WHEY PROTEIN POWDER ANALYSIS BY KJELDAHL AND MID-INFRARED SPECTROSCOPY

by

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The following individuals read and discussed the thesis submitted by student Rose Saxton, and they evaluated their presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

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ABSTRACT

There is an ever-expanding number of high protein dietary supplements marketed as beneficial to athletes, body builders, infant formulas, elder care, and animal feed. Consumers will pay more for products with high protein per serving data on their nutritional labels, making the accurate reporting of protein content critical to customer confidence. The Kjeldahl Method (KM) is the industry standard to quantitate dairy proteins, but the result is based on nitrogen content, which is an approximation of nitrogen attributable to protein in milk. Optical spectroscopy is commonly used for quality control measurements and has been identified as having the potential to complement the KM as a more nuanced testing measure of dairy protein. Infrared (IR) spectroscopy offers advantages over the KM in that IR provides an accurate representation of protein content in dairy products, and the results can be achieved very quickly. Protein analysis by IR has been used to study protein degradation in aged cheeses, and milk whey powder adulteration in whey protein concentrate supplements. The hypothesis of this thesis is that if mid-infrared (MIR) spectroscopy can be used to characterize individual whey proteins, then MIR should be applicable to qualitative analysis of protein powders and quality control monitoring of protein powder products for adulteration by inexpensive protein or amino acids. Protein powder analysis by KM revealed that the calculated total percent protein of the five protein powders tested was lower than the value stated on the product label, the percent variation between label protein content and that of the KM ranged from 2.9% to 9.5%. MIR spectroscopy spectra of four whey protein standards and four other protein

standards provided qualitative characterization of each protein by amide I and amide II peak absorbance wavenumber. Product tampering by third-party manufacturers is an issue, due to the lack of United States Food and Drug Administration regulation of nutraceutical products, permitting formulators to add low-cost nitrogen-containing components to artificially inflate the KM approximated protein content of the products. Protein powders have been found to be doped with the amino acids glycine, leucine, and glutamic acid and inexpensive proteins, like bovine serum albumin. Controlled doping experiments were conducted with each of the above listed adulterants to assess the effectiveness of MIR spectroscopy to rapidly detect product tampering. Protein doping experiments revealed that as BSA amounts were increased, the amide I/II peak shape changed from the broad protein powder peaks to the narrower BSA peaks. Amino acid doping experiments revealed that the limit of detection for MIR spectroscopy, for the three amino acids used in this study, is 25%. MIR spectroscopy results may offer product quality assurance that is complementary to dairy protein measurement by the KM.

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LIST OF ABBREVIATIONS

Ala	Alanine
Asp	Aspartic Acid
BSA	Bovine Serum Albumin
BSU	Boise State University
Cys	Cysteine
DM	Dumas Method
FDA	Food and Drug Administration
FT-IR	Fourier transform-infrared
g	Gram
GC	Graduate College
Gly	Glycine
HPLC	High-Performance Liquid Chromatography
IR	Infrared
KM	Kjeldahl Method
kD	Kilodalton
L	Liter
LC-ESI-Q-TOF MS	Liquid Chromatography-ElectroSpray Ionization-Quadrupole-Time Of Flight-Mass Spectrometry
M	Molarity
MF	Microfiltration

mG	Milligram
MIR	Mid-Infrared
mL	Milliliter
Mol	Mole
N	Normality
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
PDB	Protein Data Bank
pH	Power of Hydrogen
TDC	Thesis and Dissertation Coordinator
TP	Total Protein
SEM	Standard Error of Mean
SP	Soluble Protein
sp.	One species
spp.	Many species
STEC	Shiga toxin–producing Escherichia coli
UF	Ultrafiltration
US	United States
Val	Valine
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate
µm	micrometer

LIST OF SYMBOLS

≈	Almost equal to
±	Plus-minus
°C	The degree Celsius
°F	The degree Fahrenheit
%	Percent
α	Alpha
β	Beta
κ	Kappa
Ca^{2+}	Calcium ion
Zn^{2+}	Zinc ion
Mg^{2+}	Magnesium ion

CHAPTER ONE: INTRODUCTION TO MILK PROTEINS

Milk is a complete nutritional source of all necessary components to sustain life, and is composed of carbohydrates, lipids, proteins, vitamins, minerals, and other minor constituents. Bovine milk is composed of 87.7% water, 4.9% carbohydrates, 3.4% fats, 3.3% proteins, and 0.7% minerals. Milk can be sold on its own or made into a myriad of products that include cheese, yogurt, butter, whey protein isolates or concentrates, etc. Cheese is made from milk, beginning with the addition of acid to lower pH, causing precipitation of casein, a protein that makes-up about 80% of the protein in milk. The other 20% of protein in milk is whey, which is resistant to denaturation at low pH, and remains in solution during cheese making. Whey has historically been discarded as animal feed by food processors because it was thought to be a low value waste product, but the surge in high protein sports nutritional supplements has created consumer demand for whey protein products equivalent to a modern-day gold mine for dairy processors. Whey protein has been found to contain all the essential amino acids and is a very valuable nutritional commodity. The focus of this chapter is to detail the various proteins in milk, describe structure similarities and differences, and explain how they are utilized in dairy products.

Introduction

When milk is delivered to a processing plant it is pasteurized, and from there it can either be sold directly as fluid milk or serve as a feedstock for cheese, yogurt, whey protein powder, butter, ice cream, and many other products. Pasteurization is the process of using high temperature for a set amount of time to destroy 90% of a particular pathogen and was

first used on milk in the late 1800's to target the tuberculosis pathogen (*Mycobacterium tuberculosis*).[1] The pasteurization of raw milk resulted in a dramatic decrease in the number of incidences of tuberculosis and the process was adopted as a standard to make milk safer for human consumption. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) defines pasteurization as "any process, treatment, or combination thereof, that is applied to food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage."[2] Today, the five major pathogens that are problematic for the dairy industry are *Campylobacter jejuni*, Shiga toxin–producing *Escherichia coli* (STEC), *Listeria monocytogenes*, *Salmonella spp.*, and *Staphylococcus aureus*.[3] Pasteurization is used to keep the numbers of these foodborne illness causing bacteria to an acceptable level. The United States dairy industry uses high heat (72°C/161°F) for a short amount of time (15 seconds) to pasteurize milk.[4] A 2011 ordinance issued by the US Department of Health and Human Service and the Food and Drug Administration requires Grade "A" pasteurized milk and milk products to: be cooled/maintained at a temperature of at least 7°C (45°F), have bacterial limits that do not exceed 20,000/mL or g, have coliform counts that do not exceed 10/mL, have phosphatase levels less than 350 milliunits/L, and have no positive tests on drug residue detection methods.[5] According to the ordinance, phosphatase is naturally occurring in raw milk and levels are tested to see if proper temperatures have been reached in the pasteurization process, the phosphatase enzyme level will decrease with increasing temperature. The drug residue tests are specifically used for antibiotic detection that may have been administered to the animals prior to milking and then would be present in the raw milk. The guidelines

set by the FDA ensures that all milk meets the same set of standards and is safe for human consumption. During the pasteurization process, the milk is sent through a skimmer. The skimming process separates the raw milk into skim milk and cream through the use of centrifugation; cream has a lower density than skim milk and the two can be separated from one another.[6] Milk that is bought in the store is rated based on the percentage of milk-fat that is present in the total volume; whole milk must contain at least 3.25% milk-fat while low-fat is 0.5-2.0% and skim milk must have less than 0.5% milk-fat.[7] In all cases, skim milk is used and the cream is added back into the milk at the proper percentage. After pasteurization, most skim milk is made into cheese (mozzarella, cheddar, Swiss, etc.) or a cheese product (i.e., cottage cheese, cheese spread, cheese sauce, etc.), a process that begins by addition of starter culture and rennet to lower the pH of the milk, initiating formation of curds and whey.[8] Starter cultures such as *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus sp.* are added to convert lactose into lactic acid which lowers the pH, rennet contains the enzyme chymosin which removes the negatively charged portion of the casein protein allowing for protein aggregation and the formation of curds.[9] The curds are easily separated from solution and are used to make cheese. It is the various bacteria and sometimes fungi that are added during the initial cheese making process as well as the various time and temperature conditions as the cheese ages that determines what the final cheese will be (cheddar vs Swiss). After the curds have been separated, the remaining acidic aqueous solution contains the whey that is often further processed to extract the whey from the other solutes, mostly lactose and minerals, that remain in solution. Whey is a homogenous mixture of many proteins, the most abundant of which are β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulin G. β-Lactoglobulin is the

