Expanding the Use of High-Performance Liquid Chromatography for Varietal Identification or Verification of Selected Cereal Crops

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

Master of Science

Johnna Jorgensen

Master of Plant Science

South Dakota State University

Spring 2023

THESIS ACCEPTANCE PAGE

Johnna Jorgensen

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

E. Brent Turnipseed
Advisor Date

David Wright
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

Acknowledgements

I would like to thank my advisor Dr. Brent Turnipseed, the Plant Science Department at South Dakota State University, and my coworkers and peers from the South Dakota Seed Testing Laboratory for supporting me and encouraging my research and my pursuit of higher education. I'd also like to thank my committee members for their comments and suggestions in helping to develop my final thesis.

Thank you to my parents and my brothers for encouraging me to pursue a master's degree and challenge myself in my education. My family has been one of my strongest support systems over the years.

Finally, I want to give my deepest appreciation to my husband, Justin, for standing by me through all the challenges, and being patient when I needed it. It was his continuous support and confidence of my writing that helped to me final my research.

I greatly appreciate all the advice, guidance, and encouragement I received throughout this journey.

Thank you!

Table of Contents

List of Figures

List of Tables

Abbreviations

Å	angstrom
ACN	acetonitrile
C	Celsius
EtOH	ethanol
FGIS	Federal Grain Inspection Service
FSA	Federal Seed Act
g	gram
HPLC	High-Performance Liquid Chromatography
L	liter
LC	Liquid Chromatography
mAU	milli-absorbance unit
MeOH	methanol
min	minute
mL	milliliter
µL	microliter
µm	micrometer
PAGE	Polyacrylamide Gel Electrophoresis
PrOH	propanol
PVP	Plant Variety Protection
PVPA	Plant Variety Protection Act
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
V	v

Abstract

Expanding the Use of High-Performance Liquid Chromatography for Varietal Identification or Verification of Selected Cereal Crops

Johnna Jorgensen

2023

High-performance liquid chromatography (HPLC) is a form of analytical chemistry where adsorption is a key factor in which the purpose is to separate, identify and quantify each component in a mixture. It is the optimal separation technique of chemical and biological compounds that are non-volatile including proteins and natural products (i.e., plant extracts). As each sample component interacts differently with the absorbent material within the column, the flow rates change leading to the separation of peaks, which are plotted via computer software.

Most commonly HPLC analysis has been used for chemical manufacturing and pharmaceutical purposes. When using HPLC for crop variety identification or verification, the goal is not to look at identifying the individual protein components in each seed or to match peak height and width, as within the species the components should all be very similar. Instead, the analyst will match the peak pattern of the known variety standard in the chromatograph and the peak pattern of the unknown submitted sample, as each sample results in a unique pattern. Quantifying the peaks becomes challenging due to cultural practices (fertilization, irrigation, etc.), environmental factors (rainfall, temperature, etc.) and growth conditions (weed, pest, and disease pressure) that influence protein development and content within the seed. Due to this, the proteins among tested samples are variable, which is reflected in the chromatograms.

Currently, the method described in this document is being used to regularly confirm the variety of wheat and oat samples. The ultimate goal of this research is to develop a method that works with rice, as well as improve existing methods on other cereals. Demand for varietal verification/identification is increasing in wheat, oats, rye, triticale, and barley from producers wanting/needing to verify varieties being sold or bought.

Literature Review

1. Introduction

Most varieties of small grains (barley, oats, rice, rye, triticale and wheat) are released with a plant variety protection certificate (PVP) in the United States. The Plant Variety Protection Act (PVPA) provides legal intellectual property rights protection to breeders of new varieties of plants which are sexually reproduced, allowing the owner to recover research costs, and hopefully make a profit. Almost all wheat and oat varieties from both public and private breeding programs are released with PVP protection with Title V. Title V is a provision within the Federal Seed Act (FSA) which requires that the seed can only be sold as a class of certified seed (Foundation, Registered, or Certified class). Unfortunately, some seed of those protected varieties are marketed illegally, driving the need for variety identification or verification testing services for situations where a PVP violation is suspected. It is the variety owner's obligation whether to pursue and prosecute the violator in civil court.

Other uses for varietal testing of small grains are in quality control programs, to ensure that the farmer/seedsman has maintained accurate records of which lot is which variety. The International Seed Testing Association and the Association of Official Seed Analysts have handbooks providing electrophoretic testing methods that can be used for variety identification in many crops.

High-performance liquid chromatography (HPLC) is a form of analytical chemistry where adsorption is a key factor in which the purpose is to separate, identify and quantify each component in a mixture. It is the optimal separation technique of

chemical and biological compounds that are non-volatile, including proteins and natural products (i.e., plants extracts). The term separation refers to the process occurring as the proteins are adsorbed by the column in the HPLC and as they are eluted off the column. As each sample component interacts differently with the absorbent material within the column, the flow rates change leading to the separation of peaks, resulting in a pattern recorded by a computer software program. These patterns are unique to each individual variety, so much so, Lookhart referred to the unique patterns as “fingerprints” in April 1990 article, “Practical Wheat Varietal Identification in the United States”, published by the American Association of Cereal Chemists.

The use of chromatographic practices in separation of plant extracts is not a new concept. Chromatography, meaning “color writing”, was first introduced in 1903 by a Russian botanist as a way to separate plant pigments. It is from that point that HPLC methods have been developed for separations in a variety of industries including pharmaceuticals and chemical manufacturing.

HPLC use in the seed and grain testing industry has aided in the progression and development from the separation methods used in electrophoresis. The process of using polyacrylamide gel or starch gel electrophoresis to separate alcohol soluble protein patterns is cumbersome and difficult. However, using HPLC methods to extract and detect alcohol soluble proteins is much simpler and more efficient. The purpose of this thesis is to better explain how improved methods using HPLC can be expanded in the seed technology industry.

2. Understanding High-Performance Liquid Chromatography

2.1. High-Performance Liquid Chromatography Compared to Polyacrylamide Gel Electrophoresis

High-performance liquid chromatography serves as a suitable alternative for polyacrylamide gel electrophoresis as proven in 1986 by Lookhart, Albers, and Bietz. In this experiment, the researchers took 100 seeds of two different genotypes from the wheat variety Newton and cut them in half longitudinally. These sample seeds were obtained in even amounts from four different Foundation locations: Champagne, Illinois; Lincoln, Nebraska; Manhattan, Kansas; and Hutchinson, Kansas. One set of the halves were extracted and tested using PAGE methods, the other set were extracted and tested using HPLC methods. The authors performed side by side comparisons of the results and found that both PAGE and HPLC produced the same 42:58 ratio of genotype I and genotype II, respectively (Lookhart, 1986).

Benefits to using HPLC over PAGE include that modern HPLC methods are automated and less hands on, allowing the user to be more flexible in their work with HPLC. The chemistry behind HPLC is also more sensitive and makes the chromatograms easier to reproduce. Another benefit of using HPLC is, while the results needed for this experiment are qualitative, the results produced by HPLC methods can be used quantitatively if needed. On the other hand, getting started with HPLC does come with higher initial costs than PAGE. And finally, only one sample can be run at a time, making it more time consuming to use the HPLC method, but it is automated and does not need to be supervised to run and develop chromatograms for each sample.

2.2. The Machine

To gain a better understanding of high-performance liquid chromatography, it is most helpful to look at how a sample adsorbs and elutes through the components of the HPLC machine. Figure 1 is simplified diagram of the components of an HPLC machine. It starts with the solvent being pumped through the column to prime the column before injecting the sample. It is inside the column, after the injection, where the proteins from the sample are absorbed and then eluted. There is then a detector that the eluted proteins flow through after the column, which identifies proteins based on flow rate and sends the data to the computer program (ChemStation, OPENLABCDS – 001), which can then be reviewed and analyzed. To complete the process before moving on to another sample, more solvent will be pumped though the column to clean the column and the capillaries and collected in a waste container. Prior to running the samples, the user will program the software controlling the HPLC to run the samples as in a pre-determined order.

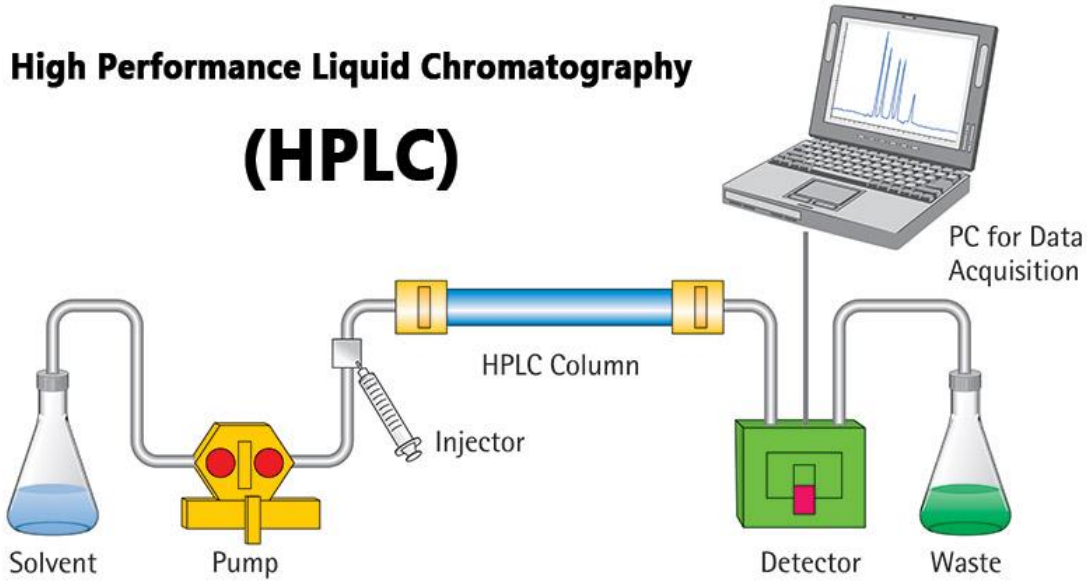


Figure 1. A simplified diagram of the components of an HPLC machine. The image is sourced from microbenotes.com (Aryal, 2019).

