THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF FOOD SCIENCE

UTILIZING PROTEIN-POLYPHENOL INTERACTIONS TO DEVELOP A GLUTEN-FREE BEER

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Fall 2017

A thesis
submitted in partial fulfillment
of the requirements
for baccalaureate degrees
in Spanish and Food Science
with honors in Food Science

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Abstract

In the United States alone, one percent of the population suffers from celiac disease, an autoimmune disorder resulting from a negative response to gluten in the small-intestine. The only current effective treatment is adherence to a gluten-free diet. However, many popular foods contain low levels of gluten, which only exacerbate the symptoms. Beer is a delicious and widely consumed beverage that contains low levels of gluten. The development of a gluten free beer would provide a safe alternative for patients with celiac disease and capitalize on a growing market for gluten-free foods. These experiments were conducted to evaluate the ability of tannic acid to remove gluten from a model beer system and Irish style ale. By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and ultraviolet–visible spectroscopy suggesting the efficacy of tannic acid to remove gliadin in beer, total quantification of protein in model beer was carried out using EZQ assay. Tannic acid in the model beer was measured using the Folin-Ciocalteu method to optimize required tannic acid in the American amber ale. The level of gliadin in model beer was reduced 90% to below 20 ppm. The total level of protein in the ale was reduced 40% at the same ratio of added tannic acid to protein. Unlike the known level of gliadin in model beer, the proteins in the ale are diverse and not purely gliadin. The findings could provide insight for breweries interested in reducing the level of gluten in beer to have fewer than 20ppm of gluten required for gluten free labeling.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express my gratitude for the advising and guidance throughout my time at Penn State. First, thank you to my thesis advisor, Dr. Elias, for continued academic support over the years. Thank you to my honors advisor, Dr. Hayes, for guidance as I entered the Food science major with many questions. I would also like to extend a thank you to Dr. Charlene Van Buiten and Benjamin Chrisfield for their mentorship and helpful suggestions. Outside of the Food Science Department, thank you to my family and friends at Penn State.

Chapter 1

Introduction

Gluten and Celiac Disease

The term "gluten" refers to the proteins known as the prolamins and glutenins from wheat, barley, rye and oats. Prolamins are alcohol-soluble proteins that are rich in proline and glutamine. Depending on the grain source, prolamins are labeled as gliadin for wheat, hordein for barley, secalin in rye or avenin in oats depending on the source grain[1].Gluten provides nutrition, but also contributes to the structure and texture of foods. Gluten can be measured or detected by an enzyme-linked immunosorbent assay (ELISA), polymerized chain reaction (PCR), and mass spectrometry (MS) [2].

A growing health concern in the United States is celiac disease. Celiac disease is an autoimmune disorder that can occur in genetically predisposed people where the ingestion of gluten leads to damage in the small intestine [3]. This differs from a wheat allergy, which results from an immunoglobulin E (IgE)-mediated reaction [2]. In celiac disease, the immune system responds to gluten by attacking the small intestine and inhibiting the absorption of important nutrients into the body. Symptoms of celiac disease include diarrhea, abdominal pain, flatulence, indigestion or weight loss, and irritability [4]. It is estimated to affect one percent of people worldwide [5]. In 2013, survey results from the National Health and Nutrition Examination Survey (NHANES) indicate that 1.69% of the population in the United States is avoiding gluten even as they do not suffer from celiac disease [6]. FDA established, among other criteria, a gluten limit of less than 20 parts per million (ppm) for foods that carry the label "gluten-free,"

"no gluten," "free of gluten," or "without gluten" [7]. The need to eat gluten-free diets to manage celiac disease has spurred a trend of gluten-free diets and foods. The US market for gluten-free products was estimated to be worth US$10 billion in 2013 and the sales of gluten free products in 2016 grew to reportedly $13.7 billion dollars [8].

Beer Protein

Beer contains roughly 300-800mg/L of protein material consisting of polypeptides in size from 5 to 100kd [9]. For commercial lager beers, the gluten determination was between 3-8.7mg/L. while wheat beers had a concentration of 10.6-41.2 mg/L [10]. Levels of gliadin specifically have also been quantified in several ales at levels 7.3 mg/L for English pale ale and 14.6–15.3 mg/L for American pale ales [11]. Much of the gluten in beer is from barley hordeins. At the same time, the gluten content of barley is variable and has been reported to range from 18.8-45.0 g/Kg [10]. Although the malt contributes gluten, less than 2% of this gluten is typically transferred to the sweet wort. From the lautering step to the addition and boiling of hops, there is a negligible reduction in gluten. The primary and secondary fermentations lead to decrease in pH, which promotes precipitation of some of the gluten. Proteins known to induce the haze in beer are largely hordeins, which are rich in proline, and some albumin or globulin derived polypeptides [12]. To remove gluten, Proline-Specific Endopeptidases have been shown to be effective in removing all immunostimulatory gluten peptides [13]. At the same time, previous work with model systems has shown that haze-active proteins contain significant amounts of proline. Meanwhile proteins that lack proline form little or no haze with added polyphenols [12].

Haze-active protein is higher in beer than the other beverages such as juice or wine [14][15]. The primary source of beer haze results from cross-linking of polyphenolic compounds

and protein, which typically is high in proline. However, some haze may arise from other factors including remaining starch and microbial sources [16]. Very little protein is needed to cause haze with previous working showing a concentration of 2mg/L being necessary [16]. Although temporary chill hazes resulting from crosslinking of low weight polyphenols with hydrogen bonds during chilling, polymerization of the polyphenols leads to permanent haze complexes [17]. Higher temperature may cause protein-polyphenol complexes to dissolve. Partial denaturation of protein during boiling in brewing exposes more polyphenol binding sites. Maximum haze occurred near pH 4 with less haze at higher and lower pH's [15].