major protein found in whey, it accounts for 50-63% of whey proteins and 12% of total milk proteins.[10] β-lactoglobulin is composed of 162 amino acids and its molecular weight is ≈18 kD.[11] α-Lactalbumin comprises approximately 20% of whey proteins and 3.5% of total milk protein.[12] α-Lactalbumin contains 123 amino acids and has a molecular weight ≈14 kD.[13] Bovine Serum Albumin (BSA) makes-up about 6-8% of whey proteins.[14] BSA is a 582 amino acid protein and has a molecular weight ≈69 kD.[15] The molecular structure of IgG is composed of two identical heavy chains and two identical light chains, with a total of approximately 1,000 amino acid residues.[16] IgG accounts for less than 1% of whey proteins.[17] IgG has a molecular weight around 160 kD.[18] To extract these whey proteins from the aqueous acidic solution, filtration followed by evaporation of the liquid provides Whey Protein Concentrate (WPC) and Whey Protein Isolate (WPI); the difference between WPC and WPI is the amount of pure protein content. WPC is defined as 34-89% protein content whereas WPI is 90% or greater.[19] WPC is the result of microfiltration (MF) while WPI is the result of MF and then ultrafiltration (UF).[20] The main difference between the two filtration types is the pore size, where MF has a larger pore size (0.08-2 μm) than UF (0.002-0.1 μm).[21] Dietary supplements containing WPI are part of an emerging market with sales driven by athletes, body builders, and cost-conscious consumers seeking to fulfill their daily nutritional value of protein effectively.

Proteins are essential dietary components for our health and wellbeing. Proteins are essential to metabolism and control everything from growth and development to causing biochemical reactions to take place. In addition, proteins are also responsible for providing mechanical support to tissues.[22] There are 21 common amino acids that our bodies need to survive, which serve as the building blocks for all the proteins contained in our bodies. Of

the 21 amino acids, 12 are non-essential, meaning that our bodies can make them in sufficient quantity to support basic function, but nine amino acids are essential, because we can't make them and need to get them from the food we eat.[23] Milk provides proteins that contain all 21 common amino acids, including the nine essential amino acids, in addition to nutrients needed to sustain life like carbohydrates, fats, vitamins, and minerals. A glass of milk of any fat content contains 8 g of protein for every one 8 oz cup.[24] Most dietary supplements contain protein in powder form that can be easily blended to make shakes or smoothies with desirable flavors.

Milk Proteins

Casein

Casein is the major protein found in milk, and accounts for about 80% of milk proteins. In raw milk, casein naturally forms into micelles.[25] The phosphoprotein families that make-up casein includes α_S1- (40%), α_S2- (10%), β- (45%) and κ- (5%) casein; each of which have multiple genetic variants and vary by species and breed of animal, with the predominant composition being α_S1- and β-casein.[26] Farrell Jr. et. al. report that the α_S2-casein family of phosphoproteins is the most hydrophilic of all the caseins and the β-casein family is the most hydrophobic. The four phosphoprotein families are also present in casein micelles that form when acid is added to milk to produce gelled milk products like yogurt and cheese. It is the different variants present in the milk that give the product its functional properties like gelling and foaming.[27] The three generally accepted models to explain the internal structure of the naturally occurring casein micelle are the sub-micelle model, the nanocluster model and the dual binding model.[28] See in **Figure 1.1**.

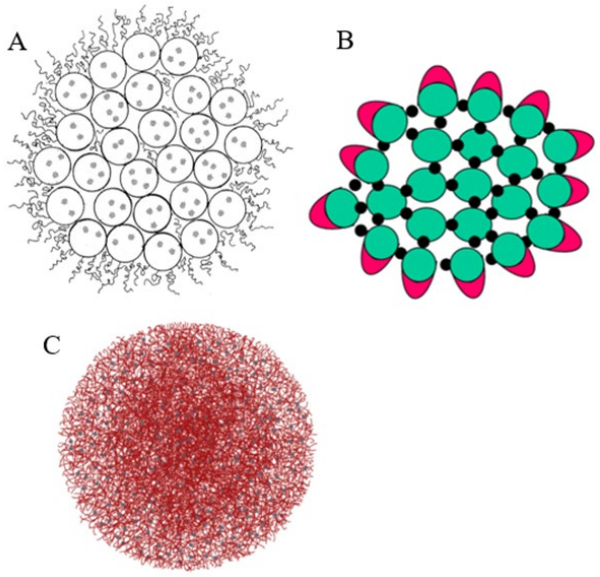


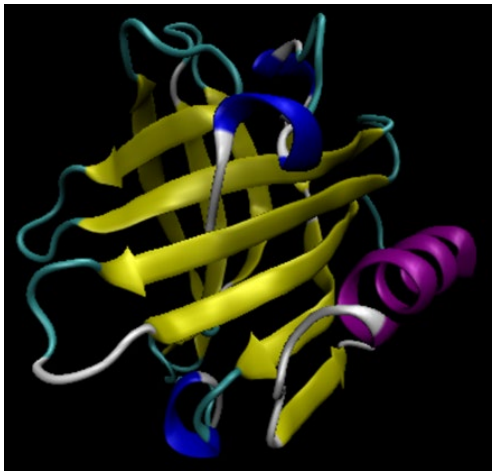
Figure 1.1. The general structure of the casein micelle. (A) Sub-Micelle Model with the submicelles represented by the hollow circles and the protruding peptide chains represented by the lines on the outside and the grey dots in the hollow circles representing the calcium phosphate. (B) Dual-Binding Model showing κ- poor regions (green spheres) linked with calcium phosphate (black dots) surrounded by κ- rich layer (green sphere attached to red triangle). (C) Nanocluster Model depicts a micelle which contains a more compact region of proteins in the middle with calcium phosphate particles (grey dots) throughout.[29-31]

The sub-micelle model suggests that two sub-micelle units of casein, one containing α_S- and β-caseins, and the other containing α_S- and κ-caseins, come together to form a bigger spherical casein micelle linked together by calcium phosphate clusters.[29] It is thought that κ-casein forms a layer on the outside, with the C-terminal portion of the protein protruding from the surface, limiting further micelle aggregation. The dual-binding model expands on the sub-micelle model by dividing the four casein phosphoprotein families into two groups: calcium-sensitive and non-calcium-sensitive. The theory is that calcium-sensitive phosphoproteins: α_S1-, α_S2-, and β-casein are surrounded by the non-calcium-sensitive phosphoprotein, κ-casein. Horne suggests that κ-casein has to be located on the outside of the micelle to stabilize the other calcium-sensitive phosphoproteins.[30] While the sub-micelle and dual-binding models both postulate that there is a definite way

that the casein micelle is structured, the nanocluster model suggests that the casein micelle is intrinsically disordered. While the other models suggest that the casein micelles are used to sequester calcium phosphate for the infant, Holt suggests the opposite, that the casein micelle is a way to purge the system of calcium phosphate.[31] Holt uses the ensemble hypothesis to describe the structure of the casein micelle, he suggests that the micelle at any one time is a cluster of proteins having various types and degrees of disproportion with a radius of 100 nm and about 800 calcium phosphate particles. The dairy industry uses casein mainly to produce cheese and cheese products, but edible casein can also be extracted from skim milk for use in a variety of products including protein powders.[32] The other 20% of proteins found in milk are the whey proteins. The four most abundant whey proteins are β-lactoglobulin (50-63%), α-lactalbumin (20%), bovine serum albumin (6-8%), and immunoglobulin G (1%).

