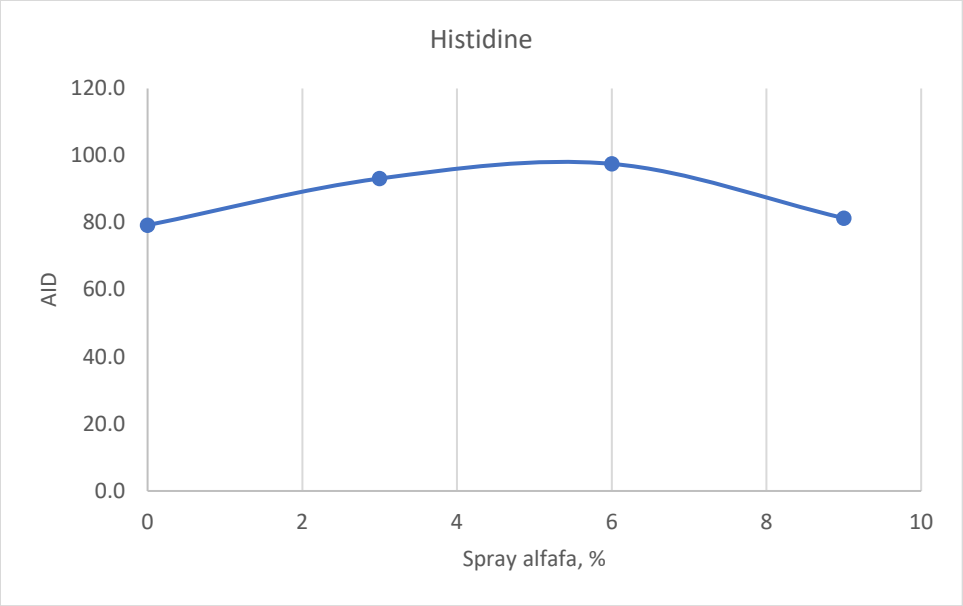
DIET	**Diet 1**	**Diet 2**	**Diet 3**	**Diet4**	
Level of Alfalfa	**0**	**3**	**6**	**9**	
Taurine	0.14	0.16	0.19	0.24	
Hydroxyproline	0.15	0.17	0.13	0.11	
Aspartic Acid	1.65	1.50	1.54	1.33	
Threonine	0.74	0.71	0.76	0.74	
Serine	0.63	0.62	0.65	0.54	
Glutamic Acid	1.99	1.86	2.38	1.83	
Proline	0.85	0.81	0.82	0.88	
Lanthionine §	0.02	0.03	0.04	0.02	
Glycine	1.15	0.89	1.19	0.94	
Alanine	0.99	0.87	1.00	0.83	
Cysteine	0.33	0.30	0.33	0.27	
Valine	0.85	0.75	0.82	0.65	
Methionine	0.19	0.16	0.19	0.16	
Isoleucine	0.70	0.61	0.66	0.51	
Leucine	1.18	0.99	1.08	0.96	
Tyrosine	0.42	0.40	0.45	0.37	
Phenylalanine	0.73	0.62	0.72	0.53	
Hydroxylysine	0.01	0.01	0.01	0.01	
Ornithine §	0.21	0.11	0.13	0.03	
Lysine	0.99	1.02	0.98	0.96	
Histidine	0.34	0.28	0.33	0.29	
Arginine	0.44	0.46	0.50	0.43	
Tryptophan	0.15	0.13	0.15	0.13	
Experimental diets for the sprayed dried alfalfa were formulated to contain the same number of amino acids.					

Table 5. Digestible coefficients of spray dried alfalfa						
Level of Sprayed Dried Alfalfa (%)	**0**	**3**	**6**	**9**	**SEM**	**p-Value**
Arginine	86.5	94.9	98.4	87.8	1.1	<0.0001
Histidine	79.3	93.2	97.5	81.3	1.7	<0.0001
Isoleucine	77.6	92.4	97.3	80.1	1.8	<0.0001
Leucine	78.8	93.0	97.5	82.3	1.6	<0.0001
Lysine	74.3	93.7	97.9	83.1	2.0	<0.0001
Methionine	87.4	95.7	98.4	88.4	1.0	<0.0001
Phenylalanine	78.6	92.6	97.3	80.1	1.7	<0.0001
Threonine	72.0	91.8	97.0	78.8	2.1	<0.0001
Tryptophan	78.8	93.7	97.6	82.0	1.7	<0.0001
Valine	74.4	91.6	96.9	76.8	2.0	<0.0001
Non-essential						
Alanine	69.0	92.7	96.6	79.0	2.6	<0.0001
Aspartic Acid	74.7	91.4	97.1	79.0	2.0	<0.0001
Cysteine	66.2	88.5	96.1	66.9	2.8	<0.0001
Glutamic Acid	80.7	93.1	97.6	82.3	1.6	<0.0001
Glycine	65.1	87.7	95.9	65.9	3.0	<0.0001
Proline	70.3	92.5	97.2	78.3	2.5	<0.0001
Serine	73.2	91.5	96.9	77.7	2.1	<0.0001
Tyrosine	78.6	92.7	97.4	80.7	1.7	<0.0001
Total	76.1	92.4	97.3	80.0	1.9	<0.0001