Figure 2 is the setup of the HPLC used by the South Dakota State University Seed Testing Laboratory with compartments of the machine defined. The prepared samples are placed in the autosampler compartment until they are individually injected as programmed. The next level of the machine setup is the column oven compartment, where the column is stored. To efficiently elute the proteins and separate them, the column is warmed to an optimal temperature depending on the compounds one wants to identify or separate. Warming the column also acts as a form of activation of the inner column chemistries. The following two compartments, the detector compartment and the binary pump compartment are as they sound, the areas where the detector and the pump are located. Typically, the user will have little interaction with these compartments. Figure 3 is the complete setup with the associated computer and software at the SDSU Seed Testing Lab.

Figure 2. The HPLC machine used at the South Dakota State Seed Testing Laboratory with parts labeled. Model number 1260 Infinity II from Agilent Technologies.

Figure 3. The complete set up of the HPLC machine at the South Dakota State Seed Testing Laboratory and the computer used to analyze results.

2.3. The Column

The column is where all separation takes place during the HPLC process, and thus, the user's choice of column is very influential in how the components in the injected sample are separated. Different types of columns are used for different separation processes, as the column type changes when comparing gas chromatography practices to liquid chromatography practices. The research presented in this paper is done strictly through liquid chromatography. The proteins are extracted through the addition of an alcohol-based solvent to the ground flour of a cleaned, randomly selected 100 pure seed sample. This extraction process, combined with previous research and the recommendations of HPLC specialists, led to the selection of using a packed column for the separation of the proteins.

The columns are packed with strings of carbon chains that interact with the natural components in the injected sample. To the right, Figure 4, is an image of the average HPLC column and a cross section of a packed HPLC column. The injected sample moves through a series of open capillaries from the point of injection to the column. In the column, the packing material, which holds the proper chemistries for a specific application, allows for the injected sample to interact, separate, and elute, based on polarity. After passing though the column, the now separated components of the sample will be sent through the

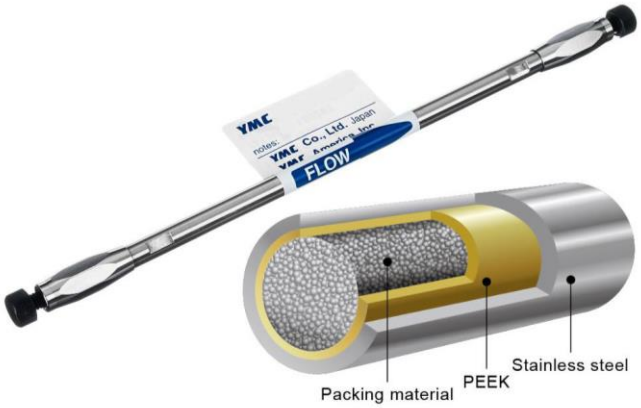


Figure 4. An image of an HPLC column and a cross section of a packed column. A packed column is typically used in most liquid chromatography practices. The image is sourced from whatishplc.com (HPLC Columns).

detector, before being discarded to the waste. Figure 5 is an illustrated representation of how the components in a sample are separated within the column.

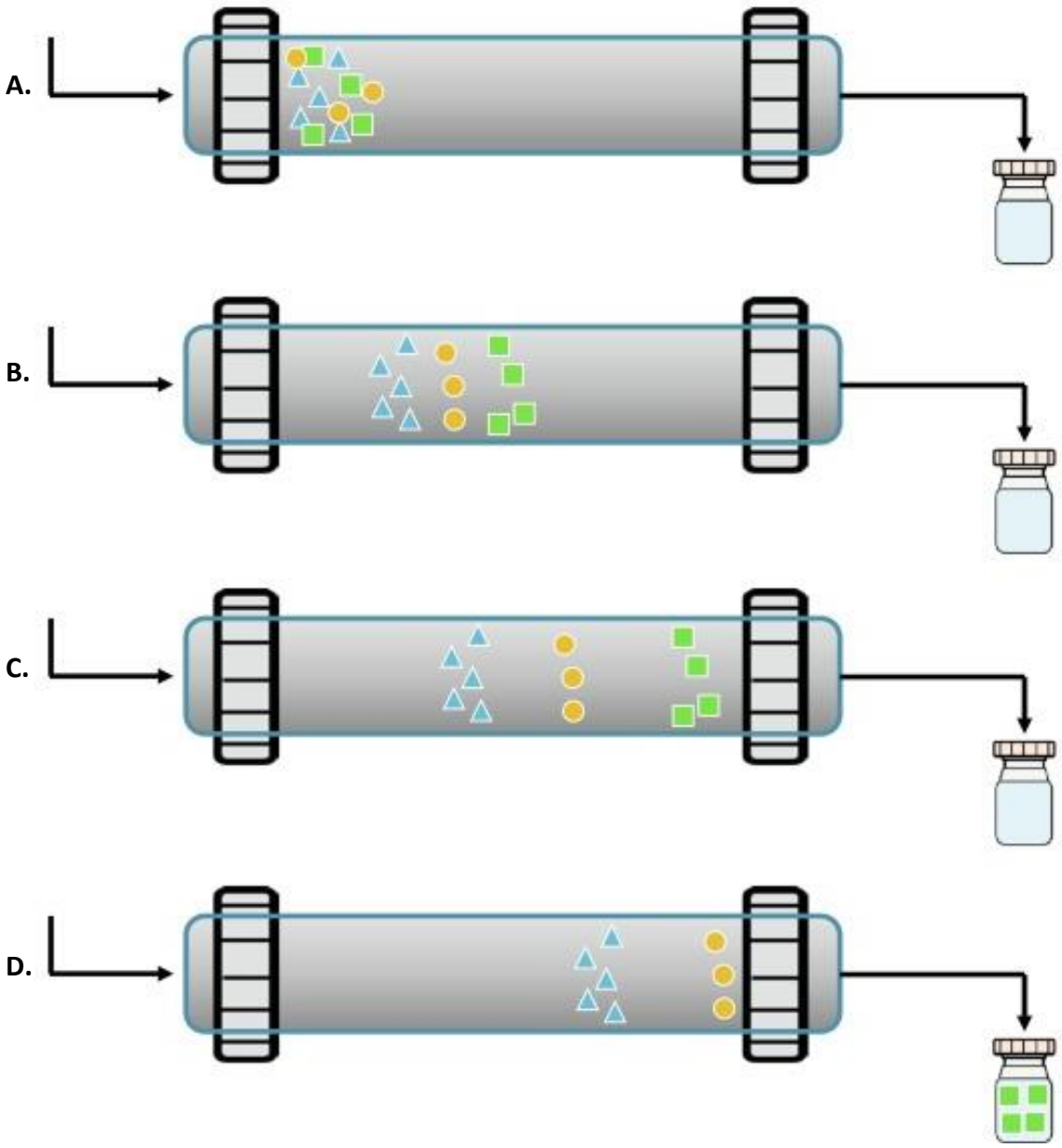


Figure 5. An illustrated representation of how a sample is separated as it moves through the column. Separating the components of the sample allows for each one to be individually identified by the detector. Separations are a result of the component's polarity. A.) The mixture enters the column. B.) The proteins separate based on interactions with the inner column packing material and polarity of the components of the mixture. C.) Different proteins adhere to the column packing materials. D.) The proteins are eluted from the packing material at different times, and detected by the detector, producing a chromatogram of proteins eluting at different times. The testing sample is then deposited as waste. The image is sourced from sciencedirect.com (Shen, 2019).

3. The Seed and the Proteins

Understanding the seed is the center of this research. Inside each seed are types of proteins, or groups of bonded amino acids, all different in nature and all in differing amounts depending on the type of crop seed, cultural factors and external factors. During the extraction process, the proteins needed from cereals are the storage proteins found the endosperm, known collectively as the prolamins and the glutelins. The prolamins are a "simple reserve of protein food found only in the seeds of cereals", meanwhile, the glutelins are a form of protein used for energy storage in the endosperm. (Hoseney, 1986). Figure 6 is a simplified illustration of a typical rice seed; however, the concept of the structures can be translated to other cereal grains.

Each crop has its own specific group of prolamins. In wheat, it is the gliadin proteins, oats, the avenin proteins, has the oryzin protein, and so forth. Determining the extraction methods from to crop is not a direct process and the method needs to be altered to better fit each type. This is better achieved through understanding the genetic makeup of the seed. Table 1 lists different cereal crops and the proteins found in the endosperm of the seed.

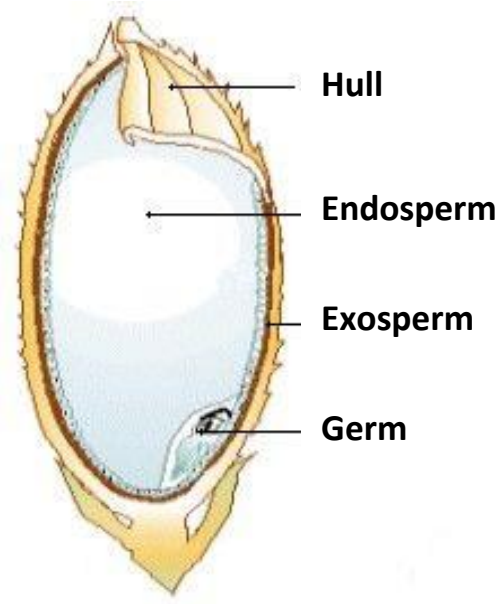


Figure 6. An illustration of a typical cereal grain seed. The storage proteins used for HPLC identification are found in the endosperm. The image is sourced and altered from gcmachines.com (Rice Bran).

	Average Protein Content	Alcohol Soluble Proteins
Wheat	13.2 %	Gliadin
Oats	11 – 14 %	Avenin
Rye	6.5 – 14.5 %	Secalin
Triticale	11.6 %	Gliadin
Barley	8 – 13 %	Hordein
Rice	2.7 %	Oryzin

Table 1. A table defining the average amount of alcohol soluble proteins found in different cereal crops.