β-lactoglobulin

The secondary structure of β-lactoglobulin is dominated by anti-parallel beta-sheet structure as seen in **Figure 1.2 A**, represented by the yellow arrows. **Figure 1.2 A** also shows the different alpha-helical aspects of β-lactoglobulin; the classic alpha-helix (purple) and the 3_{10}-helix (blue). **Figure 1.2 B** shows the amino acid sequence of β-lactoglobulin; which consists of five cysteine (C) residues.

A) 

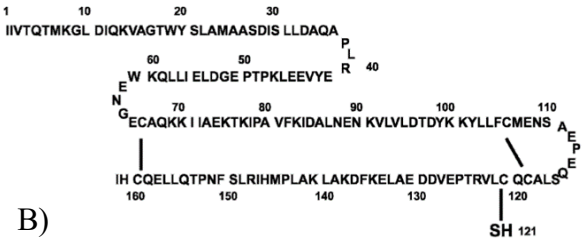
B) 

Figure 1.2. β-Lactoglobulin structure. (A) Graphical representation of β-lactoglobulin. Beta-sheet aspects of the protein are represented by yellow arrows (PDB ID 2q2m). (B) Primary structure of β-lactoglobulin. β-lactoglobulin is a globular protein that consists of 162 amino acid residues. Of importance are the five cysteine residues, four of which are linked by disulfide bonds (Cys66-Cys160 & Cys106-Cys119) and one free (Cys121).36

There are 11 variants of β-lactoglobulin, with the A and B forms being the most common in cows (*Bos taurus*).[33] There are only two amino acids that differentiate β-lactoglobulin variant A from B, at position 64 Gly in B has been changed to Asp in A and at position 118 Ala in B has been changed to Val in A as seen in **Figure 1.3**.

Gene, Protein	*B*	*A*
3065	**G**AG	
45	Glu	
3080	**C**CT	
50	Pro	
3098	**A**TC	
56	Ile	
3109	CA**G**	
59	Gln	
3982	AA*C*	AA*T*
63	Asn	
3984	G**G**T	G**A**T
64	Gly	Asp
4003	AA**G**	
70	Lys	
4027	AT**C**	
78	Ile	
5174	AA*T*	AA*C*
88	Asn	
5233	G**A**G	
108	Glu	
5263	G**C**C	G**T**C
118	Ala	Val

Figure 1.3. Comparison of *Bos taurus* β-lactoglobulin variants. β-lactoglobulin variants, variant B is the reference variant that A is compared to. Two single nucleotide polymorphisms lead to two amino acid changes; one at position 64 (changing a glycine to aspartic acid) and one at 118 (changing alanine to valine).[33]

The major function of β-lactoglobulin is to bind and transport retinol.[34] A study by Chaneton et. al. showed that β-lactoglobulin in conjunction with lactoferrin have inhibitory activity against mastitis causing bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus uberis*).[35] A study by Liu et al. demonstrated that β-lactoglobulin possess mild antioxidant properties, attributed to the free cysteine (Cys121) as seen in **Figure 1.2 B**.[36] The study used various known antioxidants such as vitamin E as well as β-lactoglobulin and compared the amount of Cu^{2+}-induced low-density lipoprotein (LDL) oxidation that occurred. It was found that while β-lactoglobulin activity was lower than that of vitamin E, there was still a mild reduction in the amount of oxidation. β-Lactoglobulin has also been shown to stimulate cell proliferation and growth.[37] Tai et al. showed that the presence of β-lactoglobulin stimulates cell proliferation, they also showed that the non-denatured form of β-lactoglobulin stimulated cell growth. The structure of β-

lactoglobulin gives the protein greater gelling and foaming properties, in comparison to the other whey proteins, which is a quality that confectioners use to their advantage.[38] β-Lactoglobulin contains one sulfhydryl group that when denatured becomes exposed and can bind to itself or other proteins, this is what makes it a great structural additive.

α-Lactalbumin

The structure of α-lactalbumin is dominated by alpha-helices as shown in **Figure 1.4,** represented by the purple helices. **Figure 1.4** also shows another alpha-helical aspect of α-lactalbumin; the 3_{10}-helix (blue).

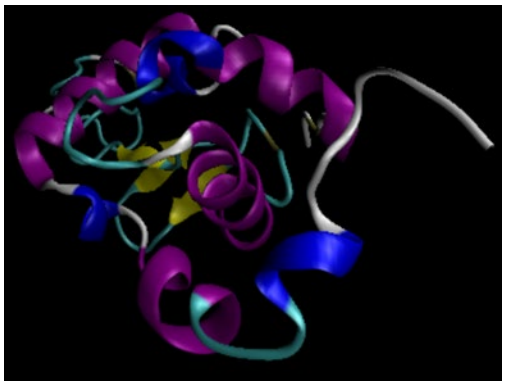


Figure 1.4. Graphical representation of α-lactalbumin. Alpha-helical aspects of the protein are represented by purple ribbons (PDB ID 1hfz).

α-Lactalbumin facilitates the synthesis of lactose from glucose and galactose by the enzyme β-1,4-galactosyltransferase.[39] Lactose is commonly referred to as milk sugar, it is the main sugar found in milk, and it is a disaccharide consisting of galactose and glucose. Glucose is generally known as blood sugar and is important for various bodily functions, including being the main source of energy for the brain.[40] To get this important sugar from mother to offspring, the monosaccharides galactose and glucose are combined by a β-1,4-galactosyltransferase catalyzed dehydration reaction to form the β-1,4-glycosidic bond in the disaccharide lactose. The presence of α-lactalbumin binds to both β-1,4-

galactosyltransferase and glucose, putting enzyme and substrate in proximity for β-1,4-glycosidic bond formation to galactose to produce lactose.[41] A secondary role for α-lactalbumin is the binding and transfer of metal ions, specifically Ca^{2+}, Zn^{2+}, and Mg^{2+}.[42] Calcium ion attraction to α-lactalbumin occurs in a region of the protein that contains several negatively charged amino acids that become available following conformational changes induced by the presence of the metal ion.[43] α-Lactalbumin also contains another binding site that associates with Zn^{2+} as well as Al^{3+}, Co^{2+}, and Cu^{2+}.[44] The Zn^{2+} binding site has also been shown to bind other metal ions, such as Mg^{2+}.[45] α-Lactalbumin is the major protein found in human breast milk; providing important amino acids like tryptophan and cysteine to the growing infant.[46] The goal of infant formula is to mimic human breast milk; with α-lactalbumin being the most abundant protein in human milk.[47] Bovine α-lactalbumin has a 74% homology to human α-lactalbumin with regard to the amino acid sequence and makes a good substitute in infant formulas.[48]

Bovine Serum Albumin

The secondary structure of BSA is dominated by α-helical structure as seen in **Figure 1.5**, represented by the purple helices. **Figure 1.5** also shows another alpha-helical aspect of α-lactalbumin; the 3_{10}-helix (blue).

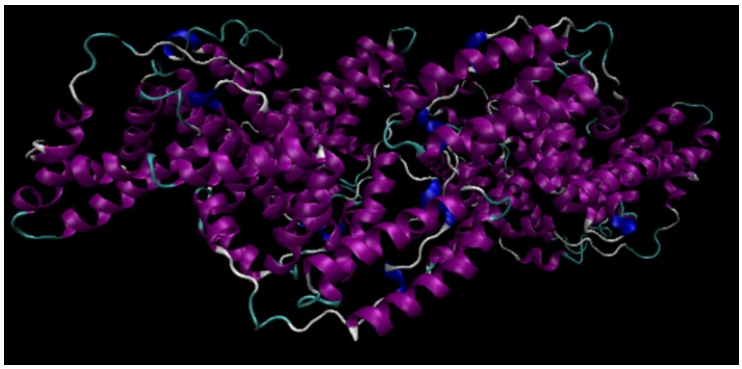


Figure 1.5. Graphical representation of Bovine Serum Albumin (BSA). The secondary structure of BSA is dominated by alpha-helical regions that are represented by purple ribbons (PDB ID 4f5s).