There are several ways to assess the protein and gluten in beer. The Bradford dye binding assay measures larger molecular weight proteins, but hardly responds to proline rich haze-active protein in beer [18]. This is possibly because Coomassie blue is biased toward basic and aromatic amino acids, which comprise only a small percentage of the haze-active protein of interest [19]. The Lowry assay may overestimate by also measuring non-protein components. Bromo pyrogallol red dye has been shown to be more effective by binding to proline rich peptides of interest, but requires removal of polyphenols [20]. Haze has been measured by turbidimetry showing tannic acid added to beer results in increased turbidity until turbidity begins to plateau at roughly 50 NTU [21]. This plateau has been observed in similar studies when either protein or polyphenol concentration increases while the other component concentration is constant [19]. When assessing the gluten content in beers by sandwich and competitive ELISA assays, Dostálek et al. found that the competitive ELISA assay reported much higher levels of gluten than the sandwich assay [10]. Siebert et. al. found that adding free proline did not increase beer haze, but smaller complexes may be more soluble and not precipitate.

Bentonite clay, silica gel, tannic acid fining, proteolytic enzyme treatment, and ultrafiltration can remove protein. However, silica is more common for beer than bentonite to reduce the removal of desirable foam active proteins [15]. Protein high in proline can form a haze that has been measured by light scattering or turbidimetry. Commercial beers contain a significant amount of haze-active protein and lower level of haze-active polyphenols when compared with wine [15]. Gliadin from wheat is a suitable protein for model beer systems because of its high proline content [12]. When assessing haze formed between gliadin and catechin versus gliadin and tannic acid, gliadin has produced more haze in combination with tannic acid. Reasons for this difference may need to do with the size of tannic acid being larger than catechin and has a higher level of hydroxyl groups for binding to protein.

Beer Phenols

In beer, nearly all the haze-active polyphenol present is likely bound to proteins in which the majority of the polyphenol binding sites are unoccupied[15]. There are several phenolic compounds in beer, but the beer polyphenols most closely associated with haze formation are the proanthocyanidins (dimers of catechin and epicatechin) [15]. Prominent proanthocyanidin species present in beer are procyanidin B3 and prodelphinidin B3 [19]. Dimers of procyanidins are preferred for forming haze complexes, but tannic acid is commercially available. There are differing opinions regarding the effectiveness of hops and malt polyphenols. Some argue polyphenols in hops are more haze active than those from malt [18] while others report hops and malt polyphenols are equally responsible [22].

To proactively stimulate protein-polyphenol complexes, tannins can be added to finished beer. Catechin can be used in place of tannic acid to from protein-polyphenol complexes, but has

been shown to be less active, potentially because of its one o-diphenol group and one m-diphenol group [12]. At the same time, catechin in beer may be desirable given the role of catechin as an antioxidant. Removing excess polyphenols can be done by polyvinylpolypyrrolidone (PVPP). PVPP adsorbs flavonoids and tannins as well as desirable antioxidants and can be added at different parts of the brewing process. Other processes including gelatin fining, egg albumin fining, casein fining, and isinglass fining can also remove polyphenols [15].

Total phenols can be measured through the Folin-Ciocalteau method and tannic acid in beer has been determined by high performance liquid chromatography with a multi-solvent gradient system [23]. One of the limitations to determining tannic acid in beer after addition of PVPP is based on a free gallic acid curve [23]. There are other factors that affect the study of the protein-polyphenol complexes. These include alcohol, hydrogen ion (pH), amino acids, metal ions, and carbohydrates. Alcohol may increase solubility of proteins and polyphenols. pH affects protein conformation–interactions between proteins and polyphenols [12], [24].

Objective

This project is significant because it explores a food processing option to enable the consumption of food products that would otherwise harm nearly 3 million individuals living with celiac disease in the United States. Moreover, results from the National Health and Nutrition Examination Surveys observed that the prevalence of individuals without celiac disease who are avoiding gluten has tripled between 2009 and 2014 [6]. If development of a gluten free all grain beer is successful, it may allow traditional brewers to enter the growing gluten-free space that has experienced 17.9% increase in sales from 2013 to 2016 [8]. The objective of this study was

to assess the efficacy of tannic acid to precipitate and remove gluten from a model beer system, and then develop a gluten-free beer based on these findings.

Chapter 2

Materials and Methods

Model Beer System

Model beer was prepared using an acetate buffer 0.01M, pH 4.5, 5% alcohol (v/v). Gliadin from vital wheat gluten was added to a final concentration of 200 mg/L. Tannic acid (TA) was added to achieve concentration of 0-2000 mg/L to the model beer. Turbidity was measured to determine the extent of aggregation of protein upon introduction of tannic acid by UV-Vis spectroscopy at 800 nm (Thermo Scientific Genesys 10S UV-VIS spectrophotometer) in triplicate.

Qualitative removal of soluble protein was observed through Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The model beer samples with the addition of tannic acid were centrifuged for 10 minutes at 10,000rpm. 20µL of supernatant was combined with an equal volume of load buffer (10 µL β-mercaptoethanol and 950 µL Laemmli buffer). Samples were incubated at 99 °C and shaken at 400 rpm for 10 minutes. Samples of 10 µL and 4 µL wide range molecular marker k494-500UL were loaded into a precast mini Protean TGX gel (Bio-Rad) containing 0.125 M Tris-HCl buffer (BioRad PowerPac HC). Samples were subjected to 150V for 45 minutes. Samples were stained with PageBlue™ Protein staining solution overnight with agitation before observation.