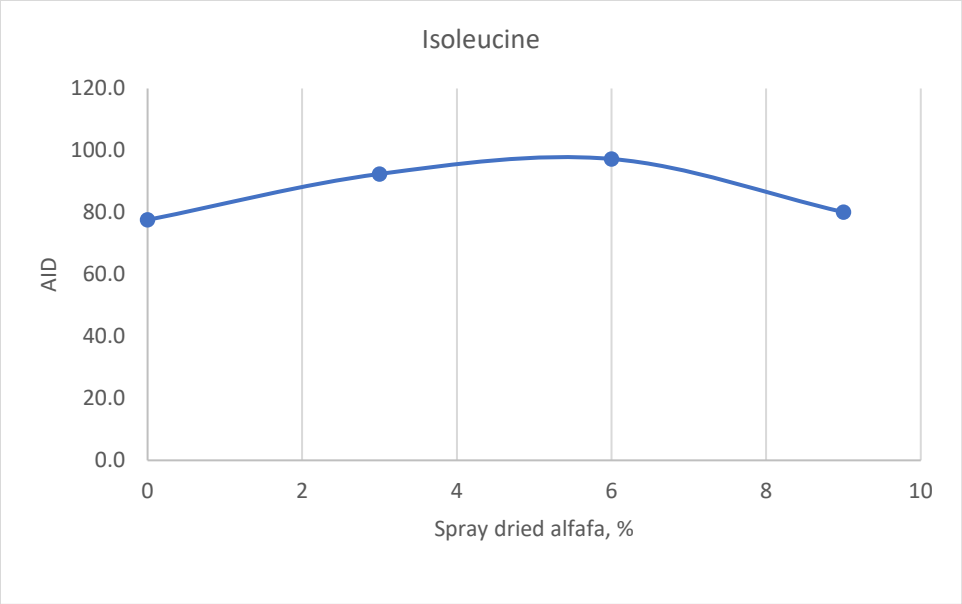
The digestible coefficients of all the amino acids decreased (P<0.01) with inclusion of Nine percent spray dried alfalfa inclusion to the diet.



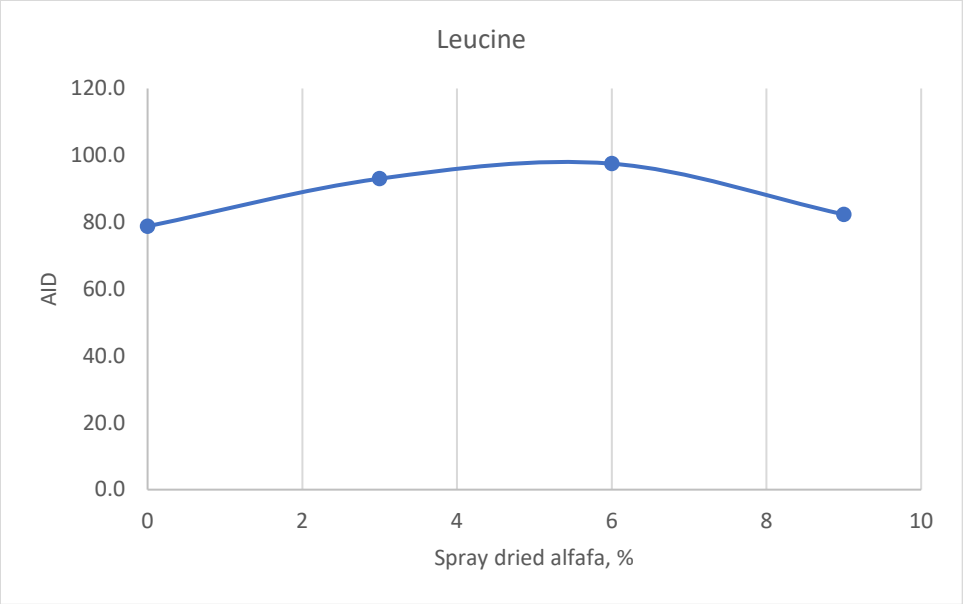
Apparent ileal digestibility of Arginine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



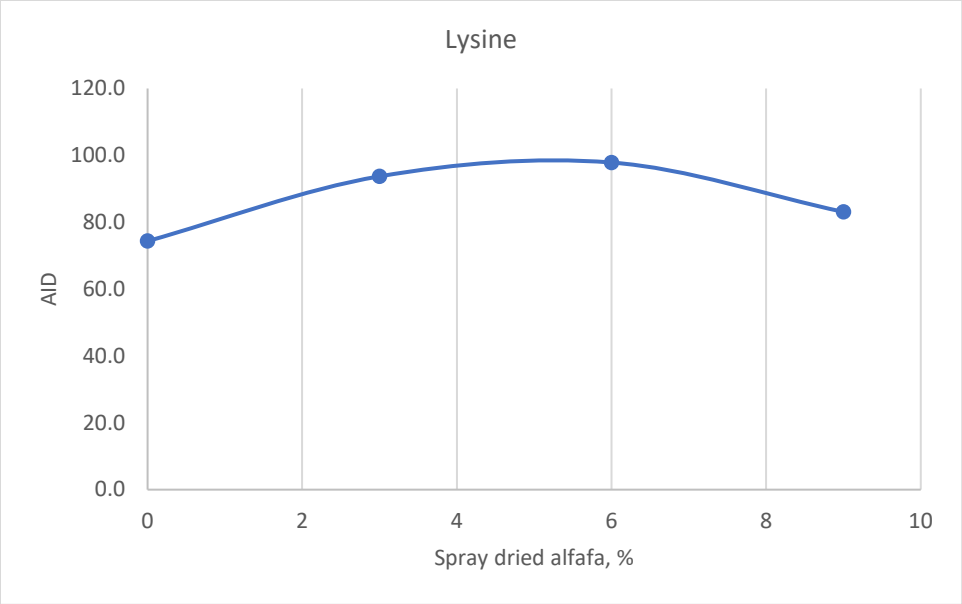
Apparent ileal digestibility of Histidine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