BSA is a blood plasma protein, synthesized in the liver, that contributes to the maintenance of osmotic pressure and facilitates the transportation of steroids, fatty acids, hormones, and other molecules in the plasma.[49] BSA is too large to exit blood vessel capillaries, creating a natural gradient for passive transport of water into the capillaries. It is the passive transport of water into the capillaries that promotes movement of blood and other materials, like hormones, throughout the circulatory system.[50] The transportation of hormones throughout the body is essential to everyday life, but two hormones that work in conjunction to produce milk during lactation are prolactin and oxytocin. Prolactin promotes milk secretion and oxytocin causes milk ejection.[51] In a study by Adeloye and Gordon, BSA was used as an emulsifier.[52] The study tested gelatin and BSA to see if they could increase the oxidative stability of an oil-in-water emulsion, BSA was found to be a better oxidative stabilizer than gelatin.

Immunoglobulin G

The primary immunoglobulin found in bovine milk is Immunoglobulin G (IgG), other immunoglobulins in bovine milk include Immunoglobulin A (IgA) and Immunoglobulin M (IgM).[53] Immunoglobulins are grouped into classes (IgG, IgA, IgM,

etc.), which corresponds to the particular constant region (Fc) that binds a particular antigen.[54] **Figure 1.6 (A)** shows the basic structure of IgG, with labeling of the antigen binding fragment (Fab) and the constant fragment (Fc) regions of the protein.[55] There are two subtypes of IgG's: IgG1 and IgG2; with IgG1 as the main immunoglobulin subtype found in bovine milk and colostrum.[56] The main difference between the two subtypes is that IgG1 possesses two disulfide bridges that connect the two heavy-chains together, while IgG2 has four disulfide bonds as seen in **Figure 1.6 (B)**.[57]

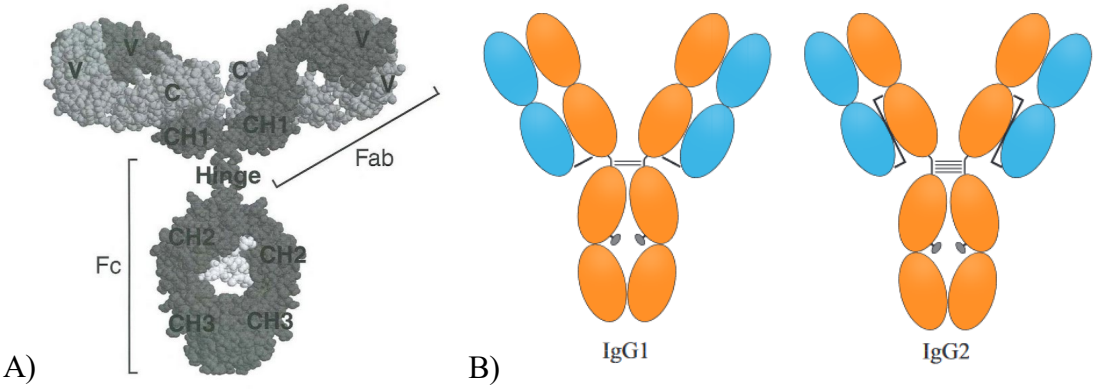


Figure 1.6. Immunoglobulin G structure. (A)Three-dimensional representation of the molecular structure for Immunoglobulin G (IgG). (B) Cartoon picture showing IgG1 and IgG2. Blue circles are light-chain contributors, orange circles are heavy-chain contributors, and black lines indicate disulfide bridges.[55,57]

The constant fragment (Fc) of IgG possesses a N-glycan region at Asn297, the structure of this N-glycan region effects the antigen-binding fragment (Fab).[58] A study by Harbison et al., used molecular dynamic simulations to test how fucosylation, sialylation, and galactosylation would affect the N-glycan region at Asn297 and ultimately the Fab region.[59] Results of the study show that while fucosylation and sialylation do not affect the conformational dynamics of the Fab region, galactosylation leads to a "closed "rather than

"open" conformation, meaning that the Fab region would not be able to bind an antigen. Immunoglobulins are antibodies that are produced by mature B cells in response to antigen stimulation and are passed from mother to offspring as the first line of defense against foreign pathogens.[60] The ingestion of milk that contains high levels of IgG by neonate ruminants and related ungulates, transfers immunoglobulins from the mother to the stomach of the infant, where it continues on to the intestines and finally into the blood stream.[61] In human neonates, immunoglobulin transfer occurs in utero by way of placental transfer and aids in protecting the infant stomach from invading pathogens.[62] In humans, bovine IgG is an important antibody that has been used for the management of enteropathy; inflammation of the intestines.[63]

Conclusions

In this chapter the major milk proteins, casein, β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulin G, were introduced. The amino acids composition, 3D-structure, and biological function for each protein was reviewed. A growing sector of the dairy industry is the emergence of high protein content nutraceutical and dietary supplement products, containing one or combinations of the primary milk proteins. The focus of this thesis is methods of detection of dairy proteins in protein powder nutritional products, and spectroscopic identification of product tampering by discovery of doping with amino acids or inexpensive protein.

CHAPTER TWO: METHODS TO EVALUATE PROTEIN IN DAIRY PRODUCTS

Milk is a complex mixture of nutrients that has been extensively studied for lipid, carbohydrate, mineral and protein composition. The focus of the current investigation is the study of dairy protein, specifically the whey proteins β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulin G. Not all protein sources are created equal, with milk, eggs, and legumes emerging as natural sources of premium protein for dietary supplements. A problem that threatens protein suppliers is the intentional adulteration of formulated products with low value amino acids or cheap proteins that artificially inflate nutritional label protein values. Proteins, particularly dairy proteins are a complete source of all amino acids in the right proportion to provide optimal benefit to the consumer. When single amino acids are introduced into a product, they are generally inexpensive, non-essential amino acids like glycine or glutamic acid, that the human body naturally produces in sufficient quantity such that additional ingestion provides no perceived benefit. Dietary supplements are not required by the United States Food and Drug Administration (FDA) to undergo quality control testing, leaving product quality verification to the manufacturer. The five proteins (casein, β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulin G) that were discussed in chapter one are used in many dietary supplements because they are a good source of complete protein. The focus of this chapter will be to evaluate five commercially available protein powders (four whey-based and one plant-based) using the Kjeldahl Method and evaluate the eight proteins that are used as protein sources in the protein powders by mid-infrared spectroscopy.

Introduction

The traditional approach to monitor protein content in food is the Kjeldahl Method (KM). The KM was developed as a means to determine the nitrogen content of organic and inorganic substances and requires three steps: digestion, distillation, and titration.[64] To calculate the protein content of a particular food item the KM uses protein conversion factors, but this protocol lacks the qualitative scrutiny to differentiate amino acids from proteins and cannot identify the difference between a high value protein product and a low value formulation. For example, a product that is made-up of pure whey-protein and one that has been doped with glycine, cannot be differentiated from one another if the nitrogen content of both is the same. There exists a need for rapid testing techniques for quality assurance and quality control measures for dietary supplements. The use of mid-infrared spectroscopy (MIR) provides one such solution, due to the relatively low instrument cost, and ease of use to acquire and interpret data. Infrared spectroscopy has been used by others for the evaluation of fat, protein, and casein content in cow's milk, as well as evaluating how lipolysis and proteolysis progress with storage.[65,66] Whey proteins were selected for this study because their use as dietary supplements continue to expand and product integrity is critical to market growth. Other studies involving the whey proteins have included quantification by reversed phase-HPLC, high-throughput LC-ESI-Q-TOF MS, and confocal Raman microscopy analysis.[67,68,69] Chapter one addressed the two classes of dairy proteins as casein and whey. Casein is the primary constituent of milk used to make cheese, while whey is used for nutritional supplements, among other products.