The prolamin proteins are alcohol soluble, making them easy to extract. Therefore, when extracting the proteins from a sample of ground and sifted flour, a 70% alcohol buffer, such as methanol, ethanol, or propanol is used. The selection of this buffer is based on the polarity of the proteins to be extracted. A more polar extracting buffer will extract the proteins more towards the beginning of the chromatogram, while a less polar extracting buffer extracts proteins more towards the end of the chromatogram. The proteins being extracted themselves also have polarity, which is demonstrated by where they are detected (time of elution from the column) in the chromatogram. Figure 7 illustrates the polarity of proteins in a chromatogram of the oat variety Rushmore. Rushmore was extracted with 70% ethanol for this chromatogram.

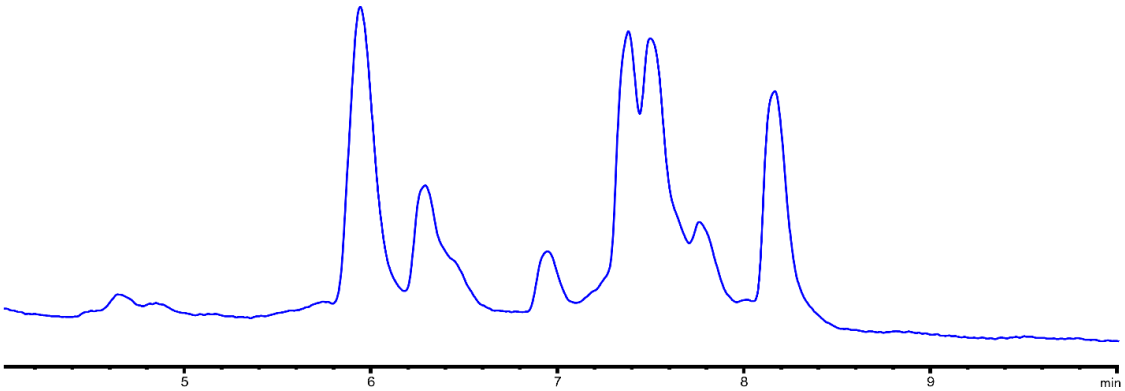


Figure 7. A chromatogram of the oat variety Rushmore, noting where proteins (peaks) are expected to extract based on polarity. The x-axis is measured in minutes, which refers to the time of elution from the column.

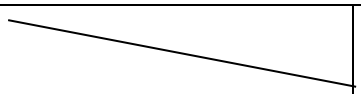
Buffer Type	Response	
Methanol		More Polar
Ethanol		Neutral
Propanol		Less Polar

Table 2. This table defines the extraction buffer and the expected response based on polarity of the buffer. When using a more polar buffer, it is expected that the extractions and detections of the proteins from the HPLC machine will happen earlier in the chromatogram.

The goal of this project was to expand the current South Dakota State University Seed Testing Lab established HPLC method to other cereal grains besides wheat and oats and develop new or better methods for all the cereal crops. This task became more of a challenge in rice as it was found that the amount of proteins naturally stored in the seed of rice is significantly less than the amount of protein found in other cereal seeds such as wheat, oats, rye, and triticale. The amount of protein in one seed compared to that of another seed of the same crop can vary based on environmental growth conditions, stresses, and soil fertility levels the plant may have endured during the growing season, but as one can see from Table 1, rice simply has less protein content.

4. Federal Grain Inspection Board of Appeals Method

The original method of extracting seed proteins with HPLC used by the South Dakota State Seed Testing Laboratory comes from the Federal Grain Inspection Service (FGIS) which based its methods on research done by cereal chemist George L. Lookhart's. The FGIS tests wheat samples using an HPLC model from Agilent Technologies, model 1260 Infinity System. Their system is a five-module system with a degasser, a binary pump, an autosampler, column oven, and a wavelength or ultraviolet detector (models GA 4225A, G 1312B, G 1329B, G 1316C, and G 1214F, respectively).

The HPLC method used by FGIS prepares the sample (bulk sample of 25 seeds) by using a coffee grinder to grind and expose the proteins from the endosperm of the seed. A U.S.A Standard No. 40 (0.0165 in, 425 μm) sieve is used to sift and separate the fine endosperm and germ grindings from the larger particles of endosperm, seed coat, and other seed chaff or debris. To extract the proteins from the ground powder, the FGIS methods uses a buffer solution of 50% n-propanol and 50% HPLC grade water, then shakes the sample in an microcentrifuge tube using a Milli-Q System Vortex Genie 2, shaking the sample for 5 minutes. The samples sit at room temperature for a minimum of three hours to allow for proper and absolute extraction of the alcohol soluble proteins. Once the extraction is complete, the samples are then centrifuged for four minutes at 13,200 revolutions per minute (RPM). The supernatant of the sample is transferred from the microcentrifuge tube to an autosampler vial, now ready to go into the HPLC machine (Liang, personal communication with Dr. Brent Turnipseed, 2017).

The FGIS method starts each run by purging the machine with a solution of HPLC grade water + 0.1% trifluoroacetic acid (TFA) for two hours with the column turn on and set to 70 degrees Celsius. This allows for the machine to equilibrate and pushes out any air bubbles that may be sitting in a capillary or tube in the machine. When starting the run, the column is turned on and allowed to heat to 70C, and the HPLC solutions, acetonitrile (ACN) + 0.1% TFA and HPLC grade water + 0.1% TFA are pumped through the machine at 1.0 mL per minute. This will continue throughout the course of the run, with solution proportions changing as programmed. The autosampler will now draw 10 μL from the sample in the filter vial and it will move with the HPLC solvents through the column and the detector, before going to waste. The detector will

send the gathered information from the HPLC machine to a software computer program that will be used to produce chromatograms and allow for comparison the samples, or to a known standard. It takes approximately 39 minutes to complete running one sample using the method from FGIS (Liang, personal communication with Dr. Brent Turnipseed).

5. Lookhart's Role in Developing HPLC Methods

George L. Lookhart, a now retired cereal chemist from the USDA-ARS Grain Marketing and Production Research Center in Manhattan, Kansas, was a major contributor to the development of seed protein extraction and detection methods using HPLC technology. The research and work he published has aided in the development of new methods and the improvement of previously established methods. Similarly, the method used for gliadin extraction by the South Dakota State University is derived from Lookhart's publications.

Lookhart viewed HPLC not only as a way of varietal identification, but also as a way of analyzing the purity of a seed lot, as well as analyzing the quality of a seed lot, understanding that peak height variations are a direct result of variations in protein amounts, which can be attributed to variety genetics, soil type, farming practices, and weather (rainfall, temperature, etc.).

Lookhart also understood that the preparation of a sample was key in having accurate and repeatable results. The only unknown variable should be the sample itself. In preparing the sample, the extracting solvent should be "of the highest possible purity" (Shewry). Any impurities in the solvent could be detected by the UV detector and could result in altering or skewing the resulting chromatogram.

Column selection is critical in HPLC, and given the protein molecules are of the larger variety, a wide-pore column, such as a C8 or C18, is the best fit. The C in the column size refers to the number of carbons in a carbon chain inside the column, which contributes to sensitivity and detectability. Lookhart, as well as other key HPLC researchers, found that using a shorter column, combined with higher flow rates, a steeper gradient, or higher temperature would produce comparable and acceptable results. As a result, cost, solvent consumption, and analysis time usually declined (Shewry).

Materials and Methods

6. Introduction

Below is the basic list of equipment and chemicals needed for this research.

6.1. Materials

Known Seed Standard Samples

Pure Seed Samples

Forceps

Seed Counter

Coffee Grinder (Mr. Coffee Grinder set to fine grind)

U.S.A. Standard Test Sieve, No. 40 (0.0165 in, 425 µm)

Weigh Paper

1.5 mL Graduated Microcentrifuge Tubes

Extraction Buffers

- HPLC Grade Water
- Methanol
- Ethanol
- Propanol
- Sodium Dodecyl Sulfate

Pipettes

Pipette Tips

120 V Fisher Pulsing Vortex

Hermle Benchmark Z 216 M Centrifuge

0.45 µm Filter Vials

HPLC Machine (1260 Infinity II)

HPLC Column

HALO 1000 Å, Diphenyl Column

Zorbax 300 SB – C8 Rapid Resolution Column

H_20 + 0.1 % TFA

ACN + 0.1 % TFA

ChemStation Computer Software, OPENLABCDS – 001

6.2. Seed Materials

Samples of known wheat, oats, rye, triticale and barley varieties were provided by the South Dakota Seed Testing Laboratory, located at the Young Brothers Seeding Technology Laboratory in Brookings, South Dakota. These known samples were obtained by the lab from various seed companies in various locations of the United States as part of the lab's routine variety identification program. A Foundation class of each seed variety is preferred as it should be the purest source for known check samples. The samples of rice varieties used in this research were provided by Supreme Rice, LLC, located in Crowley, Louisiana.

6.3. Sample Preparation Materials

To prepare the samples for the extraction process, 100 random pure seed units were counted out and ground using a Mr. Coffee grinder, model number IDS77, set to fine grind. A Mr. Coffee grinder does not explicitly need to be used, but is what was used in preparing all sample for the following experiments. A U.S.A Standard No. 40 Test Sieve (0.165 in, 425 μm) is used to separate the course grindings from the fine powder, which is saved for extracting the proteins.

6.4. HPLC

The model of HPLC machine used for this thesis was the 1260 Infinity II from Agilent Technologies. It was paired with ChemStation, OPENLABCDS – 001 for analyzing the results.

Two columns were used to complete these experiments, the Zorbax 300 SB – C8 Rapid Resolution column (4.6 x 150 mm, 3.5-Micron), a product of Agilent

Technologies, and the HALO 1000 Å Diphenyl column (2.7 µm, 4.6 x 150 mm), a product of Advanced Materials Technology, Inc.