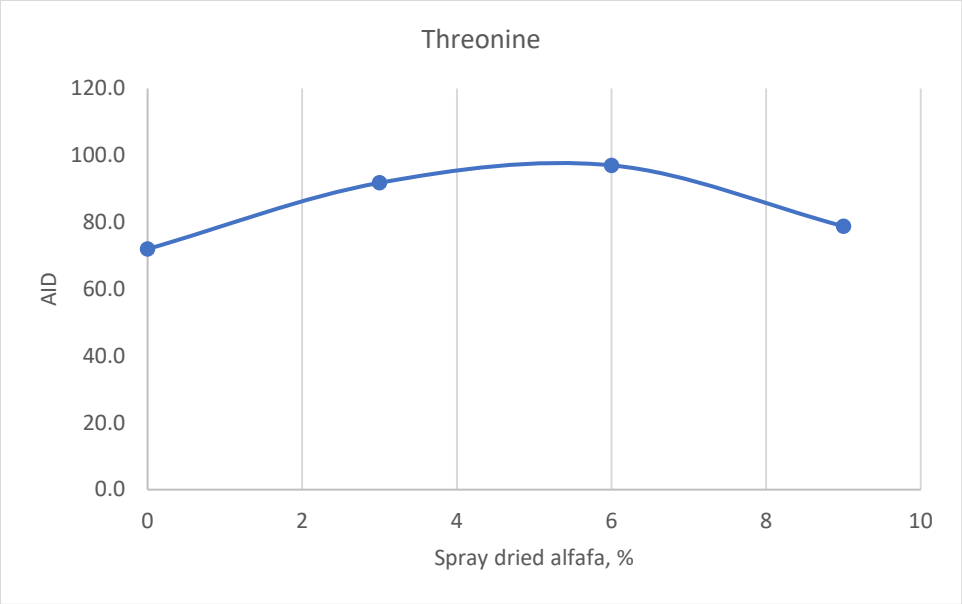
Apparent ileal digestibility of Isoleucine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



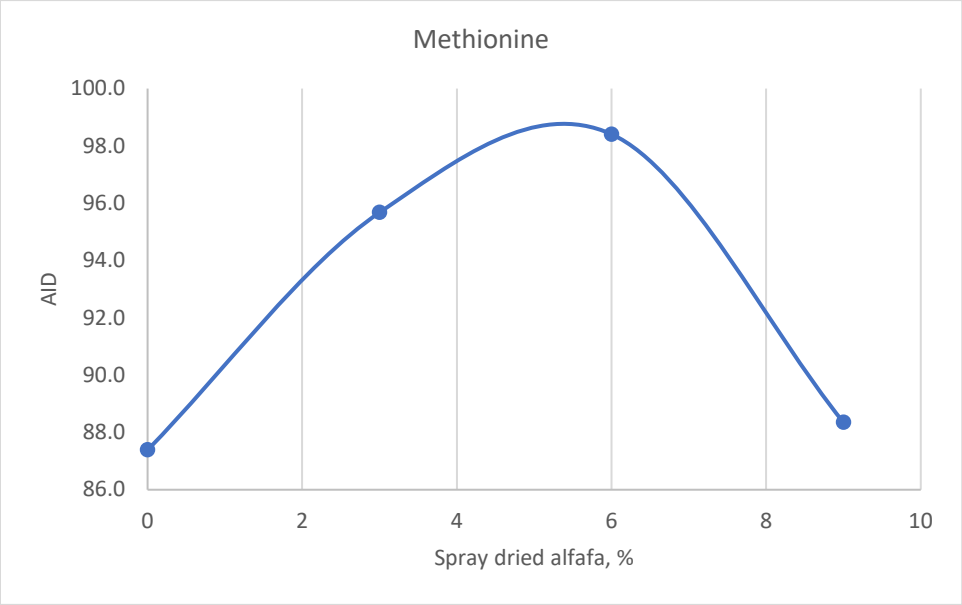
Apparent ileal digestibility of Leucine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



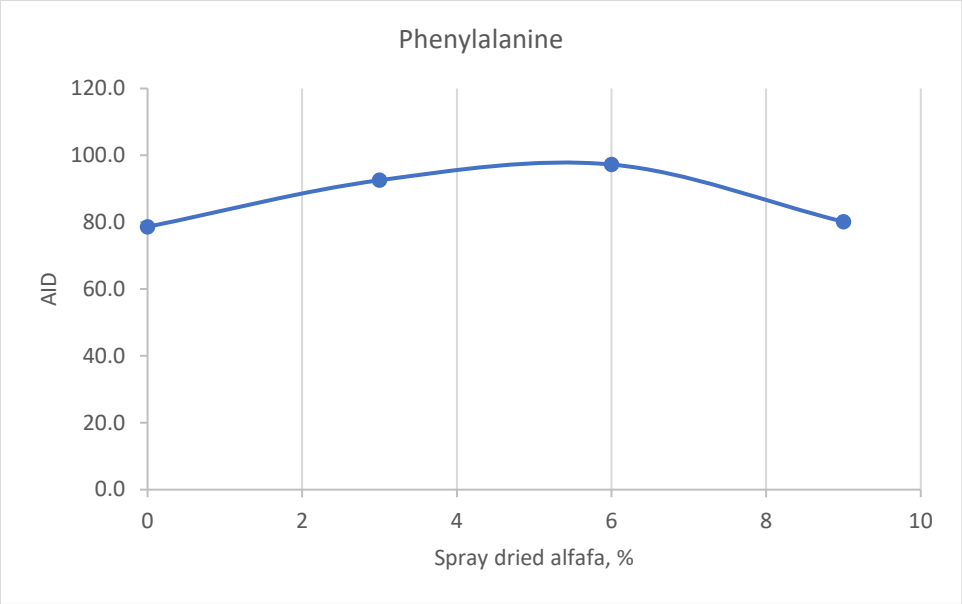
Apparent ileal digestibility of Lysine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