The Kjeldahl Method

The Kjeldahl method (KM) was first developed in 1883 to study proteins during malt production associated with beer-making.[70] During the malting process, storage proteins are broken down by enzymes, making them accessible for digestion by yeast. The soluble protein (SP) to total protein (TP) ratio is used to determine a sufficient amount of time, or malting, for the storage proteins to be converted into usable proteins.[71] The malting process involves five steps that are critical for proper malt production, the first of these include cleaning and drying of the raw barley.[72] When the barley is ready to begin its journey to be made into malt, it is first steeped in a water bath to bring the total moisture content up to between 42-46%.[73] The water content is critical in allowing for the embryo to obtain the proper amount of oxygen and start the germination process. The barley embryo is then allowed to germinate for a prescribed amount of time, depending on the particular malt desired.[74] After the barley has been allowed to germinate, the growth process is halted by a two-step drying method called kilning.[75] The amount of time that a grain is malted for impacts the starch to enzyme ratio and each step of the process has to be timed just right to get the best quality malt. If the barley grain is allowed to steep too long, this results in an early and longer germination time and allows for the enzymatic breakdown of the starchy endosperm material and its contents including protein.[76] The KM is used to determine the total protein content of the malt and used to calculate Soluble Protein to Total Protein (SP/TP) ratio. The SP/TP ratio is dependent on when the germination process is stopped, because as germination is allowed to happen, storage proteins are broken down into useable amino acids; the amino acids go into solution (wort),

while the storage proteins stay in the malt (mash) and the SP/TP ratio increases. As with the beer industry, the dairy industry uses the KM to calculate total protein.

In the present study, the KM was used to calculate the nitrogen content for the amino acid lysine, and dietary supplements according to the three-step protocol: digestion, distillation, and titration.[77] The amino acid lysine was chosen because a review of the literature suggested using it as a standard for the KM.[78] In the digestion step, the protein contained within the sample is broken-down using concentrated sulfuric acid and heat, a temperature of 400°C was maintained for 1 hour 45 minutes. The whole protein structure is denatured by the heat, which allows the concentrated sulfuric acid to further break-down the individual amino acids into ammonium ions. The digestion results in the formation of an ammonium sulfate solution. In the distillation step, concentrated (40%) sodium hydroxide is used to convert the ammonium ions into ammonia gas; for every 5 mL of concentrated sulfuric acid used in the digestion step, 20 mL of concentrated sodium hydroxide is used in the distillation step. The addition of a strong alkali allows for the neutralization of the acid and liberation of ammonia gas from the digested sample, steam distillation is then used to condense water which carries the ammonia gas into a receiving vessel. The receiving vessel contains a standard 4% boric acid solution with the pH indicator bromocresol green-methyl red mixed indicator. The boric acid captures the ammonia gas forming an ammonium-borate complex, and a mixed indicator allows for the visualization of the pH change that occurs when the ammonia gas is transferred into the boric acid solution. The receiving solution must be above the distillation outlet in order for all ammonia gas to be captured; in this case 150 mL of boric acid was used. An acid-base titration is then performed, with a known concentration (0.1 M) and amount of hydrochloric

acid, to determine the concentration of ammonium ions in the ammonium-borate complex. The concentration of ammonium ions in the sample is then used to calculate the percent nitrogen of the sample according to **Equation 1**.[79] The percent nitrogen is then multiplied by a conversion factor to yield a final percent protein of the sample; for the whey-based protein powders the conversion factor for milk and dairy (6.38) was used, and for the plant-based protein powder the conversion factor for brown rice (6.25) was used.[80]

$$\%\ Nitrogen = \left(\frac{(Standard\ Acid\ (mL) - Blank(mL)) * \left(\frac{0.001L}{1mL}\right) * (N\ of\ Acid) * \left(\frac{14.007g}{mol}\right)}{Weight\ of\ Sample\ (g)}\right) * (100)$$

(Eq. 1)

In **Equation 1**, the standard acid mL and blank mL are the volume of titrant required to reach the endpoint of the titration, the blank was experimentally determined to be 10 mL, the normality of the hydrochloric acid was 0.1 N, and the weight of sample corresponded to either 0.5 g, 1.0 g or 2.0 g of protein powder used in the digestion step.

The 6.38 conversion factor that has been used for all milk and dairy products since the 1800's, is based on the nitrogen content of the two major proteins that make-up milk, casein (15.9%) and lactalbumin (15.4%), and their approximate quantities in milk of 80% and 20%, respectively.[81] For whey protein powders, the conversion factor of 6.38 is used to calculate protein content even though, the products don't contain casein protein. It is now known that the original approximation of lactalbumin content in dairy of 15.4% used for KM calculations, represents a complex mixture of whey proteins, including β-lactoglobulin, α-lactalbumin, BSA, Immunoglobulin G (IgG), and many other minor constituents.

Materials and Methods

Equipment

Protein powder weights were taken with a Torbal AGZN200 top loading balance to the nearest 0.0001 g. Protein powder digestion was achieved using a FOSS DT208 Labtech digester consisting of an eight by 250 mL tube block; digestion temperature was set to 400°C, and digestion was performed for 1 hour and 45 minutes. Distillation was performed with a FOSS KT200 Kjeltec distillation unit, followed by manual titration to determine nitrogen content.

Materials, Samples, and Standards

Commercially available whey protein powders were purchased from BodyBuilding.com and the plant-based protein powder was purchased from a local grocery store. All chemicals were purchased from Fisher Scientific, including sodium hydroxide pellets (Catalog #S318-500), boric acid powder (Product #A74-1), hydrochloric acid (Catalog #A144S-500), L-lysine monohydrochloride (98.5-100.5%, Catalog #BP386-100), and ammonium sulfate (99.999%, Catalog #AA1063909).

Reagents for Kjeldahl

Unless otherwise stated, all reagents were purchased from Fisher Scientific. The reagents used for the Kjeldahl Method included concentrated sulfuric acid (95-98%, Product #A484-212), and Kjeldahl catalyst tablets (FisherTab™ CT-37 Kjeldahl Tablets, Product #K3011000); each tablet has a mass of 3.9 g and consists of 3.5 g K_2SO_4 and 0.4 g $CuSO_4$. The protein digestion mixture used for Kjeldahl experiments was prepared by combining two FisherTabs™, 5mL, 10 mL or 15 mL of sulfuric acid, and a 0.5 g, 1.0 g or 2.0 g sample of protein powder. After digestion, deionized (DI) water was added to dilute

the mixture to prevent precipitation. Solutions (weight/volume) of 40% sodium hydroxide, 4% boric acid, 0.1 M sodium hydroxide, and 0.1 M hydrochloric acid were prepared. To 1.0 L of 4% boric acid receiving solution, was added 1.5 – 2.0 mL of a bromocresol green-methyl red mixed indicator (Product #B0120100ML).

Protein Powder Analysis

The Kjeldahl Method was used to obtain total protein content in blank and protein powder samples. Blank samples contained all reagents, but no protein, which permitted baseline zero-point correction for non-protein nitrogen sources. Endpoint titration was then used to calculate percent nitrogen, see **Equation 1**. The percent protein was then calculated from the percent nitrogen calculation by multiplying by the corresponding conversion factor of 6.38 for milk and dairy and 6.25 for plant, and 6.07 for BSA.[82]

Digestion

To each 250 mL digestion tube was added two Kjeldahl catalyst tablets, a sample of protein powder that was 0.5 g, 1.0 g or 2.0 g, and 5 mL, 10 mL or 15 mL concentrated sulfuric acid. The tubes were place in a preheated (400°C) block digester for 1 hour 45 minutes, followed by cooling of the tubes to room temperature, and finally 40 mL of DI water was added to prevent precipitation.