All of the HPLC methods used at the South Dakota State University Seed Testing Laboratory use a gradient method of two solvents: A) H_2O + 0.1% TFA and B) ACN + 0.1% TFA. A gradient method uses changing ratios of solvent A and solvent B during the extraction process that aids in properly separating and detecting the different proteins for each seed. The gradient methods used is presented in Table 3.

Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate (mL/min)
0.00	75.0	25.0	1.500
1.25	65.0	35.0	1.500
10.75	50.0	50.0	1.500
11.00	5.0	95.0	1.500
14.00	5.0	95.0	1.500
14.10	75.0	25.0	1.500

Table 3. The gradient method and flow rate used with the HPLC at South Dakota State University Seed Testing Lab.

6.5. Extraction Buffers

The extraction buffers used were all HPLC grade from Fisher Scientific. They included water, methanol, ethanol, and propanol. Variations of 70% and 80% concentrations were created with the appropriate combination of buffer and HPLC grade water. 70% ethanol + 2.0 g sodium dodecyl sulfate (SDS) was also tested using to extract the proteins. Table 4 identifies which extraction buffer works best with which crop.

	70% Methanol	70% Ethanol	70% Propanol
Wheat		X	
Triticale	X		
Rye	X	X	
Barley	X		
Oat		X	X
Rice		X	X

Table 4. A summary of which buffer extracts proteins the best for each crop.

6.6. Methods

All of the following methods were originally derived from the FGIS method and altered to better fit the crop from either further research and testing, or from advice from Dr. Merlin K. L. Bicking (ACCTA, Inc.), an expert in HPLC and chromatography method development and troubleshooting.

6.6.1. Method One

The following method was used regularly on wheat and oats at the South Dakota State Seed Testing Laboratory and was extended to use on triticale, rye and barley prior to May 2021.

To prepare the samples, begin by selecting 100 pure seeds randomly from a given sample. This sample is then ground using the coffee grinder and sieved using the U.S.A Standard No. 40 sieve. Discard the course grindings and save the fine flour. A portion of 0.060 g of ground and sieved flour is then weighed and transferred into a 1.5 mL graduated microcentrifuge tube.

For extraction of the proteins, 700 µL of 70% ethanol is added as the extraction buffer to the graduated microcentrifuge tubes. All crops are extracted with the same buffer. The samples then agitate or shake for five minutes using a Fisher Pulsing Vortex, 120V (model: 9454FIPSUS). To properly and completely extract, the samples are left to sit at room temperature for a minimum of three hours. If extracting overnight, the sample will be placed in a freezer set to -23C.

After the extracting sample has set for an appropriate amount of time, whether for a minimum of three hours at room temperature or overnight in the freezer, the graduated

microcentrifuge tubes are then placed evenly and centrifuged in the Hermle Benchmark Z 216 M centrifuge for eight minutes at 13,200 RPM.

After the centrifugation has completed, use a pipette to pull 500 µL of the supernatant from the graduated microcentrifuge tube and transfer into an appropriately labeled 0.45 µm filter vial. The extracted samples are then ready to be placed in the HPLC autosampler tray in an organized fashion, (a known check vial first, followed by the submitted sample vial) for separating and analyzing the protein chromatograms. The two will be overlaid and compared to each other, confirming if the submitted sample is a match or not.

Prior to each run, the HPLC is prepped by opening the valve in the binary pump compartment and running a 50% H_2O + 0.1% TFA/50% ACN + 0.1% TFA (solvent A/solvent B, respectively) purge for about 5 minutes. The organized run in the autosampler tray is entered into the ChemLAB software program and the HPLC machine will run as programmed through completion. The machine cleans and idles automatically following the completion of the run. The autosampler pulls 6 µL from each sample to be injected for separation and detection by the HPLC machine. The results can then be viewed and analyzed, and the vials will be appropriately stored or discarded, depending on the need. This method was designed to run one sample every 17 minutes. Data is collected at wavelengths of 210, 220, 280, 300. All chromatograms are analyzed at a wavelength of 210.

6.6.2. Method Two

In May 2021, Dr. Merlin K. L. Bicking (ACCTA, Inc.) consulted with the South Dakota State Seed Testing Laboratory to help the improve used methods and to gain a

better understanding of the HPLC machine. The following method is the result of Dr. Bicking's visit.

To prepare the samples, select 100 seeds at random from a submitted sample and grind into a fine powder and sieve. Weigh out 0.060 g ground powder and place into a graduated microcentrifuge tube. Add 700 µL of the appropriate extraction buffer as per Table 1.4 and shake. Dr. Turnipseed and Dr. Bicking suggested experimenting with different extraction buffers to better the extraction of the proteins of different crops. Let sit at room temperature for a minimum of three hours, or if extracted overnight, set in a freezer set to -23C.

Whether extracting at room temperature or overnight, the next step to prepare the samples is to centrifuge them. The samples were placed evenly and balanced in the centrifuge. After they have been centrifuged, transfer 500 µL of the supernatant from the graduated tubes via a pipette into an appropriately labeled filter vial.

Load the prepared filter vials into the autosampler tray and prep the HPLC by first purging the machine for a minimum of five minutes or until there are no air bubbles visible in the capillaries of the binary pump compartment. Then program the software to fit the prepared run in the correct order. Once completed, the results can be viewed and analyzed using the offline version of ChemLAB.

This method differs from method one listed previously as it is utilizing various extraction buffers that better fit the different crops tested, and it runs each sample for 18 minutes, adding an extra minute to the cleaning process at the end of each sample run. This extra minute ensures that the column and capillaries are fully cleaned of any

potential contaminant from the previous sample that may pollute or taint the detector for the next sample in line.

6.7. Rice Trials

The following trials were done to help develop a method for extracting and analyzing the proteins from rice. Samples of rice were first tested with a 0.1% tetrazolium solution to test viability prior to HPLC testing. Live, viable seed is important when extracting the proteins, as dead seed does not have extractable proteins. The following is a list of the nine rice varieties provided by Supreme Rice, LLC, to be used in the experiments:

Chenies	Gemini	Mermentau
CL – 151	Hybrid	PUL – 1
CL – 153	Jupiter	Titan

6.7.1. Column Selection

During these experiments, two columns of differing chemistries were used for comparisons, the Zorbax 300 SB – C8 Rapid Resolution Column made by Agilent Technologies, and the HALO 1000 Å, Diphenyl Column made by Advanced Materials Technologies, Inc. The HALO column has a chemistry that is more sensitive to separations within the column, which leads to more prominent peaks in the chromatographs, ultimately making it the best selection when testing rice with HPLC methods.

6.7.2. Buffer Experiments

Rice samples were extracted using five different buffers: 70% methanol, 70% ethanol, 70% propanol, 70% ethanol + 2.0 g SDS, and HPLC grade water. A sample of

100 seeds were then randomly selected, prepared, extracted with each of the five buffers, and analyzed in the same process as described above in the previous section. The rice extracted with buffers of HPLC grade water and 70% methanol showed no peaks during the analysis and was rejected as a possible extraction method. Both 70% ethanol and 70% propanol resulted in some peaks and these extractions buffers were noted as possible methods to build upon, creating a baseline of which further trials were compared to. The chromatogram featuring 70% ethanol + 2.0 g SDS did not improve the extraction and separation of the proteins compared to those extracted with only 70% ethanol, causing rejection of this extraction method. Due to the lower quantity of protein available from the rice embryo, the resulting peaks on the chromatogram are lower and more difficult to analyze, creating a challenge in developing a method for using HPLC to identify rice varieties.