The total protein was assayed fluorometrically using EZQ® Protein Quantitation Kit (R33200) (Thermo Scientific Fluroskan ascent FL) with a minor adjustment of sample for standard curve selection of gliadin rather than ovalbumin. The solutions were centrifuged to separate precipitate. Samples for the gliadin standard curve ranged from 0-200 mg/L.

Total phenols were quantified via the Folin-Ciocalteu assay with a calibration curve prepared using gallic acid. Model beer was centrifuged for 10 minutes at 10,000 rpm. 20µL of supernatant was mixed with 1,5800 µL water and 100 µL of Folin-Ciocalteu reagent (Sigma, St. Louis, MO). The assay mixtures were shaken for 5 min, followed by the addition of 300 µL Na_2CO_3 solution was then added. Samples were incubated for 30 minutes at 37 °C. The absorbance at 765 nm was measured using a Hitachi U-2000 Spectrophotometer (Hitachi, Tokyo, Japan). Tannic acid concentration was compared between the tannic acid stock and model beer to assess tannic acid consumption and excess.

Table 1. Model beer system samples for UV VIS, EZQ, and Folin-Ciocalteu. All samples were prepared with 200 mg/L gliadin. Controls of tannic acid were prepared were prepared for each sample without gliadin as noted Ac, Bc, etc.

Sample with 200 mg/L Gliadin	Tannic Acid Concentration (mg/L) for turbidity	Tannic Acid Concentration mg/L for SDS-PAGE, protein and phenol measurements
A	0	0
B	25	100
C	50	400
D	100	500
E	150	1600
F	200	2000
G	250	
H	300	
I	400	
J	500	

Ale

Ale was brewed using barley and amber dry malt extract**.** Willamette hops were added at the beginning of the 45-minute boil. Yeast was pitched at 40°C. The primary fermentation was completed over 14 days followed by a secondary fermentation of four days before analysis.

Tannic acid was dosed into the ale at levels of 200 mg/L and 200 mg/L before vortexing and resting for ten minutes prior to analysis. As not all tannic acid is used in beer stabilization, the total phenol level was compared between the beer with and without added tannic acid. Samples containing tannic acid were centrifuged for 10 minutes at 10,000rpm. Following the centrifugation, the supernatant was decanted off of the precipitate. The ale total protein and total phenols were quantified using the using EZQ® Protein Quantitation Kit and Folin-Ciocalteu described above.

Removal of phenols was also considered. The addition of PVPP was also necessary due to natural phenols present already in the ale, whose concentration exceeded the range of the method. The samples series was tested with the addition of PVPP at the recommended levels of 200g/L used to enhance the stability of beer during production and storage [16]. Samples containing tannic acid were centrifuged for 10 minutes at 10,000rpm. Following the centrifugation, the supernatant was decanted off of the precipitate. PVPP was added directly to the supernatant before analysis by EZQ® Protein Quantitation Kit and Folin-Ciocalteu. The treatments were compared by a one-way ANOVA and grouping by Tukey method using Minitab..

Chapter 3

Results and Discussion

The gliadin-tannic acid complexes form a white precipitate that sediments below the solution. When vortexed, these complexes form a characteristic haze indicated by higher absorbance at 800nm in Figure 1. Minimal tannic acid is needed (25 mg/L TA for 200 mg/L gliadin). The peak turbidity was seen with the addition of 200 mg/L of tannic acid. The decrease in absorbance with higher additions of tannic acid may occur due to flocculation given the thermodynamic instability as well as polymerization of the complexes with many binding sites as proposed by Siebert et al. [19]. Additional variability in particle size may also impact the turbidity of the model beer system.