Distillation

To each distillation vial was added 20 mL, 40 mL or 60 mL of 40% NaOH, depending on amount of sulfuric acid used, and allowed to distill into 150 mL of 4% boric acid (with bromocresol green-methyl red mixed indicator), as the receiving solution. The mixed indicator gave the 4% boric acid solution a red color, which changed color from red

to green following distillation, indicating a rise in pH. The distillation time was set to 10 minutes for all trials.

Titration

A 50 mL burette with 0.1 M hydrochloric acid was used to titrate each distilled sample. Titration was deemed finished when the color of final solution changed from green to pale pink, indicating that all ammonia in solution was neutralized. The total amount of acid titrant required to neutralize the ammonia generated by distillation permitted calculation of percent nitrogen from each protein powder sample using **Equation 1**. The percent nitrogen calculation was then multiplied by the respective (6.38 or 6.25) conversion factor, to calculate percent protein.

Ammonium Sulfate Chemical Check

Ammonium sulfate was used to test the distillation unit and reagents used in the distillation step. [83] To a 250 mL Kjeldahl tube was added 2.0 g ammonium sulfate (99.99%), 75 mL DI water, 50 mL of 40% NaOH and the solution was distilled into 150 mL of 4% boric acid (with bromocresol green-methyl red mixed indicator), as the receiving solution. The resulting ammonium-borate complex was titrated as described above, and percent nitrogen was calculated as described above. A percent recovery could then be calculated from the resulting percent nitrogen according to **Equation 2**.

$$\%\, Recovery = \left(\frac{\%\, Nitrogen}{21.09}\right) * (100) \qquad \text{(Eq. 2)}$$

Results

Kjeldahl Method evaluation of protein powders at 0.5 g, 1.0 g and 2.0 g provided the following results shown in **Figure 2.1**. With all the protein powders tested, as sample sizes were increased, the percent nitrogen also increased. This result was unusual as percent nitrogen for a particular protein powder should be the same, no matter what the sample size is.

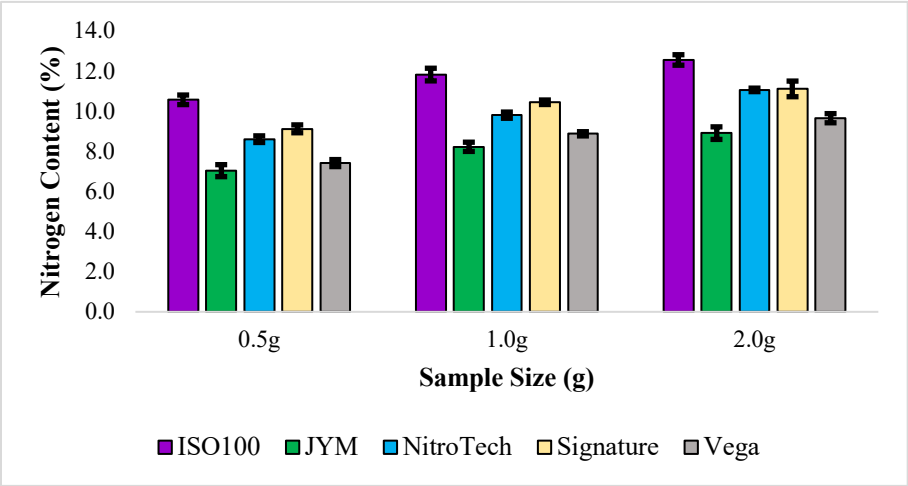


Figure 2.1. Bar graph of mean percent nitrogen for five protein powder products, at three sample sizes: 0.5 g, 1.0 g and 2.0 g. Standard deviations are shown for each, error bar based on a sampling size of 14 measurements (means with Standard Error of Means (SEM)). = ISO100 protein powder, = JYM protein powder, = NitroTech protein powder, = Signature protein powder and = Vega protein powder.

Testing of 99.99% pure ammonium sulfate was used as a chemical check and verification of the distillation and titration steps. A 2.0 g sample of ammonium sulfate resulted in 99.99% recovery of ammonia. The 98.5% purity of commercially available L-lysine monohydrochloride was used to verify the digestion step and provided a theoretical maximum nitrogen content of 15%. Experimentally, the KM was used to determine the percent nitrogen values for lysine; the 0.5 g, 1.0 g and 2.0 g samples calculated to 8.6, 12.3 and 14.7%, respectively, showing better agreement with the theoretical value with

increasing sample size. Both the ammonium sulfate and lysine experimentally verify that the 2.0 g sample shows the true percent nitrogen content for each of the protein powders; the 2.0 g sample of ammonium sulfate resulted in a 99.99% percent recovery of ammonia and the 2.0 g sample of lysine resulted in a 98% percent recovery of nitrogen.

When looking at the 2.0 g samples, **Figure 2.2.** shows three basic categories (low, medium, and high), for protein content, that the five protein powders fall into.

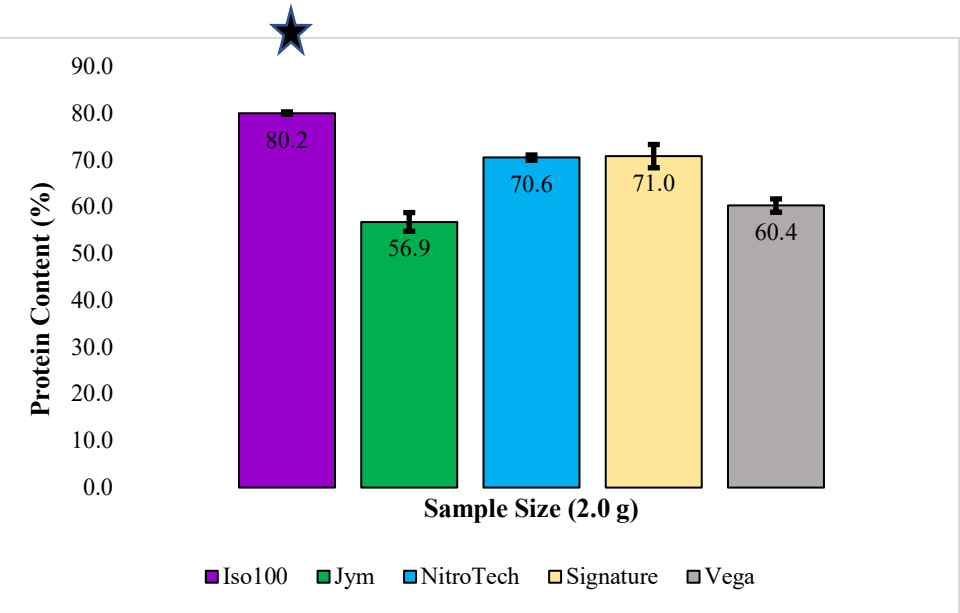


Figure 2.2. Bar graph of mean percent protein for five protein powder products, 2.0 g sample size. Star represents the significant difference found between ISO100 and all the other protein powders. Standard deviations are shown for each, error bar based on a sampling size of 14 measurements (means with SEM). ■ = ISO100 protein powder, ■ = JYM protein powder, ■ = NitroTech protein powder, ■ = Signature protein powder and ■ = Vega protein powder.

The whey-based protein powder (JYM) and the plant-based protein powder (Vega) fall into the low protein content category, with a protein content of 56.9% and 60.4%, respectively. The other three whey-based protein powders fall into the medium (NitroTech & Signature) and high (ISO100) protein content categories, with a protein content of 70.6%, 71.0% and 80.2%, respectively. When a one-way ANOVA test was performed on

the five protein powders, a significant difference was found at the 0.05 critical alpha value with a p-value <0.001. The ANOVA test confirmed that there was a significant difference between the ISO100 protein powder and the four other protein powders.