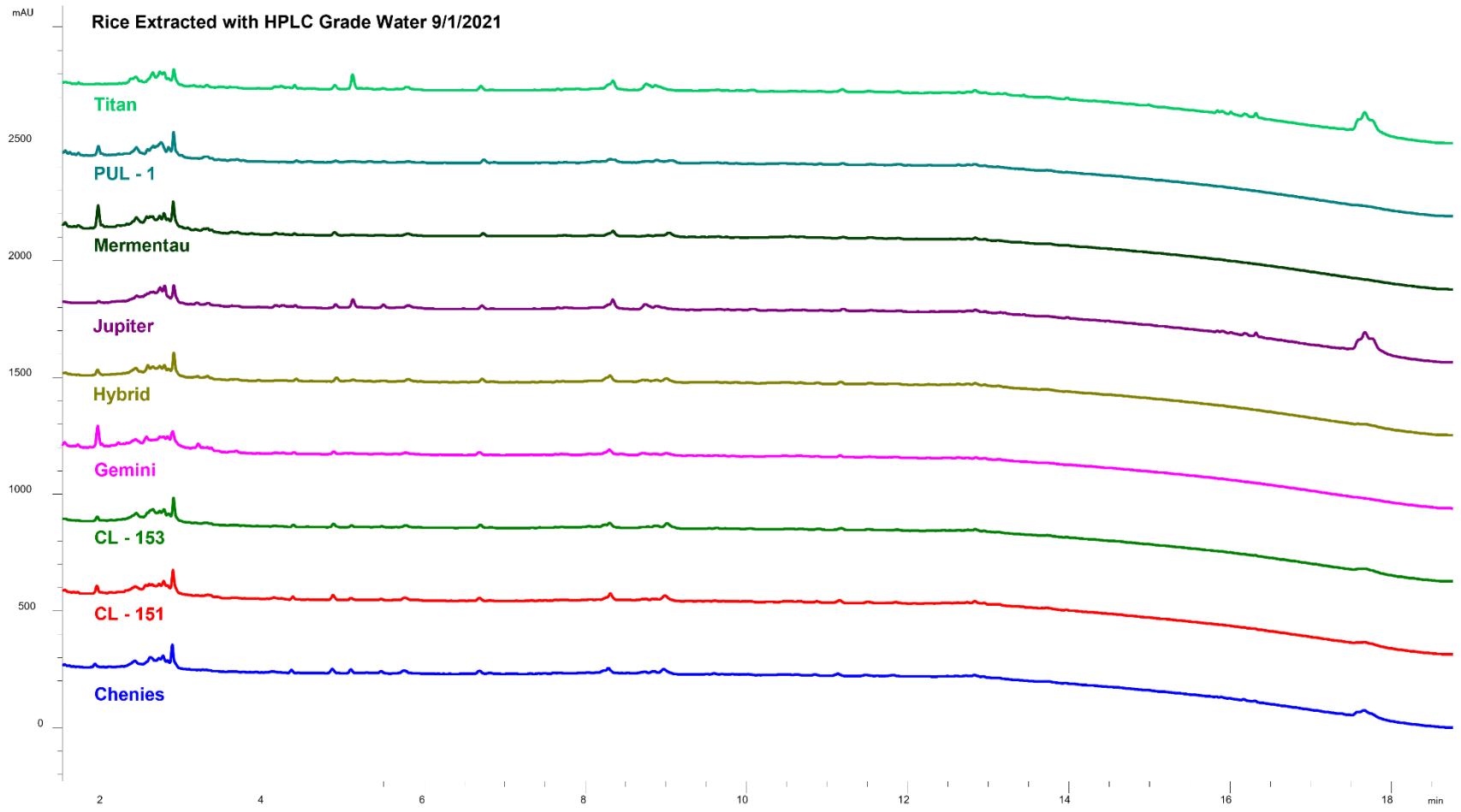


Figure 8. A chromatogram of nine rice varieties, extracted with water and tested with the HALO Diphenyl column. The Y-axis is milli-absorbance units, and the X-axis is in minutes.

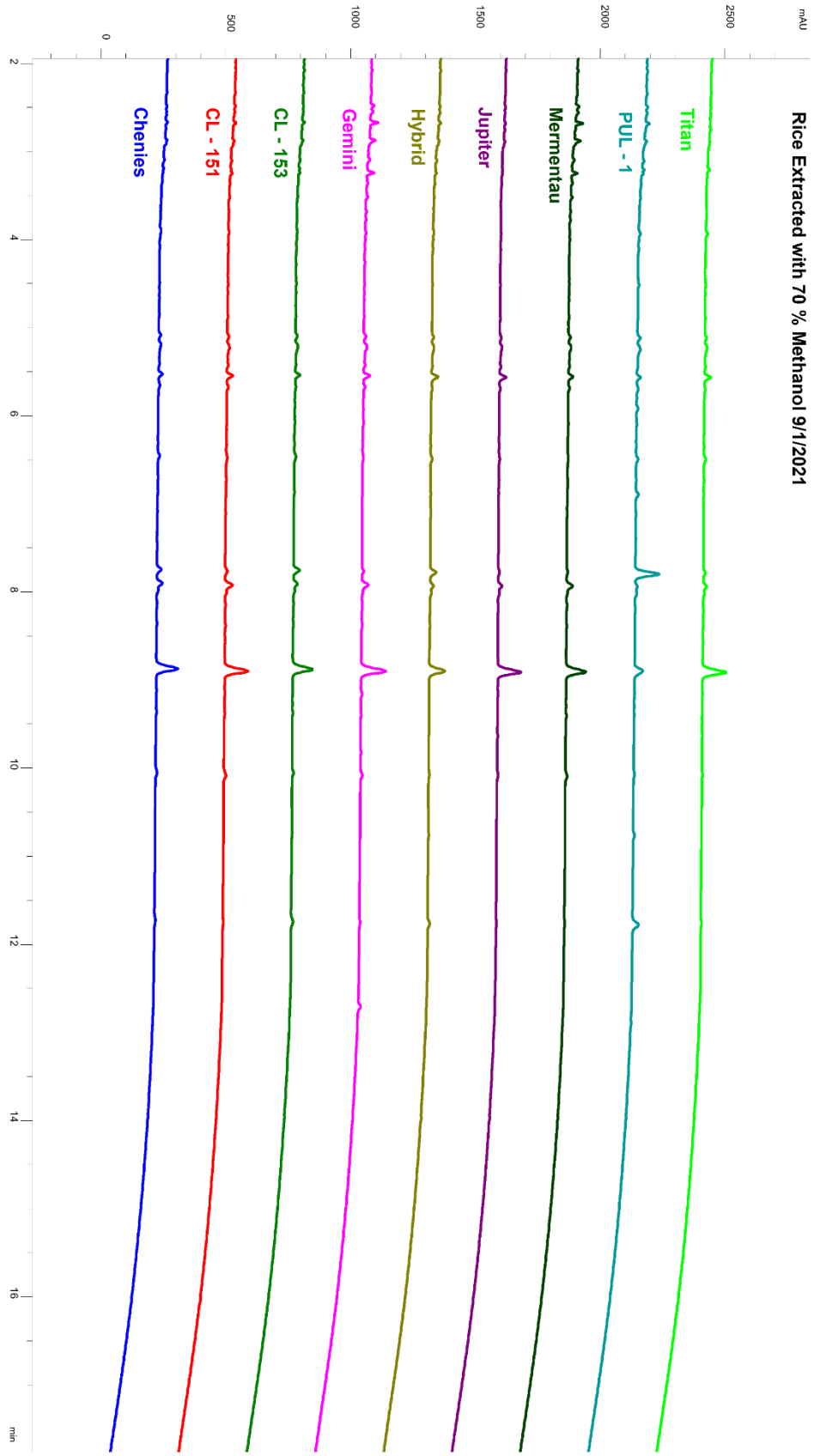


Figure 9. A chromatogram of nine rice varieties, extracted using 70% methanol and tested using the HALO Diphenyl column. The Y-axis is milli-absorbance units, and the X-axis is in minutes.

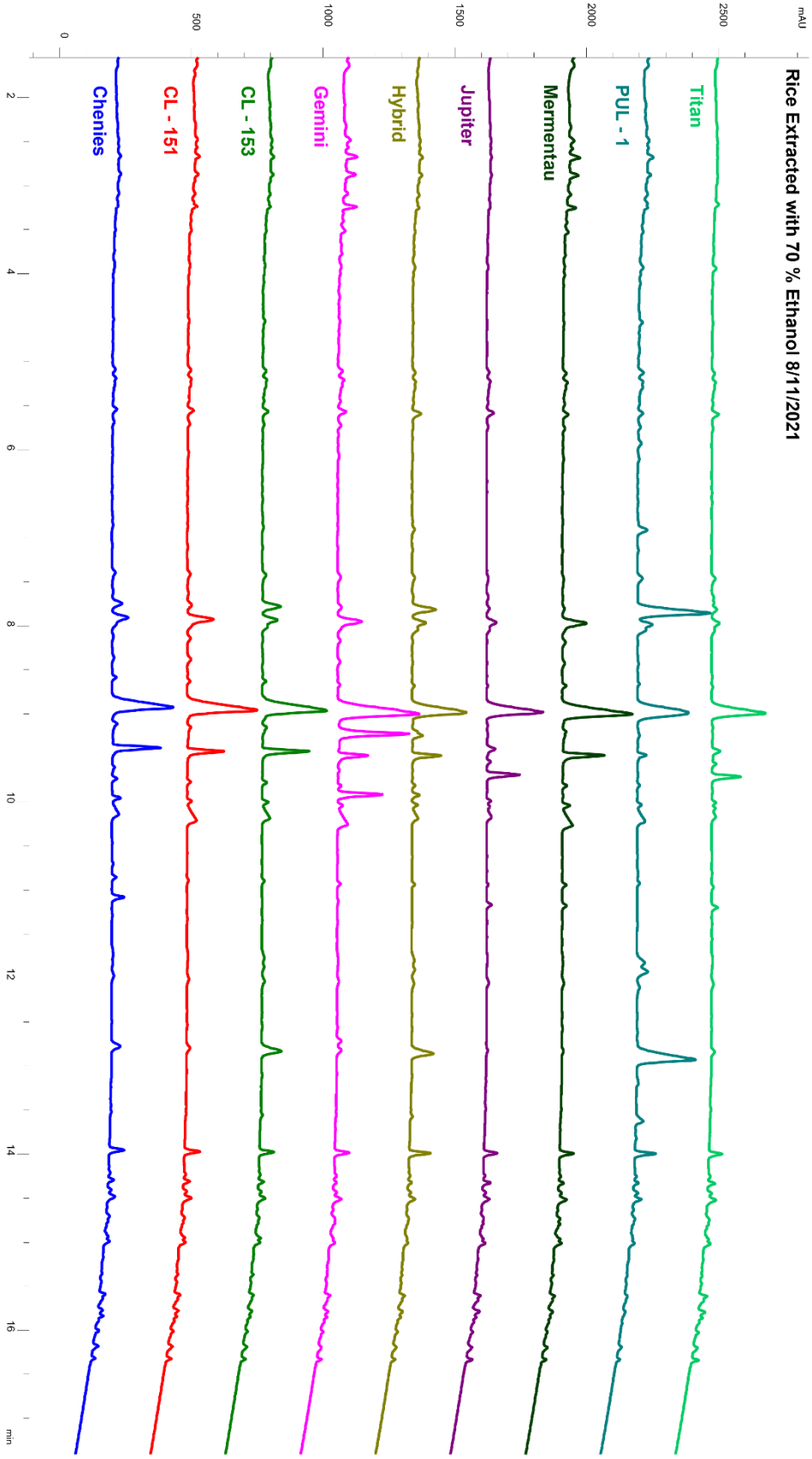


Figure 10. A chromatogram of nine rice varieties, extracted using 70% ethanol and tested using the HALO Diphenyl column. The Y-axis is milli-absorbance units, and the X-axis is in minutes.