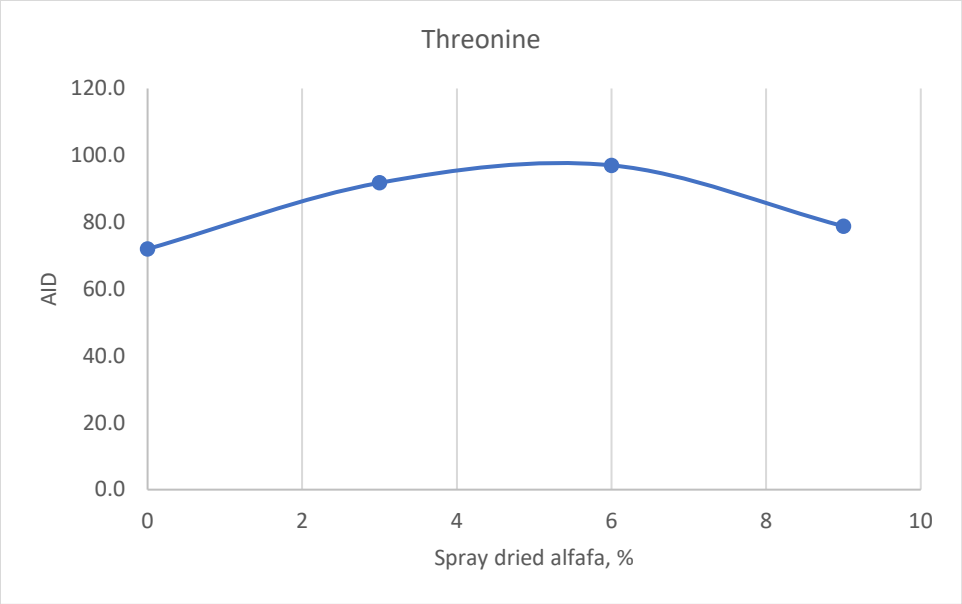
Apparent ileal digestibility of Threonine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



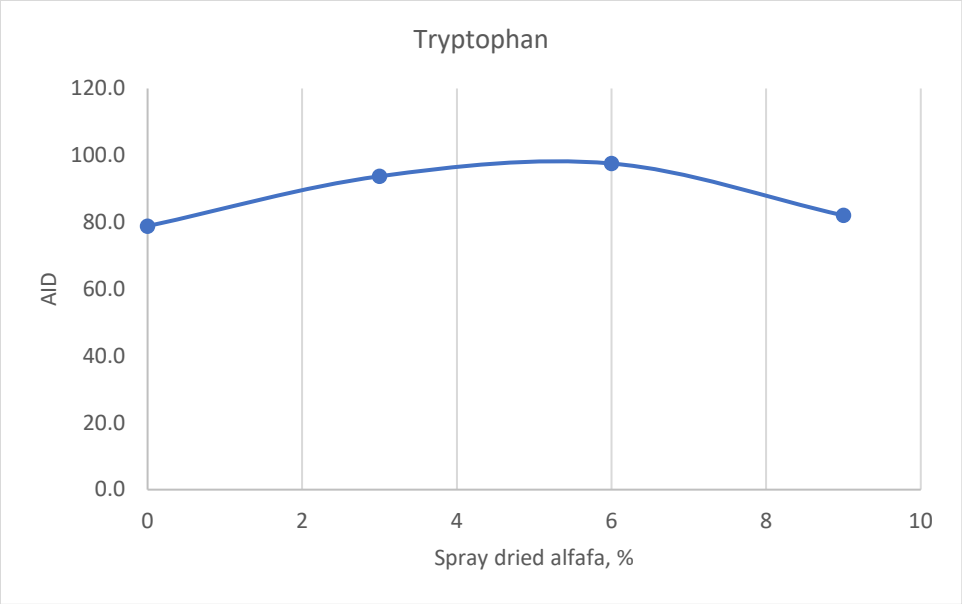
Apparent ileal digestibility of Methionine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