To compare the KM results to product labeling, the 2.0 g data was used. The 2.0 g sample size was chosen because both the ammonium sulfate and lysine recoveries showed that this sample size provided the most accurate results. Based on the label, a 30.0 g serving of ISO100 was expected to contain 25.0 g of protein. When the 80.2% protein calculation, from the 2.0 g sample size KM protein percent results, was applied to the 30.0 g scoop of ISO100, a total of 24.1 g of protein per serving was calculated. The data in **Table A.1** show that all the protein powders tested by the KM had lower protein per serving than what was reported on their labels, with differences ranging from 0.7 g-1.9 g. When the standard deviations were applied to the respective protein powders, there was no significant difference in protein content, between what was measured by the KM and what was reported on the labels, see **Figure 2.3**. This is to say that when all possible nitrogen contributors were taken into account and conversion factors were applied, product labels reflected the protein content that was determined by KM when 14 trials were combined.

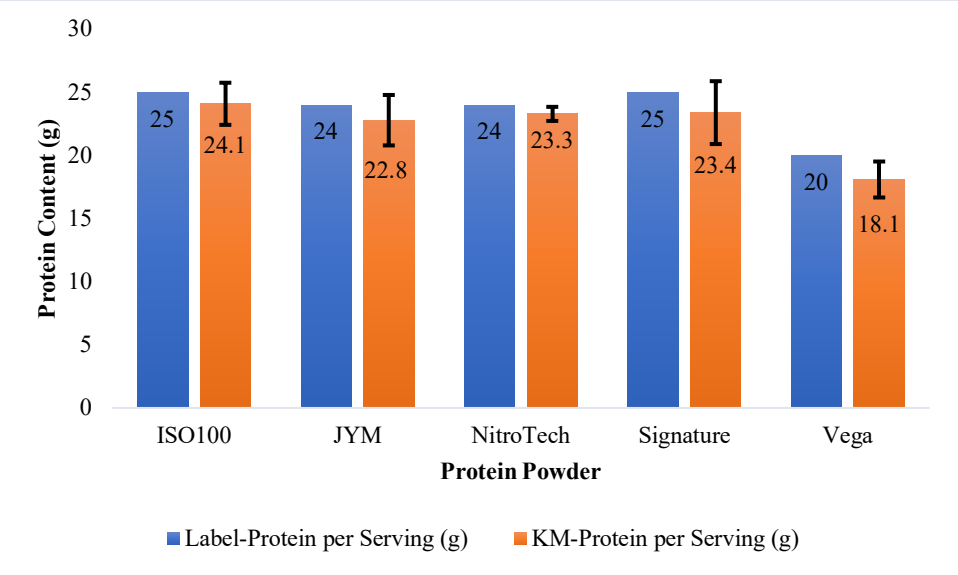


Figure 2.3. Bar graph comparing protein content as stated on the labels to experimental data. Standard deviations are shown for each, error bar based on a sampling size of 14 measurements (means with SEM). ■ = Protein per serving (g) as stated on the label, ■ = Protein per serving (g) found using the Kjeldahl Method.

When the KM total protein values were used to calculate the amount of protein per container for each of the protein powders, the NitroTech protein powder contains the most protein with 722.3 g, because NitroTech has more servings per container out of all products tested, with 31 servings (**Table A.1**). When the price of the products was considered, the whey-protein powder Signature was the best value out of the five protein powders, because this protein powder is the lowest priced protein powder of the products tested, at $20.24 for a two-pound container. When the cost of the container is divided by the protein per container, the cost of protein per container can be found. The whey-protein powder Signature is again the best value at $0.03 per gram of protein, because this protein powder has the most protein per container (631.8 g) at the lowest price ($20.24).

Mid-Infrared (MIR) Spectroscopy

Protein Analysis

Infrared spectroscopy offers rapid analysis of amide bond absorbance at precise wavenumbers for amino acids in proteins and can differentiate proteins by monitoring signature absorbance frequencies. MIR spectroscopy passes light at wavelengths ranging from 25 to 2.5 μm (wavenumbers: 4000 – 400 cm^{-1}) through a sample, permitting detection of absorbance frequency based on characteristic bond vibration. When infrared light hits the bonds of a protein, wavelengths of light are absorbed characteristic to the vibrational frequency of the bonded atoms and a signal is produced. The absorbance signature of one protein can be differentiated from other proteins based on the unique absorbance pattern for each protein. When differentiating proteins by MIR, the two specific sections for characterization are the amide I (1700-1600 cm^{-1}) and amide II (1580-1510 cm^{-1}) regions of the spectrum.[84] Gallagher describes the amide I band is due to the carbonyl stretching vibration between 1700-1600 cm^{-1}, and the amide II band is due to the N-H bending vibration between 1580-1510 cm^{-1}. The MIR literature associated with protein product analysis suggests proteins may be distinguished from one other using two other product components; including lipids (≈1743 cm^{-1}) and carbohydrates (≈1080 cm^{-1}).[85] The lipid peak at 1743 cm^{-1} is characteristic of the ester C=O stretching from triglycerides.[86] The carbohydrate peak at 1080 cm^{-1} is due to the C-O stretch common to all polyhydroxy aldehydes and ketones.[87] The fingerprint region (1200-700 cm^{-1}) is used for structural confirmation.[88] The five regions discussed (amide I, amide II, lipid, carbohydrate, and fingerprint) are shown in **Figure 2.4**.

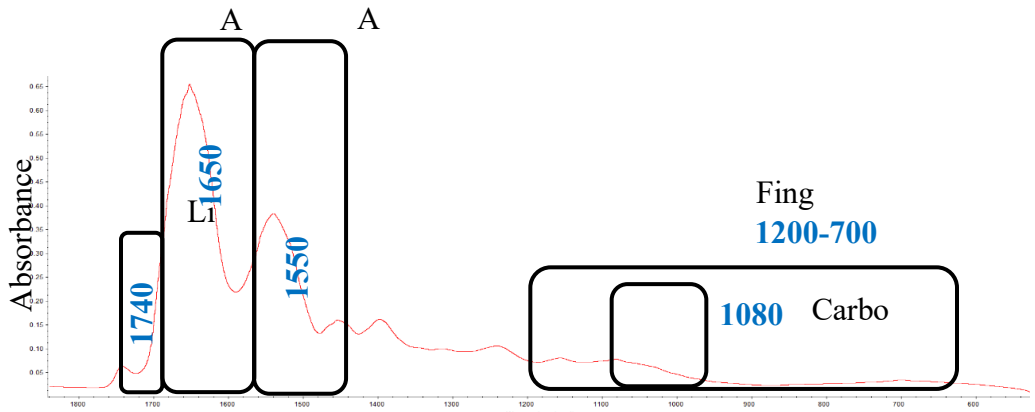


Figure 2.4. Full pea protein mid-infrared spectrum, with wavenumber identifiers. Mid-IR spectrum highlighting regions discussed: lipid region (1740 cm^{-1}), amide I region (1700-1600 cm^{-1}), amide II region (1580-1510 cm^{-1}), fingerprint region (1200-700 cm^{-1}), and carbohydrate region (1080 cm^{-1}).