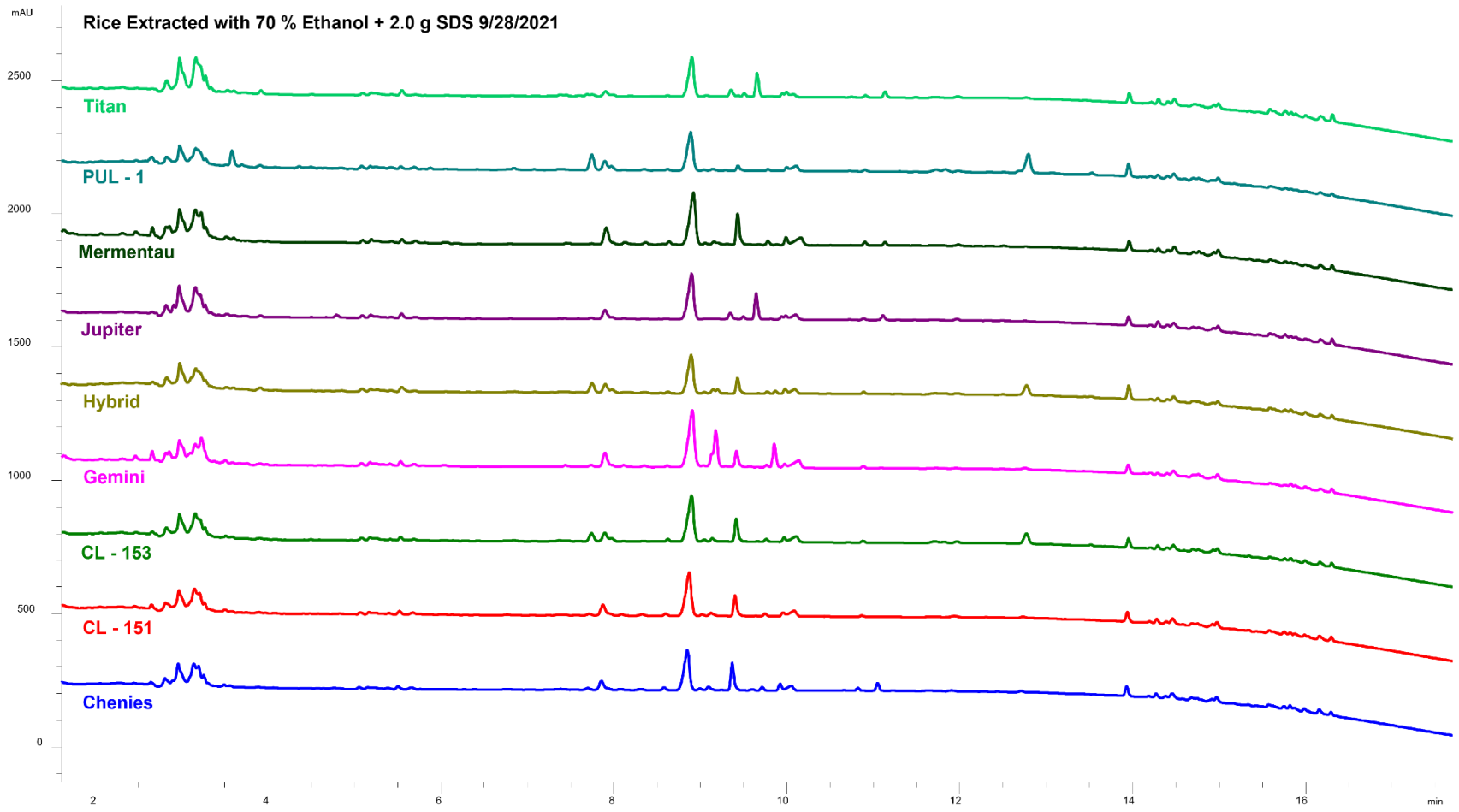


Figure 11. A chromatogram of nine rice varieties, extracted using 70% ethanol + 2.0 g SDS and tested using the HALO Diphenyl column. The Y-axis is milli-absorbance units, and the X-axis is in minutes.

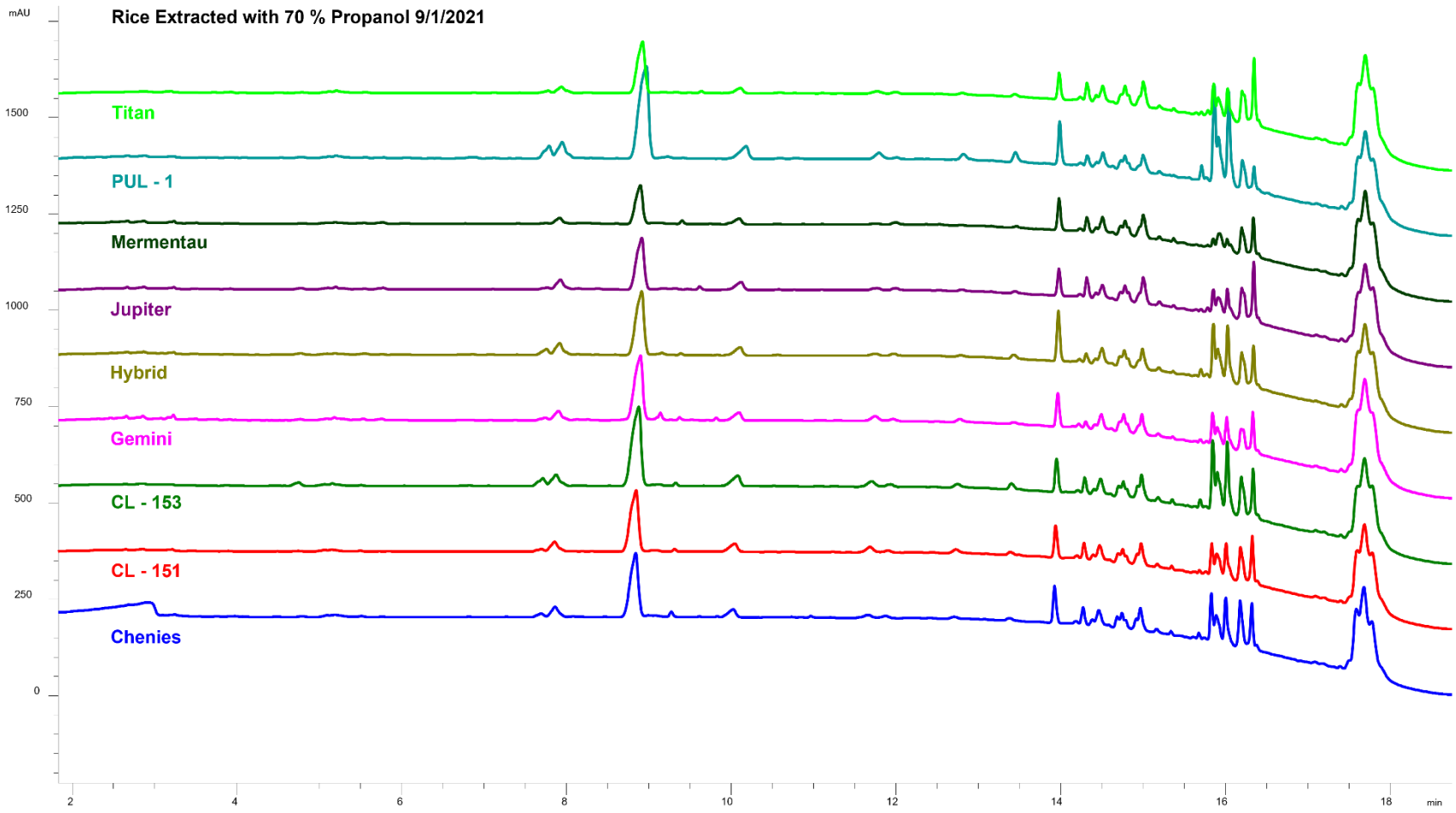


Figure 12. A chromatogram of nine rice varieties, extracted using 70% propanol and tested using the HALO Diphenyl column. The Y-axis is milli-absorbance units, and the X-axis is in minutes.

The chromatograms (Figures 8-12) feature the nine varieties of rice that were used to develop methods for extracting and analyzing proteins for verification. These particular chromatograms were tested using the HALO Diphenyl column, the more sensitive of the two columns. Notice that the most pronounced and defining peaks in the middle of the chromatogram all have similar patterns, making it challenging to definitively distinguish the varieties apart.

6.7.3. Hulling the Rice Seeds

Six sets of 100 rice seeds were counted out for the following experiment. The rice seeds were hulled using a barley pearler and a South Dakota Seed Blower was used to separate the chaff from the seeds. The purpose of hulling the seeds was as an attempt to minimize the excess chaff that could be ground and mixed with the desired powder. The lemma and palea on the rice seed do not contain alcohol soluble protein, and excluding them during the grinding process would lead to a purer ground flour to be weighed and extracted. Six hulling trials were completed, three trials were hulled for 7.0 seconds and three were hulled for 8.0 seconds.

Time Hulled	Seeds Left Intact			Average
7.0 s	15	37	24	25.3
8.0 s	2	12	13	9.0

Table 5. The data collected during the rice hulling experiments. Unfortunately, after running the hulled seeds through the HPLC prepared in the same way as the unhulled process, the hulling trials proved themselves unnecessary.

After the seeds were hulled, they were then prepared, extracted with the two extraction buffers (70% ethanol and 70% propanol), and analyzed the same way as the baseline sample. Hulling proved to not be beneficial to the experiments and instead consumed more time and was more cumbersome.

6.7.4. Extraction Flour Weight Trials

The next experiment involved testing different quantities of the ground rice flour to add into the graduated microcentrifuge tube for extraction. This experiment had its limitations as the need to create a greater concentration of proteins in the graduated microcentrifuge tube, the amount of extraction buffer had to stay constant. By increasing the ratio of ground powder to extraction buffer, but not changing the amount of extraction buffer, there was less available supernatant to draw from the graduated microcentrifuge tube and put into the filter vial.

The following amounts of powder were weighed and put into a graduated microcentrifuge: 0.060 g, 0.120 g, 0.240 g, and 0.480 g. Ultimately, it was found that the highest weight, 0.480 g of powder extracted with 700 µL of an extraction buffer, led to the taller and more defined peaks from the rice extraction trials. This was the expected result as increasing the

protein concentration would lead to more defined peaks in the chromatogram. Table 6 lists the

appropriate weights to be measured for each crop to ensure a proper extraction.