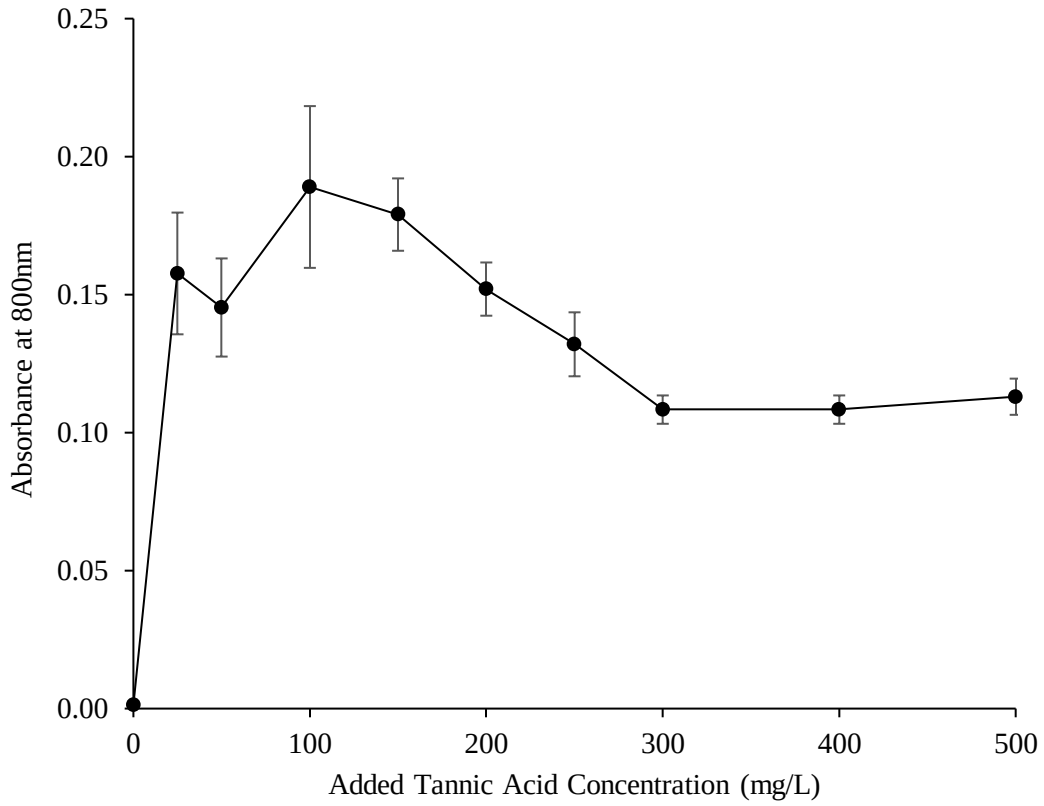


Figure 1. UV-Vis Absorbance of model beer system at 800nm with the addition of TA.

To qualitatively confirm the removal of protein from the model beer system, Figure 2 shows the reduction in observed protein from A (200 mg/L gliadin without tannic acid) with the addition of tannic acid. The multiple bands for channel A highlight that gliadin consists of multiple protein fragments. In channel C there was virtually no visible protein. The blue smears, particularly in samples E, F, and Fc, suggest that components other than protein have been stained. The tannic acid causes a blue smear even without the presence of gliadin. SDS-PAGE for these samples was repeated and produced the same smearing.

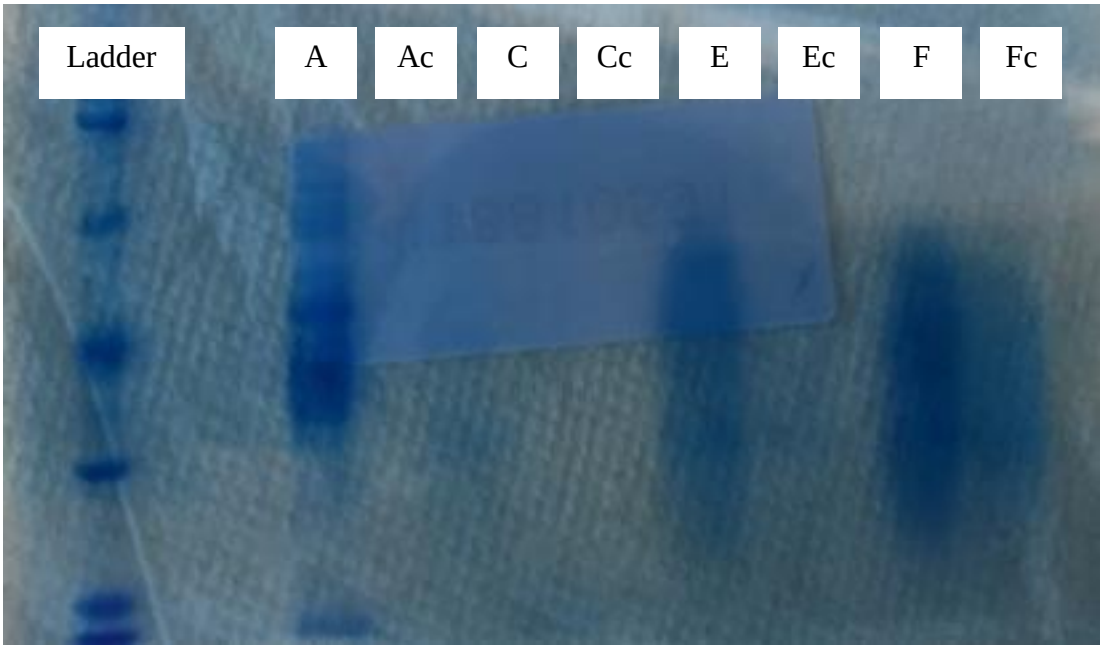


Figure 2. SDS-PAGE gel of model beer with the addition of tannic acid.

Figure 3 shows a plot of both total phenols and gliadin in the model beer system. It reflects the relationship between gliadin concentration in the model beer and uptake of tannic acid to form complexes. Corresponding to the peak turbidity shown when tannic acid is added at 200 mg/L to the model beer system, there is a sharp decline in gliadin present in solution marked with a dashed line. The decline in gliadin concentration is very pronounced until a level of 500

mg/L tannic acid is reached, at which point the reduction in soluble gliadin is too low to be accurately quantified.

The addition of tannic acid to the model beer system has a slower increase in observed total phenols than the pure tannic acid control. Tannic acid, added at a low-level, binds to available gliadin sites. However, the level of total phenols in the model beer system never achieves a level close to the pure tannic acid solution despite having exceeded the binding capacity of the gliadin. Some tannic acid may become trapped within the gliadin-tannic acid precipitate and removed during centrifugation. Moreover, at higher concentrations of tannic acid, solubility is reduced. Although appropriate dilutions within range of precision were used, total phenol measurements recorded at the upper range may reflect a greater margin of error.