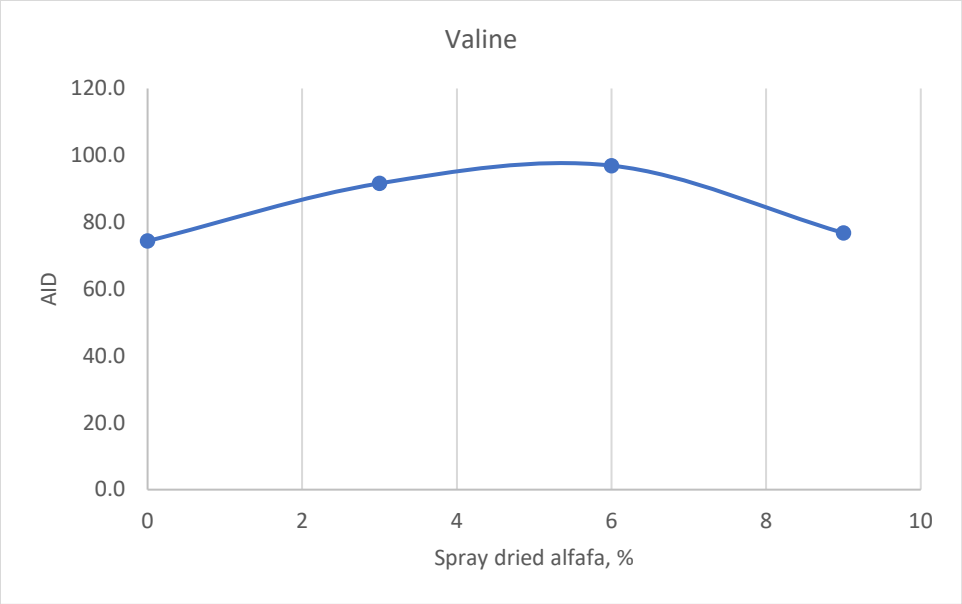
Apparent ileal digestibility of Phenylalanine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



Apparent ileal digestibility of Threonine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



Apparent ileal digestibility of Tryptophan in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.



Apparent ileal digestibility of Valine in sprayed dried alfalfa was significantly ($P<0.01$) improved at 6% inclusion rate.

Conclusion:

The study evaluated the efficacy of spray dried alfalfa in ileal digestibility in young pigs. The sprayed alfalfa was included in the diet at 0, 3, 6 and 9 percent of the diet. Digestibility coefficients for all the amino acids indicated that the optimum inclusion rate of spray dried alfalfa is 6 per cent. The overall ileal digestibility results of the present study shows inclusion of 9% spray dried alfalfa in young pig diets may influence performance. A pig nursery performance study could be performed to confirm the inclusion rate. Spray dried alfalfa could be equally efficacious to spray dried plasma, since the pig's performance were not affected with the experimental diets were formulated with no sprayed plasma. A nursery growth performance study to compare spray dried alfalfa and spray dried plasma will justify the replacement of spray dried plasma with spray dried alfalfa.

APPENDIX G

Supply Chain Phase 1 - Steve Olson Consulting

LCCMR Value-Added Alfalfa Commercialization Phase 1 Report
Prepared by Steve Olson Consulting, LLC
March 16, 2020

AURI's Scope of Work for this LCCMR project is to: Develop value-added processes and products for profitable alfalfa marketing focusing on three areas:

1. Implementation of advanced chopping & sealing mechanisms to reduce moisture-related spoilage and nutrient leaching of alfalfa due to rain;
2. New applications for alfalfa; and
3. Develop supply chain connections and identify new market opportunities through exploration, development and management of pilot scale projects with private businesses to test and commercialize new products and technologies.

Of the five outcomes for this project, this report focuses on #4) "Upgrade alfalfa leaf extract for use in non-ruminant and aquaculture feeds and identify and capture value-added opportunities in these sectors."

Summary

Phase one focused on defining the commercialization scope. Information gathered in Phase 1 confirmed potential success for value-added alfalfa product commercialization in monogastric animals (swine & poultry), aquaculture and nutraceuticals. Using a combination of literature reviews and conversations with experts in swine & poultry nutrition and agronomy alfalfa production we've learned:

- Saponins, a chemical compound found in alfalfa and legumes and other plants, may not be the barrier, as originally thought, to using alfalfa-derived products in monogastric animals, specifically swine & poultry.
- Low saponin alfalfa varieties exist; and
- Research literature indicates saponin may have cholesterol lowering capabilities and therefore human health uses
- According to the U of MN Extension "There are more than 1 million acres of alfalfa in Minnesota, but the goal of maximizing yield isn't always realized. The University of Minnesota Alfalfa Variety Trials reported average yields in the range of 6 to 7 tons per acre (dry matter) across all locations in recent years. However, the statewide average during that time hovered around 3 tons per acre of dry matter (Figure 1)" https://extension.umn.edu/planting-forages/alfalfa-establishment-steps-maximize-yield#alfalfa-yields-in-minnesota-702861

The following section identifies next steps and consideration for the primary components for commercialization:

1. Product Characteristics
2. Alfalfa Production
3. Alfalfa Processing
4. Product Distribution
5. Markets

Next Steps, Questions & Considerations

Product Characterization

1) Continue Literature Review
    i) Product Questions:
        (1) Saponin
            (a) Clarify – does saponin have a negative impact on mono-gastric?
            (b) Potential to use genetic engineering to pull out saponin
            (c) Does the chemical separation process damage or change saponin compounds?
        (2) What information is needed to evaluate potential market?
        (3) Is there an enzyme to break down i.e. Phytate/Phytase?
        (4) More measurement better to know how to use
            (a) Can it be measured consistently?
            (b) Can product attributes be measured in the field by alfalfa producers?
    b) Characterize product content
        i) Develop specification sheet
            (1) Analysis
                (a) Macro nutrients
                (b) Saponin, Glyphosate, Alkaloids levels
                (c) Xanthophyll levels
                (d) Soluble vs Insoluble
        ii) Include analysis for mold & mycotoxin
        iii) Compare to soybean, corn gluten & marigold
            (1) Ratio of Amino Acids to Crude Protein
        iv) Product Availability
    c) Prove efficacy on product & process
        i) Bench to scale up
2) Value proposition for crop producer
3) Value proposition for livestock producer

Production

- Growing region in MN – is there an optimal region or restrictions on production?
    - Currently MN farmers raise about 1 million acres of alfalfa, mainly in central MN. Yield ranges from 3 -7 tons/acre dry matter. Findings from this project could incentivize farmers to put more emphasis on maximizing yield.
- What is the value proposition for crop producer?
- Is there a concern for nitrate leaching? – consider combining with plant protein - broom grass
- Water quality – will/what incentives are needed for farmer to produce?
- What is the optimal plant stage for harvesting to maximize yield, product content, and profitability? ID nutritional contents at stage of maturity (alfalfa plant)
- Particle size important for feed and differs for poultry (500-900 micron); and swine (300-600 micron)
    - What's the density coming into the processing plant and coming out?