Two of these weight trials were completed, once with 700 µL of 70% ethanol and once with 700 µL of 70% propanol. Ultimately, there was little difference between the two buffers. Referring to Figure 10, in section 6.7.2 the chromatogram pictured is the result of extracting with 70% ethanol. The defining peaks are more centrally located, as ethanol is a neutral extraction buffer. Propanol is a less polar extraction buffer, which emphasizes proteins in the later part of the chromatogram. Conversely, methanol is a more polar extraction buffer, emphasizing the proteins that are featured during the earlier

time of the separation. Ultimately, if the extraction buffer is unknown, ethanol is the best option as an extraction buffer creating a good baseline for further development.

The weight trials were then extended to test a smaller range of flour weights, starting at 0.240 g and increasing by increments of 0.030 g until reaching 0.480 g. The theory behind extending this part of the experiment was that there would be weight where the definition of the peaks would be maximized and then would start to diminish. However, it was once again discovered that 0.480 g is the best amount of ground and sieved flour to extract from when working with rice. In Figure 13, it can be observed that the peaks in the line for 0.480 g have more defined peaks than the other lines in the chromatogram. This portion of the experiment was completed using the Halo Diphenyl column.

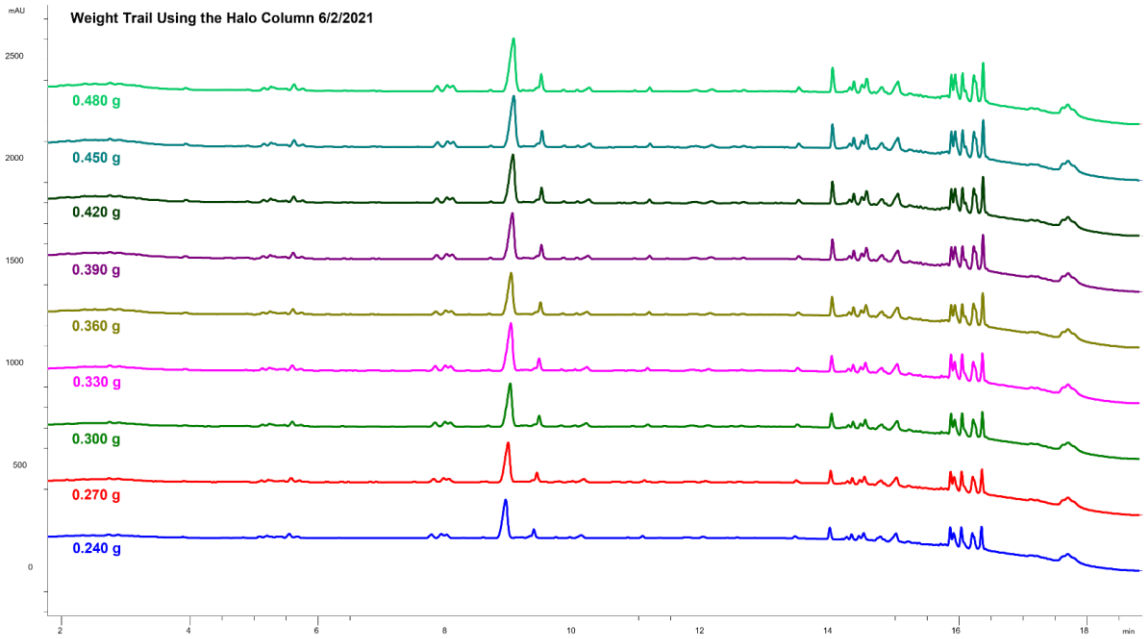


Figure 13. This chromatogram is the result of testing a smaller range of rice flour measurements. With each line in the chromatogram, the measurement of the ground rice flour increases by increments of 0.030 g. The top line of 0.480 g has the most defined peaks compared to the other lines. This was completed using the more sensitive Halo Diphenyl column.

	Weight (g)
Wheat	0.060
Triticale	0.060
Rye	0.060
Barley	0.060
Oat	0.060
Rice	0.480

Table 6. A table noting the appropriate amount of flour to weigh when preparing HPLC extractions.

6.7.5. Altering the Time Allowed for Extraction of Proteins

The extraction time refers to the time the sample will need to sit after the extraction buffer has been added to the microcentrifuge tube of ground and sieved flour. According to the FGIS method previously used, it is recommended to leave the samples to extract for a minimum of three hours at room temperature, or if overnight, in the freezer at -23C.

Eight microcentrifuge vials are filled with 0.480 g of ground and sieved rice flour and extracted with 700 mL of 70% propanol. A known rice variety was not used as an effort to save the limited amount of seed available. Instead, a large bag of mixed unknown rice varieties from an unknown year was used. This bag was sourced from the cold room in the SDSU Seed Testing Laboratory. After adding the propanol, the samples are shaken using the 120 V Fisher Pulsing Vortex and then left at room temperature throughout the duration of the experiment. One microcentrifuge tube is centrifuged, and the supernatant is pulled and pipetted into a filter vial every hour for eight hours.

Extraction Period (hr)	Centrifuge Time
0	8:32 AM
1	9:32 AM
2	10:32 AM
3	11:32 AM
4	12:32 PM
5	1:32 PM
6	2:32 PM
7	3:32 PM
8	4:32 PM

Table 7. The eight times that samples were pulled from extracting and were centrifuged and the supernatant was pipetted into a filter vial to prepared to run through HPLC.

The assumption behind experimenting with the time allotment of the extracting time was to see if proteins are extracted more by being left at room temperature longer, resulting in taller or more defined peaks. Ultimately, there were no dramatic results from this experiment. The three-hour extraction sample was used as the baseline for comparison. The peaks of the one-hour extraction were approximately half of that of the three-hours extraction. The peaks of the eight-hour extraction were only slightly larger than those peaks in the three-hour extraction. Also note there were no new peaks appearing as the samples sat longer. Figure 14 shows the chromatogram results from this experiment. The area on the chromatogram noted by the dashed box is the peak which was most defined and the easiest to compare for the experiment.

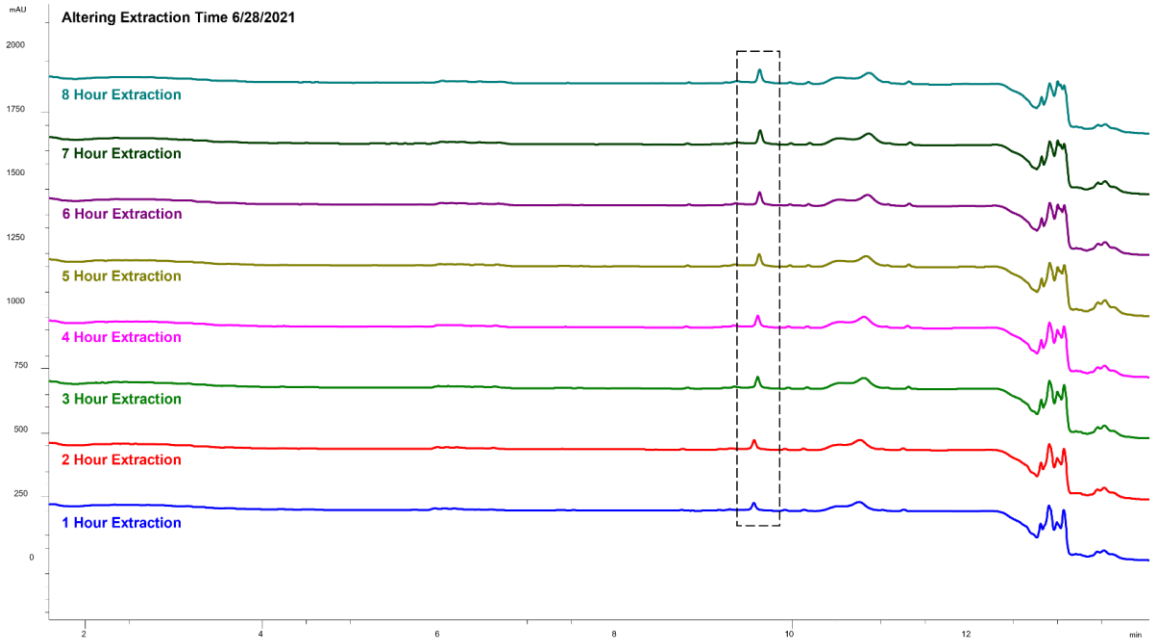


Figure 14. The chromatogram results of testing shorter and longer extraction times. The green, three-hour extraction line is the baseline of which all other lines were compared. The boxed section of the chromatogram is the peak used to best compare the results.

6.7.6. Altering the Time Allowed for Separation of Proteins

The correct time allotment allowing for total separation of the proteins is crucial for identification. The separation time is referring to the number of minutes the HPLC machine is programmed to run one sample, not including the time allotted for the HPLC to clean the previous sample from the column before starting the next sample. Based off the FGIS method, a 17-minute run, from injection to completing the clean was used. HPLC consultant, Dr. Merlin K. L. Bicking (ACCTA, Inc.), worked with the South Dakota Seed Testing Laboratory in May 2021, and suggested extending the sample run from 17 minutes to 18 minutes, adding the extra minute to the clean. Refer to Figure 15, a chromatogram of wheat which shows an example of a sample run from the initial injection to the end of the clean. Notice for wheat that most proteins are separated between minutes two and ten, with the clean starting at minute twelve and a half.