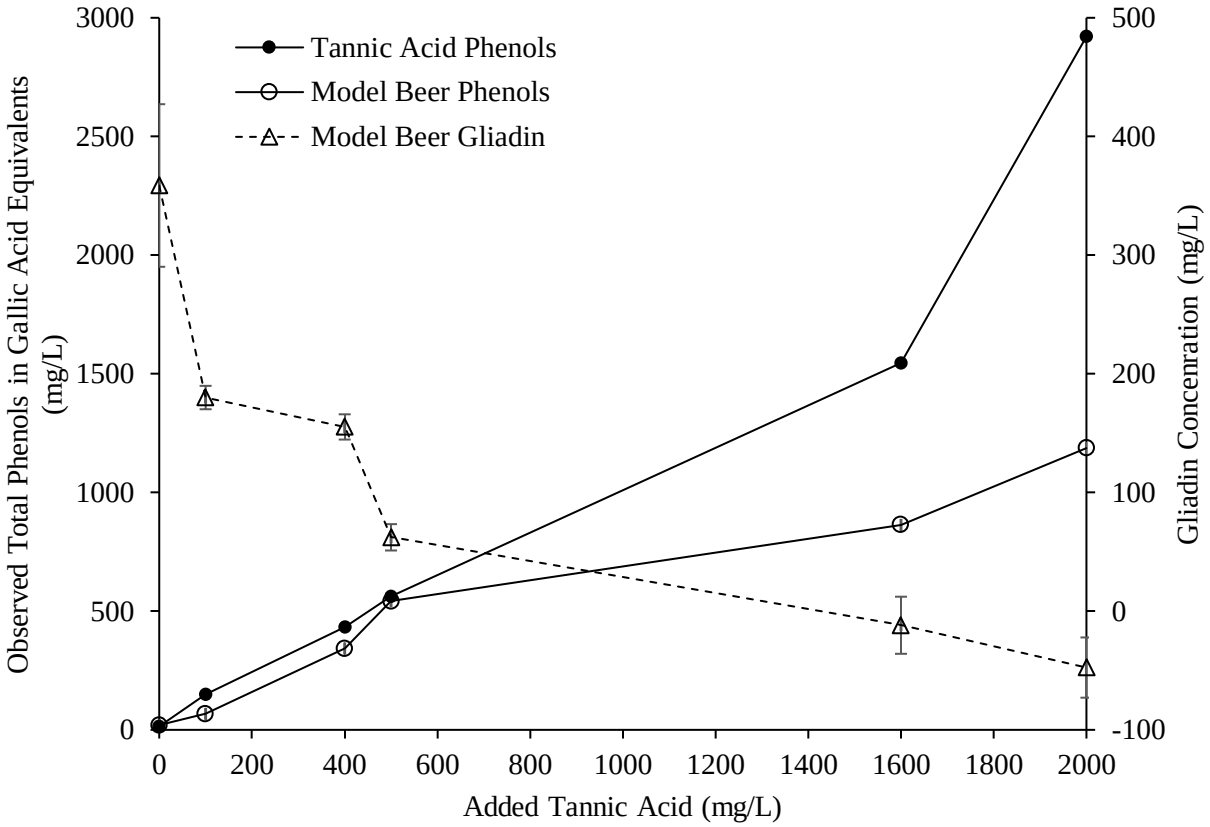


Figure 3. Model beer phenol concentration with increasing levels of added tannic acid plotted alongside model beer protein concentration.

Following the patterns exhibited in the model beer system, the initial total protein concentration in the ale was 227 mg/L. The total soluble protein in the beer was reduced by nearly 50% with the addition of 200 mg/L, but did not significantly decrease at a higher level of tannic acid of 200 mg/L. Due to the variety of proteins and peptides in beer, the remaining protein may not consist of gluten. Although the addition of tannic acid greatly impacted the total protein content, it constituted a marginal increase in total phenols relative to the initial concentration of phenols in the beer. The addition in PVPP at 200 mg/L reduced the total phenols by over 30% to a level lower than initial phenol content in the ale. PVPP likely extracted both excess tannic acid and other phenols. The differences between total phenol concentration for the ale, ale with tannic acid, and ale with tannic acid and PVPP were significant ($p<0.05$).

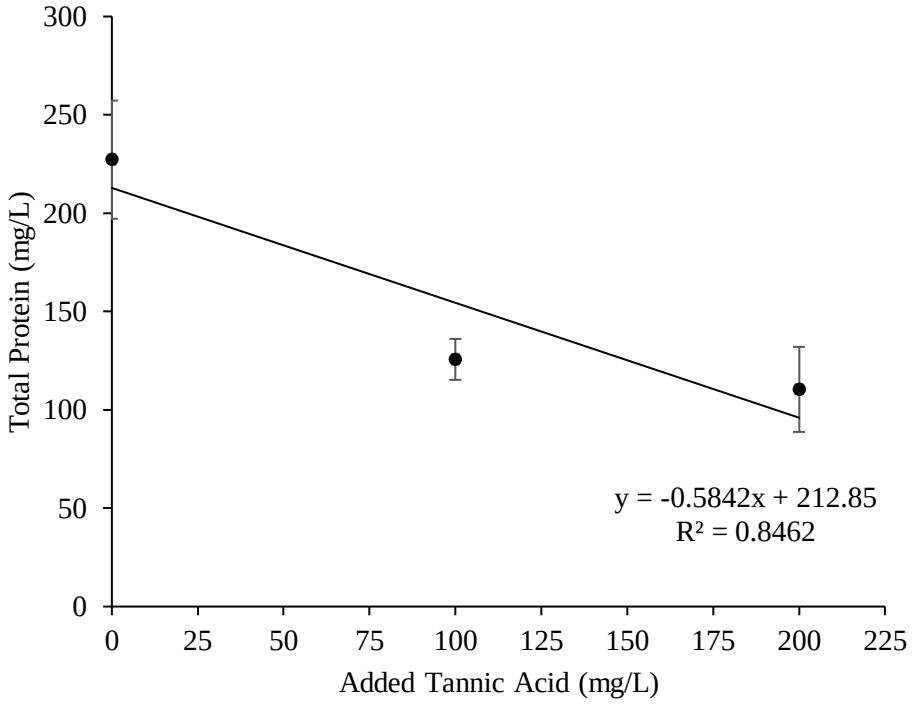


Figure 4. Ale total protein concentration with addition of tannic acid only.