- What are options for production arrangement with alfalfa growers?

Processing Consideration

- What are factors in determining feasibility to co-locate with existing ethanol plant, pea protein facility, or other processing facility?
    - What logistics are needed for proper handling & storage of raw and finished product at processing facility?
    - Rail accessibility
- What capitalization is needed?
- Magic Valley – use a process to shrink down alfalfa & get consistency
- ID processing to achieve consistent composition
- Investigate parameters to maximize fermenting:
    - Look at effect of Ph adjustments on chemicals
- Particle size important for feed and differs for poultry (500-900 micron); and swine (300-600 micron)
    - What's the density coming into the processing plant and coming out?

Distribution – go to market

- Evaluate distribution options:
    - Direct
    - Distributor
    - Depends on competition – soybean and corn
    - Depends on market

Markets

1. Evaluate size of each market
2. What information is needed to evaluate potential market?
3. Value proposition to supply chain & end user

- Biofuels
- Feed – swine, eggs, turkeys, broiler (in Midwest to lesser extent)
    - Possible use in young pig diets to replace higher priced alternatives i.e. fish oil
    - Space within diets – nutrient pack
    - Ratio of Amino Acids to Crude Protein – is it better or same as soybean meal?
    - Monogastric – Air Dry vs Ruminant – Dry Matter
    - Use in poultry withdrawal diet prior to harvest – clean up gut
    - Gut health
    - Xanthophyll content?
        - Less concern for broilers
        - More important for egg layers (table egg)
    - Aquaculture – consider floating characterization – tilapia, shrimp
    - Particle size important for feed and differs for poultry (500-900 micron); and swine (300-600 micron)
    - Feed trials by species
        - Connect with U of MN for invitro digestion studies
- What's the density coming into the processing plant and coming out?

- Co-product
    - Fiber
        - Source for dairy
        - Ensiled
- Human
    - Microbial- gut health
    - Cholesterol reduction supplement
- Food safety –
    - use in pre-harvest feed withdrawal – microbial/pathogen/bacteria reduction
- Organic/non-organic

APPENDIX H

Supply Chain Phase 2 - Steve Olson Consulting

A

ALFALFA PROTEIN COMMERCIALZATION REPORT

Steve Olson

STEVE OLSON CONSULTING, LLC SteveOlsonConsulting.com

AURI Alfalfa Protein
June 17, 2022

Summary:

To investigate the market potential for alfalfa protein, we engaged with poultry and swine nutrition experts from the University of MN and private sector[i]. They assisted in identifying opportunities, barriers, and questions to be answered for commercialization and uses in animal diets. This report includes a summary of those conversations as well as the alfalfa protein analysis.

In short, alfalfa protein has potential viability for several markets, under the right circumstances – swine (baby pigs), poultry, pet food, aquaculture, ethanol production, as a soil amendment, and human uses. More research is needed to better refine alfalfa protein's fit for each market. Furthermore, a potential value-added opportunity exists for alfalfa producers to process in field, thereby reducing yield loss.

Product Attributes (Refer to AURI product sheets)

For specific product attributes please refer to AURI product sheets.

Market Segment: Animal Nutrition

Animal nutrition offers the greatest potential near term market because supply chains exist for handling alfalfa product, and nutritionists are familiar with it as a feedstock with ruminant diets. A mindset shift is needed by nutritionists, and poultry, and livestock producers to expand their knowledge of alfalfa protein to regularly consider in their ration. Likewise, the supply chain will need to use different equipment to process raw and finished products.

Swine

AURI initiated research conducted by Dr. Sam Baidoo at the University of Minnesota's SROC (Southern Research & Outreach Center)[ii] with nursery pig diets showing that 6% inclusion of spray-dried alfalfa protein in the diet was optimal and had no significant negative effect on performance, good nutrient absorption. Furthermore, the trial found good absorption of nutrients by the piglets.

Additional research is needed to answer:

1. Is damaged lysine digested/absorbed available protein synthesis?
2. Is there mineral toxicity?
3. Sprayed dried alfalfa is comparable to blood plasma, how does the lack of immunoglobulin in alfalfa affects the diet and economics of spray-dried alfalfa?
4. Does the methionine to protein ratio fit for organic diets?
5. Drying alternatives to Maltodextrin

Poultry

For use in poultry diets alfalfa spray dried protein is approved for up to 10% inclusion. The Xanthophyll and Vitamin A profile when added to egg-type laying hens improves the yellowing in egg yolks and skin pigmentation in broiler meat. Spray dried alfalfa also has value to poultry starter diets (broiler) and as pre & pro-biotic. Potential barriers to use in poultry diets are the:

- Fat to energy ratio
- High potassium to phosphorus ratio in antibiotic free (ABF) diets
- Storage - feed mill bin space
- Lysine bioavailability for protein synthesis
- Turkey finishing diets
- High fiber
- Anti-nutritional factors

Pet Food

This market is familiar with pelleted alfalfa and a major potential Minnesota industry supply chain partner is processing and serving this market.