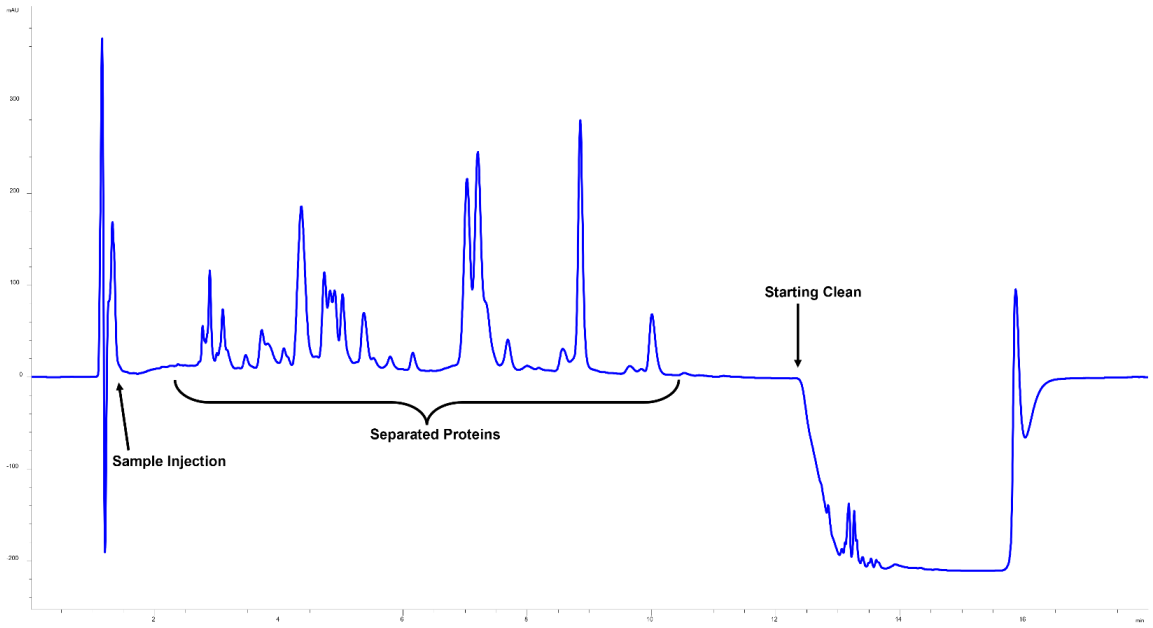


Figure 15. A chromatogram of spring wheat Ascend - SD. This is an example of an 18-minute run sample.

When running the rice with the 18-minute programmed run, the proteins of the rice took longer to be separated and detected by the HPLC machine, so the run sequence

for rice was extended to 23 minutes total with the clean starting at minute 17.5. This allowed more time for the proteins to separate without running into the clean on the chromatogram. The injection amount was also changed from 6 µL to 10 µL for the rice.

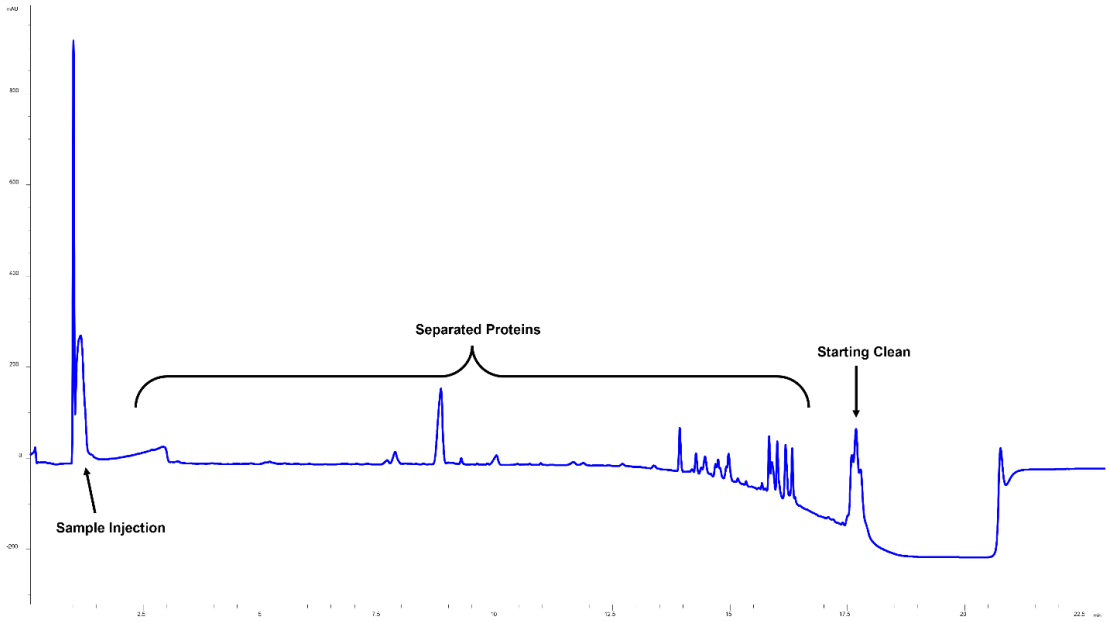


Figure 16. A chromatogram of rice variety Chenies. This is an example of a 23-minute run sample.

6.8 Results

After completing all the above-mentioned experiments, it was found that rice proteins can be extracted, separated, and detected by the HPLC machine. To do so, select 100 rice seeds at random, with or without the hull is acceptable. Grind the seed using a coffee grinder and sieve the seed with an U.S.A. Standard Test Sieve, No. 40, leaving a fine flour. Weigh the flour out to 0.480 g, put into a microcentrifuge tube and add 700 µL of 70% ethanol or 70% propanol (based on preference). Shake using a 120 V Fisher Pulsing Vortex for five minutes, and then allow the sample to sit at room temperature for a minimum of three hours. Centrifuge the samples, pipette the supernatant from the microcentrifuge tube and deposit into a filter vial. Prepare the HPLC machine by purging to remove all air bubbles in the capillaries. Run the sample on a column with sensitive

chemistry similar to the HALO Diphenyl column for 23 minutes, with an injection of 10 µL. The sample can then be analyzed using a program like ChemStation Computer Software.

7. Discussion

The research and methods presented by this paper have room for improvement and further exploration. Looking at the chromatograms presented in section 6.7.2., one will notice that even on the chromatograms exhibiting extractions by 70% ethanol and 70% propanol, it is difficult to find distinguishing peaks to identify the rice varieties when comparing to each other. Because of this, an analyst cannot definitively tell the varieties apart, preventing the SDSU Seed Testing Laboratory from testing rice varieties commercially for producers at present.

Aside from working with rice varieties, further HPLC research could include expanding the use of the machine to other field crops, native grasses, and potentially, legumes. The original purpose behind HPLC was to find an easier and quicker varietal identification method compared to polyacrylamide gel electrophoresis. Ultimately, the machine and the current methods have proved themselves incredibly successful with testing cereal crops such as wheat, oats, barley, rye and triticale.

Works Cited

Agilent Technologies. “HPLC Basics: Fundamentals of Liquid Chromatography.”

Aryal, Sagar. *HPLC - Definition, Principle, Parts, Types, Uses, Diagrams*. 2019, MicrobeNotes.com. microbenotes.com/high-performance-liquid-chromatography-hplc/. Accessed 6 Feb. 2021.

Bicking, Merlin K. L., ACCTA, Inc. Personal Consultation. May 2021.

Bietz, Jerold A. "High-Performance Liquid Chromatography of Cereal Proteins." *Advances in Cereal Technology*. Vol. VIII, American Association of Cereal Chemists, 1986, pp. 105-170. *Google Scholar*. Accessed 16 Feb. 2021.

Boligon, Aline A., and Margareth L. Athayde. "Importance of HPLC in Analysis of Plants Extracts." *Austin Chromatography*. Austin Publishing Group, 2014. Accessed 7 Dec. 2020.

Branlard, Gerard, and Emmanuelle Bancel. "Protein Extraction from Cereal Seeds." *Methods in Molecular Biology*. Humana Press Inc., 2007, pp. 15-25, Google Scholar. Accessed 10 Dec. 2020.

Dong, Michael W. *Modern HPLC for Practicing Scientists*. 2006.

Hay, R.L., K.H. Sutton. “Identification and discrimination of New Zealand bread wheat, durum, and rye cultivars by RP-HPLC.” *New Zealand Journal of Crop and Horticultural Science.* 1989, 1990. Published online 2012.

Hoffman, Walter F. "An Alcohol-Soluble Protein Isolated from Polished Rice." *The Journal of Biological Chemistry*. Division of Agricultural Biochemistry, University of Minnesota, St. Paul, 1925, pp. 501-04, Google Scholar. Accessed 30 Jan. 2021.

Hoseney, R.C. “Chapter 3: Cereal Protein.” Principles of Cereal Science and Technology. *American Association of Cereal Chemists, Inc*., 1986, pp. 69-87.

HPLC Columns: Understanding the Basics, Types, and Applications. whatishplc.com. whatishplc.com/hplc/hplc-column/. Accessed 10 Feb. 2021.

Liang, Joshua Y. Federal Grain Inspection Service. Personal communication with Dr. Brent Turnipseed. 14 Nov 2017.

Lookhart, G L., and J A. Bietz. "Practical Wheat Varietal Identification in the United States." *Cereal Foods World*. Vol. 35, American Association of Cereal Chemists, 1990, pp. 404-07, Google Scholar. Accessed 28 Jan. 2021.

Lookhart, G. L., et al. "Comparison of Polyacrylamide Gel Electrophoresis and High-Performance Liquid Chromatography Analyses of Gliadin Polymorphism in the Wheat Cultivar Newton." *American Association of Cereal Chemists, Inc.*, vol. 63, no. 6, 1986, pp. 497-500.

Rice Bran Oil Manufacturing Process. AGICO Group. www.gcmachines.com/rice-bran-oil-manufacturing-process.html. Accessed 10 Feb. 2021.

Shen, Chang-Hui. *Quantification and Analysis of Proteins*. 2019, Academic Press. www.sciencedirect.com/science/article/abs/pii/B9780128028230000080. Accessed 7 Feb. 2021.

Shewry, Peter R., and George L. Lookhart. *Wheat Gluten Protein Analysis*. St. Paul, Minnesota, AACC International, Inc., 2003, pp. 61-89.

Sutton, K.H. “Seed Quality Measurements by RP-HPLC.” Seed Symposium 1991.