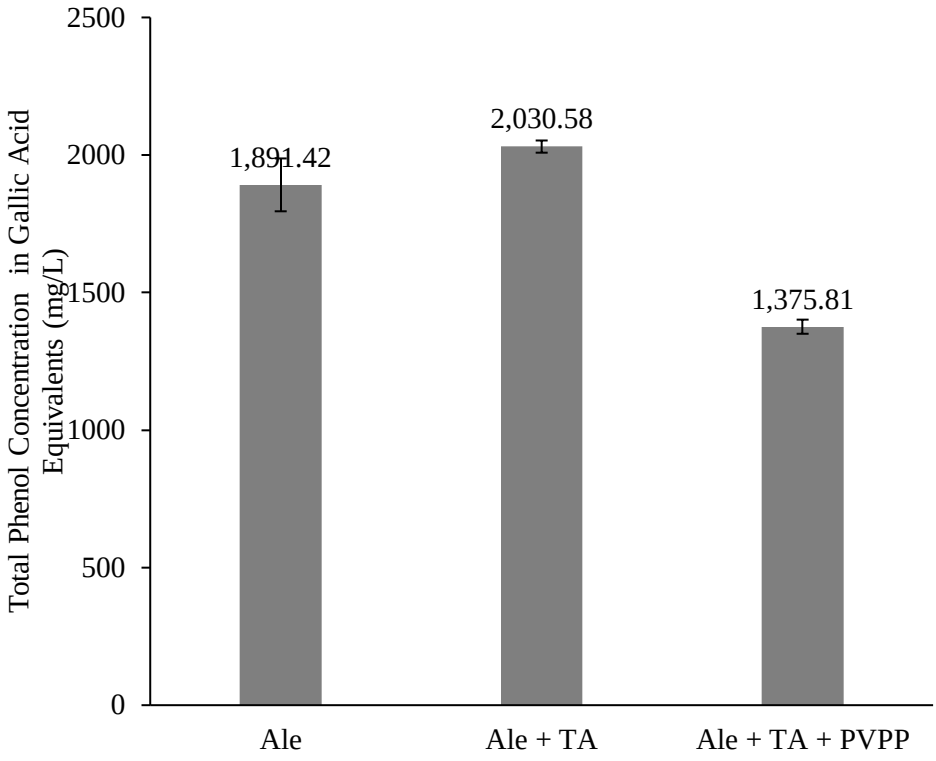


Figure 5. Ale phenol concentration with additions of TA at 200 mg/L and PVPP at 200 mg/L.