General Opportunities

General potential uses applicable across animal nutrition segments is use as a pellet binder, as a natural source of methionine spray dried alfalfa is attractive for use in organic production (the methionine to protein ratio needs to be further determined).

General questions to be answered:

- Sugar fractionation info?
- Ensile and transport as needed to prevent sugar degradation
- Features of new strains (USDA-ARS)
- What is the effect of ensiling and heating on saponin?

	Opportunities	Barriers	? to be Answered
Swine	• Nursery pig diet – no significant effect on performance (Baidoo research) • Good absorption of nutrients • Pellet binder	• Digestibility in Baidoo research looks good but doesn't account for damaged lysine (which can be digested/absorbed but is not available to be utilized for protein synthesis. • If juice is dried, then just an alternative ingredient competing with other proteins • Alfalfa lacks immunoglobulins found in blood plasma	• Mineral toxicity? • Growth performance trial comparing spray dried alfalfa juice as replacement for blood plasma in piglet diet. • Dilution of spray dried alfalfa juice with Maltodextrin (researchers had to dilute to get it dried down to spray) • Methionine to protein ratio – especially for organic diets
Poultry	• Up to 10% inclusion allowed	• Fat to Energy ratio limitations	• Omega 3 & 6 levels • Effects of pressing on profile

	• Dehydrated alfalfa meal (40% the nutritional value (energy) of corn) • Egg yolk pigmentation (Vit A & Xanthophyll) @<10% • Broiler – yellow skin pigmentation @< 10% • Pellet binding characteristics w/ nutrient value • Pre-Starter Diets (meat birds) • Pre/Pro-biotic (juice is spray dried) • Natural methionine source – organic diets • Feed mill bin space - @ 2-20% inclusion (depending upon packaging)	• High Potassium to phosphorus ratio ABF diets • Feed mill bin space • Lysine bioavailability (Maillard reaction) • Cost prohibitive for turkey finishing diet • High fiber • Anti-nutritional factors	• What are the vitamin E levels in juice or dried soluble? • Methionine to protein ratio – especially for organic diets
Cattle	• Pressed meal (post-liquid removal) is ideal cattle feed	• Need for drying or re-ensiling pressed meal (expense)	•
Aquaculture	• See U of MN research appendix	•	•
Pet Food	• Currently much of the pelleted alfalfa currently going into the pet food market – market familiarity • MNVAP currently serves the pet food industry • Concentrate protein	• High fiber content	• Blend powder • Nutrition analysis • Back calculate liquid analysis from moisture level • Continue discussions with potential commercialization partners

Market Segment: Non-Animal Nutrition

Spray dried alfalfa also has opportunities for use in non-animal nutrition markets. Again more research is needed to determine potential specific potential in each market.

Ethanol

Spray dried alfalfa good starch source; as a perennial alfalfa qualifies for the Minnesota bio-incentive grant program; and can be certified as organic for use in specialty organic vodka. Potential barriers are having enough quantity available for consistent utilization; storing the alfalfa juice; does not have the sugar content to stand alone as a feedstock; and only fresh alfalfa is an option.

Further research is needed. AURI is collaborating with a potential industry partner on bench top study analyzing fermentation and sugar; the need and ability to filter out solids; and inaction with protein and yeast.

Plant Nutrition

Another potential opportunity is using alfalfa juice as a foliar-applied plant nutrient for organic production. Like other markets, barriers are product availability, application method(s), and product storage.

	Opportunities	Barriers	? to be Answered
Ethanol	• Good starch source • Qualifies for MN bio incentive grant program • Juice is certified organic for organic alfalfa vodka production	• Product quantity availability. • Alfalfa juice storage. • Applies to fresh alfalfa only • Sugar content too low to be a viable stand-alone option	• Fermentation/sugar analysis? • Shelf-life? • Year-round availability • Ability/need to filter-out solids? • Inaction with protein and yeast? • Results from ethanol plant bench top study? • Economics? • Increased efficiency with ensile product that's been frozen?
Plant Nutrition	• Alfalfa juice may provide a valuable foliar feeding for organic production.	• Application methods and material handling • Product availability	• Potential for foliar application

[i] Dr. Sally Noll, University of Minnesota, Dept of Animal Science, Poultry Nutrition and Dr. John Goihl, Agri Nutrition, Swine Nutrition

[ii] Dietary evaluation of spray dried fermented alfalfa juice on nutrient balance, ileal digestibility, and performance of weaned pigs. Dr. Sam Baidoo, University of MN Southern Research & Outreach Center, December 2021

APPENDIX I

AURI Application Information Sheets

Alfalfa: High-Value Products

Alfalfa Juice, Post-Ensiling

Agricultural Utilization Research Institute

Material Overview

Minnesota-grown alfalfa hay was ensiled for storage, and after a period of fermentation the alfalfa was pressed to extract liquid. The "juice" contains high levels of potentially useful sugars and nutrients, and can also be extracted from fresh, non-ensiled alfalfa for use in applications requiring an unfermented ingredient for product development.

AURI is exploring potential high-value uses in the animal feed, human nutrition, renewable energy and biobased products sectors for this and other alfalfa-based ingredients.