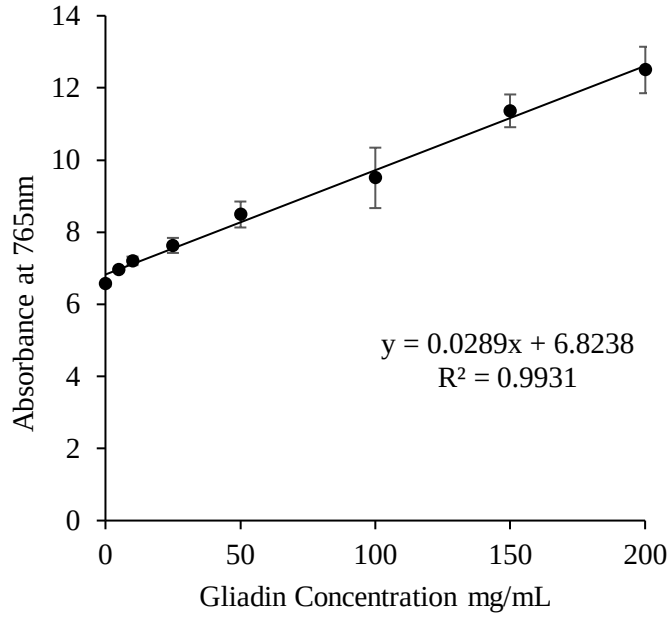
Chapter 4

Conclusions and future directions

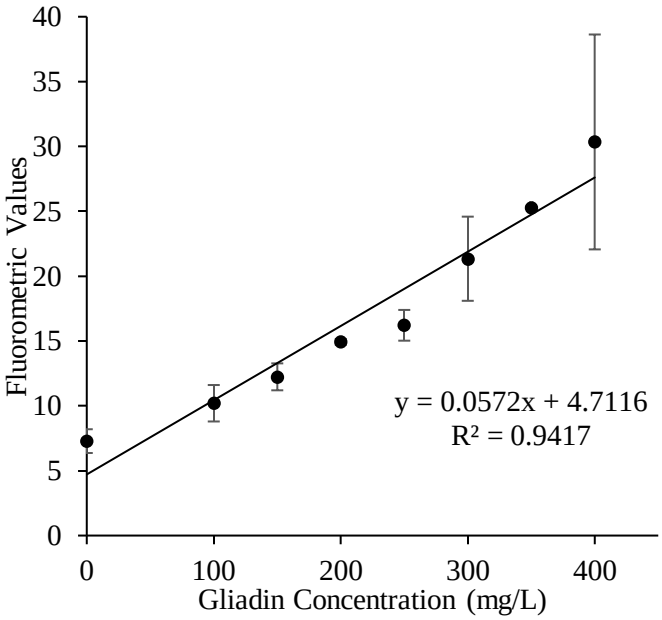
The results for the model beer and Irish style ales showed tannic acid can be effectively used to reduce the levels of gluten in beer. Tannic acid effectively removed gliadin from the model beer system via complexation to form a precipitate at low levels of addition. The plateau in protein level indicated the addition of excess tannic acid. Although the exact mechanism is slightly unclear, the model proposed by Siebert et al. was supported through these experiments [19]. The solid was removed and discarded by means of centrifugation and also yielded a brighter beer. Filtering the beer by a means other than centrifugation is also a possibility. While removing protein from the beer, it remains a question if all protein removed consisted of gluten fragments or other proteins and if levels of gluten remain below the limits of detection. PVPP effectively countered the excess tannic acid in the beer. PVPP may have removed both tannic acid and other phenols related to stability, foaming, and flavor.

The next steps include quantifying the protein after both tannic acid addition and PVPP addition. It would also be advantageous to consider protein assessment using bromo pyrogallol for assessment of the smaller immunogenic peptides. Although the EZQ® Protein Quantitation Kit measured total protein, it was also unable to discriminate gluten from hordeins or other proline-rich peptides. Several further methods of gluten analysis would address this challenge. One concern not explored is the change in taste and texture of the beer after the additives as well as sensory analysis as a whole. A shelf life study of the beer would also capture changes resulting from the reduction in gluten and other phenols during storage.

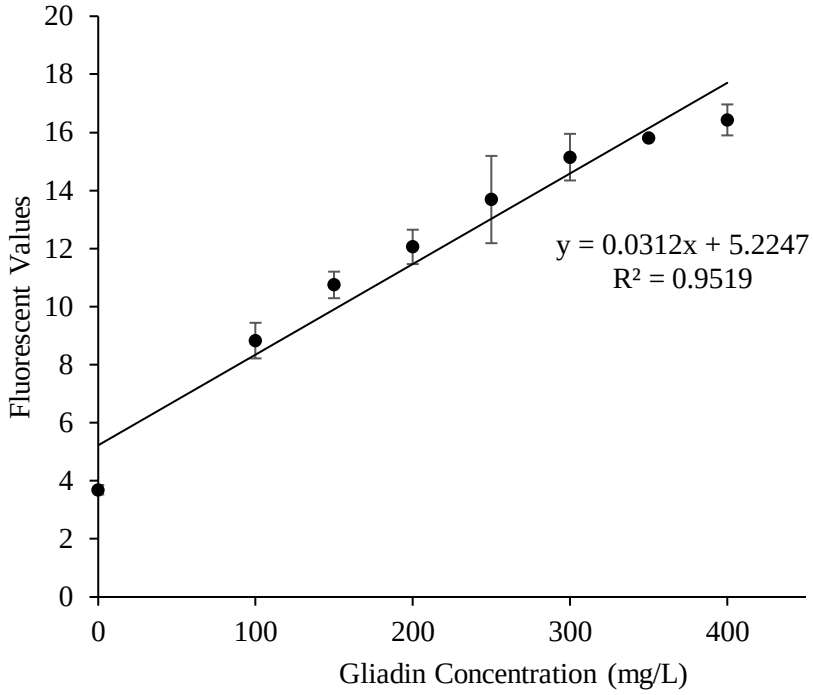
Appendix



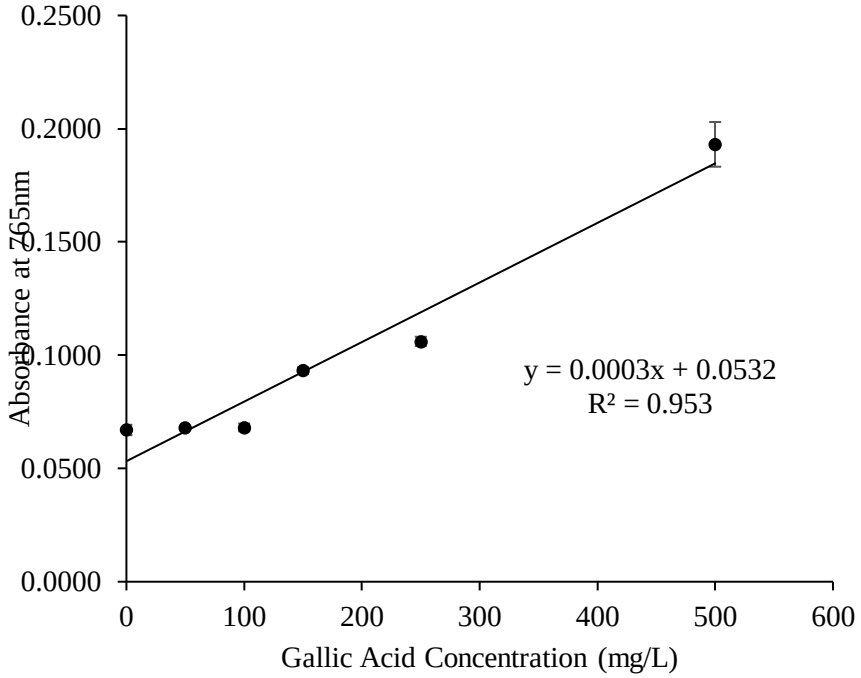
A. Standard gliadin for EZQ assay protein analysis of model system for 2000 mg/L TA.