Material Analysis

Nutrient		
Moisture		87.30%
Dry Matter		12.70%
pH		4.90
		Dry Basis
Crude Protein	%DM	37.64
Sugar (WSC)	%DM	8.50
Fat (Acid Hydro.)	%DM	2.05
Calcium	%DM	2.13
Phosphorus	%DM	0.55
Magnesium	%DM	0.71
Potassium	%DM	7.32
Sulfur	%DM	0.79
Sodium	%DM	0.12
Zinc	ppm	71.00
Iron	ppm	252.00
Manganese	ppm	63.00
Copper	ppm	16.00
Boron	ppm	79.00

AURI Tech Notes:

- Alfalfa haylage was ensiled and allowed to ferment for a minimum of 60 days.
- Alfalfa hay was ensiled at higher than normal moisture levels to increase juice volume when pressing.
- Juice was extracted using a screw press.
- Moisture and plant maturity at harvest may affect the nutrient content in juice.

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Funding for this project was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (LCCMR).

Alfalfa: High-Value Products

Post-Press Alfalfa Haylage

Agricultural Utilization Research Institute

Material Overview

Minnesota-grown alfalfa hay was ensiled for storage. After a period of fermentation the alfalfa was pressed to extract liquid, and this "juice" contains high levels of potentially useful sugars and nutrients.

After pressing to extract liquid, the remaining alfalfa was re-ensiled. While the pressing process extracted a portion of the alfalfa's nutrients, the post press material still contains nutrient levels that offer potential value for animal nutrition uses.

Material Analysis

Nutrient		
Moisture		59.63%
Dry Matter		40.37%
pH		4.79
		Dry Basis
Crude Protein	%DM	15.26
AD-ICP % of CP	%CP	9.51
ND-ICP w/SS	%CP	13.76
Protein Sol.	%CP	45.35
Ammonia-CP	%CP	8.94
ADF	%DM	43.34
aNDF	%DM	49.62
aNDFom	%DM	47.72
Lignin	%NDFom	21.92
Sugar (ESC)	%DM	1.32
Sugar (WSC)	%DM	1.67
Starch	%DM	1.49
Fat (EE)	%DM	4.37

AURI Tech Notes:

- The re-ensiled haylage maintained nutrient quality during continued storage.
- A reduction in moisture and crude protein was observed due to liquid extraction.

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Material Profile

Post Press Alfalfa Haylage

Agricultural Utilization Research Institute

		ADF	OARDC	MLK 2013
TDN	%DM	55.14	54.22	56.57
Nel 3x	Mcal/cwt	56.03	54.93	57.59
Neg	Mcal/cwt	28.41	27.70	31.10
Nem	Mcal/cwt	53.96	53.19	56.90
Milk per ton	lb/ton DM			2412

Ash	%DM	8.47
Calcium	%DM	1.58
Phosphorus	%DM	0.28
Magnesium	%DM	0.32
Potassium	%DM	1.85
Sulfur	%DM	0.22
Lactic Acid	%DM	<0.01

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Alfalfa: High-Value Products

Spray-Dried Alfalfa Powder

Agricultural Utilization Research Institute

Material Overview

Minnesota-grown alfalfa hay was ensiled for storage and after a period of fermentation the alfalfa was pressed to extract liquid. The liquid was then spray dried into a high-protein powder.

AURI is exploring potential high-value uses in the animal nutrition sector for this alfalfa-based ingredient.

Material Analysis

Nutrient		
Moisture		10.07%
Dry Matter		89.93%
		Dry Basis
Crude Protein	%DM	37.95
AD-ICP % of CP	%CP	0.48
ND-ICP w/SS	%CP	0.88
Protein Sol.	%CP	94.26
ADF	%DM	1.43
aNDF	%DM	0.89
aNDFom	%DM	0.12
Sugar (WSC)	%DM	8.31
Starch	%DM	0.58
Fat (EE)	%DM	2.31
Ash	%DM	19.75
Calculations		
NFC	%DM	39.99
NSC	%DM	8.89
Adjusted Crude Protein	%DM	37.95

Material Profile

Spray-Dried Alfalfa Powder

Agricultural Utilization Research Institute

		Dry Basis
Crude Protein	%DM	37.95
Total Amino Acids	%DM	21.61
Total Amino Acid	%CP	56.94
Lysine	%CP	3.53
Methionine	%CP	0.76
Cysteine	%CP	0.58
Alanine	%CP	4.37
Aspartic Acid	%CP	12.94
Glutamic Acid	%CP	4.82
Glycine	%CP	2.40
Isoleucine	%CP	3.03
Leucine	%CP	4.08
Proline	%CP	5.27
Threonine	%CP	2.32
Valine	%CP	3.98
Arginine	%CP	1.00
Histidine	%CP	1.03
Phenylalanine	%CP	2.45
Tyrosine	%CP	1.84
Trytophan	%CP	0.55
Serine	%CP	1.98

		Dry Basis
Calcium	%DM	1.85
Phosphorus	%DM	0.49
Magnesium	%DM	0.75
Potassium	%DM	6.43
Sulfur	%DM	0.29
Sodium	%DM	0.11
Zinc	ppm	52.00
Iron	ppm	302.00
Manganese	ppm	65.00
Copper	ppm	7.00
Boron	ppm	54.00

AURI Tech Notes:

- Alfalfa haylage was ensiled and allowed to ferment for a minimum of 40 days.
- Alfalfa hay was ensiled at higher than normal moisture levels (72% vs typical 65%) to increase juice volume when pressing.
- Pressed, fermented alfalfa juice has high sugar content and may serve as a base for development of other high-value alfalfa applications.
- Final analytical profile of the spray dried material may vary based on varietal of alfalfa, conditions during harvest and processing methods after cutting.

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