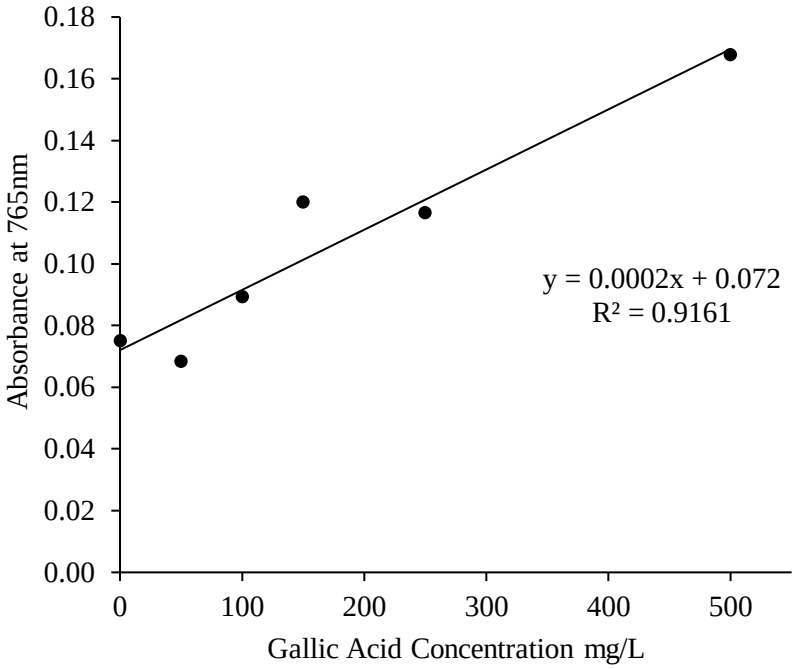
B. Standard gliadin curve for EZQ assay of protein of model beer system for 500 mg/L TA.



C. Standard gliadin curve for protein of ale and model beer system for 500 mg/L TA.



D. Standard gallic acid curve for total phenol analysis of model beer system.



E. Standard gallic acid curve for total phenol analysis of ale by Folin-Ciocalteu assay.

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- Completed pilot plant trial to produce base cakes and managed hand sandwiching variables
- Analyzed results from focus groups and roundtable to provide qualitative consumer learnings
- Shared recommendations with stakeholders and senior leaders in poster and presentation

D.D. Williamson and Co. Inc., Science and Innovation Intern, Louisville, KY *May 2016 – July 2016*

- Evaluated color applications in meat, bakery, beverage, and nutraceutical products
- Co-devised bench-top procedures and documentation in a fast-paced environment
- Handled the operation and troubleshooting of the spray dryer

Campbell Soup Company, R&D Co-op, Camden, NJ *July 2015 – Jan. 2016*

- Collaborated with cross-functional teams to develop beverage line extension
- Designed and executed quality project between manufacturing plants
- Coordinated pilot plant run and processed ingredients to specification
- Received "employee of the first quarter" award

RESEARCH EXPERIENCE

Department of Food Science, Undergraduate Research Assistant, Penn State *Aug. 2014 – May 2015*

- Analyzed droplet size and protein composition via SDS PAGE and EZQ assays
- Prepared emulsions, model beer, and model wine for HPLC analysis
- Distributed samples for sensory tests after successful ServSafe certification

Summer Research Opportunities Program, Purdue University, West Lafayette, IN *June 2014 – July 2014*

- Researched *S. enterica* migration in chicken shell eggs for 40 hours/week
- Applied cold atmospheric plasma technology to whole chicken shell eggs
- Presented work at Purdue University's poster symposium

Penn Vet Working Dog Center, Summer Intern, Philadelphia, PA *June 2013 – Aug. 2013*

- Prepared dogs for the University of Pennsylvania ovarian cancer study
- Improved fitness of dogs, recorded video data, and assisted in husbandry

LEADERSHIP

Sigma Alpha Sorority, Treasurer, Penn State *Jan. 2014 – Dec. 2017*

- Managed $6,000 budget and involvement documentation system (Dec. 2016 – present), emerald scholar

Food Science Club, Penn State *Aug. 2013 – Dec. 2017*

- IFTSA College Bowl Team—2017 national champion, PSU Ag Springboard — 2015 2nd place

Schreyer Honors College Student Council, Service Chair, Penn State *Aug. 2013 – Dec. 2017*

- Organized philanthropy events, park clean ups, and Relay For Life (Aug. 2014 – May 2015)

SCHOLARSHIPS AND AWARDS

- Academic Excellence Scholarship – Penn State
- Agriculture Future of America Industry Sponsorship
- Beliasov Family Scholarship
- Boyd E. Wolff Gotcha Fund
- College of the Liberal Arts Enrichment Award
- Dahle Chester Memorial Scholarship
- Emmett C. Dawson Jr. Memorial Award
- Penn State Sigma Alpha Alumni Association Award
- PMCA Student Honoree
- Provost Award – Penn State
- Schreyer Ambassador Travel Grant
- Virginia Todd Chapel Executive Internship Award

MEMBERSHIPS

• Phi Beta Kappa • Gamma Sigma Delta • Institute of Food Technologists