The amino acid composition of every protein contributes to a three-dimensional structure that can be measured as a distinct IR "fingerprint", which can be differentiated from other proteins by the maximum absorbance wavenumber and absorbance signal magnitude. For example, both α-lactalbumin and BSA are dominated by α-helical secondary structure, but their size and structural differences are sufficiently diverse to provide amide I absorbance bands of unique wavenumber for them to be characterized by MIR.[89] Quality assurance for a whey protein dietary supplement that has been doped with amino acids can be readily achieved using MIR spectroscopy, because the amino acids do not have peptide bonds of unique signature absorbance, whereas the whey proteins do. Although amino acids absorb light within the amide I and amide II regions of the spectrum, their absorbance is less distinctive and more variable in pattern, making them easily differentiated from proteins.[90]

Materials and Methods

Equipment

Protein powder weights were taken on a Torbal AGZN200 top loading balance to the nearest 0.0001 g. Infrared (IR) spectra were recorded using a Nicolet™ iS20 FT-IR

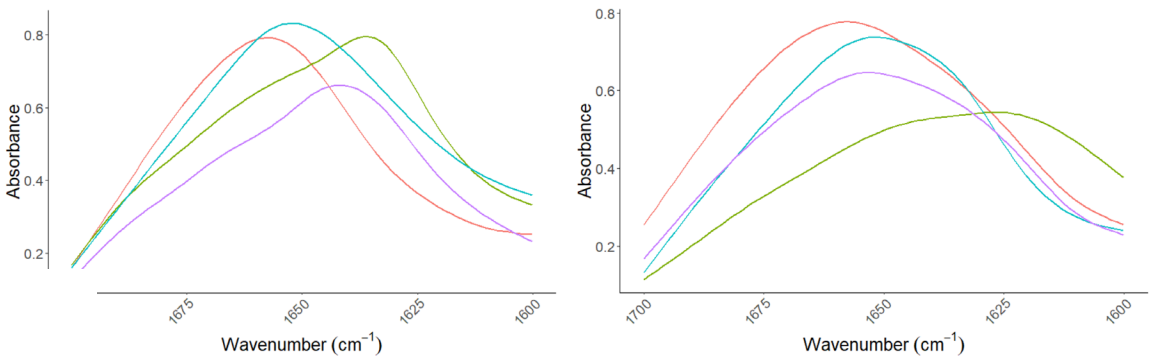
spectrometer equipped with a Nicolet™ iZ10 module and OMNIC 9 Software Suite. The Nicolet™ iS20 FT-IR spectrometer with a Nicolet™ iZ10 module equipped with an attenuated total reflectance (ATR) diamond plate was cleaned with isopropanol, allowed to dry, and a background spectrum recorded prior to sample runs. In each case, the background spectrum was subtracted from the protein powder spectrum, to generate a true sample spectrum. Protein powder samples were loaded on the surface of the ATR accessory and a force probe was tightened to ensure adequate contact with the crystal; a total of three spectra were collected for each protein powder, after every sample analysis the crystal was cleaned with isopropanol and a new sample was analyzed. Collection parameters included 512 scans at a resolution of 4 cm^{-1}, with data spacing at 0.482 cm^{-1}, using a DTGS KBr detector and KBr beam splitter. Spectra were collected using Blackman-Harris apodization and Mertz phase correction. After data collection, the advanced ATR-correction and auto optimization features of Thermo Scientific™ OMNIC™ Software were applied to all spectra. The Blackman-Harris apodization increases the signal:noise ratio and the Mertz phase correction ensures that a true sample spectrum is generated. The advanced ATR-correction feature includes correcting for variations in the depth of penetration and absorption band shifts between samples and the auto optimization feature includes baseline correction, blanking the saturated peaks, and smoothing and normalizing each spectrum. To plot data into graphs, CVS files were downloaded from OMNIC™, consolidated into one file, and saved as Excel files. The Excel files were imported into RStudio where figures were constructed.[91]

Materials, Samples, and Standards

Commercially available whey protein powders were purchased from BodyBuilding.com and pea protein powder was purchased from a local grocery store. The protein standards β-lactoglobulin (≥90%, Catalog #L3908-5G), α-lactalbumin (≥85%, Catalog #50-176-5110), and Immunoglobulin G (≥95%, Catalog #I5506-10MG) were purchased from Sigma Aldrich. The protein standard Bovine Serum Albumin was purchased from Fisher Scientific (Catalog #BP9700100). The pea (80%) and brown rice (80%) proteins were purchased from Amazon.com and both were sourced from Terrasoul Superfoods.

Results

MIR spectra for four whey proteins show the individual proteins can be differentiated from one another by analysis of the amide I region. When the amide I region is viewed, the individual proteins can be differentiated; **Figure 2.5 A** shows the spectral overlay for the four whey protein standards, and **Figure 2.5 B** displays the spectra of brown rice, casein, egg albumin, and pea proteins from 1700 – 1600 cm^{-1}. The peak absorbance wavenumbers for the amide I region of the eight proteins are listed in **Table 2.1**.



A) B)

e 2.5. Amide I region of MIR spectrum for all protein standards. (A) MIR spectra of four whey proteins— = β-lactoglobulin, — = α-lactalbumin, — = BSA, — = IgG, comparing the amide I spectral region (1700-1600 cm^{-1}), and (B) MIR spectra of — = Brown Rice, — = Casein, — = Egg Albumin, — = Pea, comparing the amide I spectral region (1700-1600 cm^{-1}).

The spectral overlay for the amide II region, from 1580-1510 cm^{-1} for the whey protein standards was reviewed, **Figure 2.6 A** shows and **Figure 2.6 B** shows the spectral overlay for brown rice, casein, egg albumin, and pea proteins over the same range. The peaks in the amide II region are less discernable than the amide I region, however, the casein protein had a peak that was distinct from the two plant proteins (brown rice and pea) and egg albumin at 1516 cm^{-1}, and the whey protein α-lactalbumin had a noticeably different peak shape and maximum wavenumber (1550 cm^{-1}) than the other three whey proteins (β-lactoglobulin, BSA, and IgG).

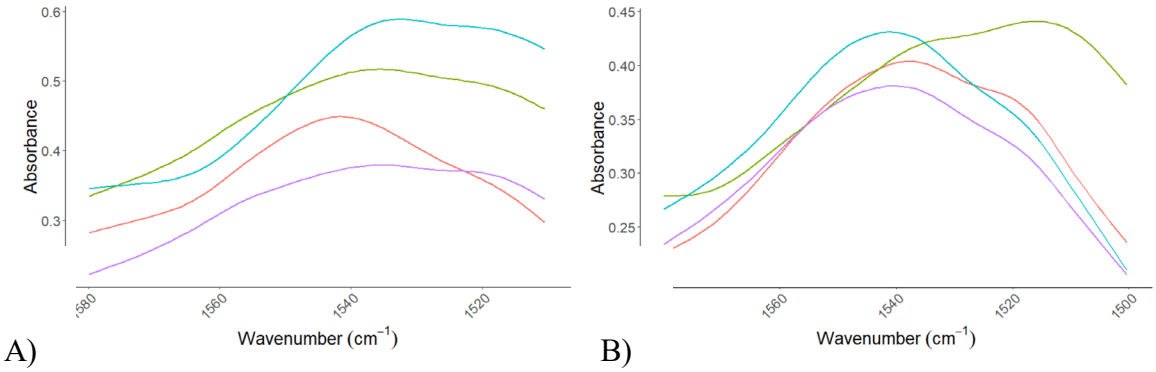


Figure 2.6. Amide II region of MIR spectrum for all protein standards. (A) MIR spectra of the whey protein standards = β-lactoglobulin, = α-lactalbumin, = BSA, = IgG, comparing the amide II spectral region (1580-1510 cm^{-1}), and (B) = Brown Rice, = Casein, = Egg Albumin, = Pea, comparing the amide II spectral region (1580-1500 cm^{-1}).

Next, the lipid and carbohydrate signature regions of the MIR spectrum for each of the eight proteins were evaluated (brown rice, casein, egg albumin, pea, β-lactoglobulin, α-lactalbumin, BSA, and IgG) (**Figure 2.7**). When looking at the lipid peak, **Figure 2.7** (**A** and **B**) of the eight proteins, the pea protein was found to have a distinct lipid peak at 1743 cm^{-1}, in contrast to the other seven proteins tested. When looking at the carbohy-drate peak, **Figure 2.7** (**C** and **D**) shows that IgG, brown rice, casein, egg albumin, and pea proteins have a carbohydrate peak at the wavenumbers 1075 cm^{-1}, 1080 cm^{-1}, 1074 cm^{-1}, 1079 cm^{-1} and 1082 cm^{-1}, respectively, which is not observed for the other three dairy proteins. The exact wavenumbers for the lipid and carbohydrate peaks for brown rice and pea proteins are summarized in **Table 2.1**.

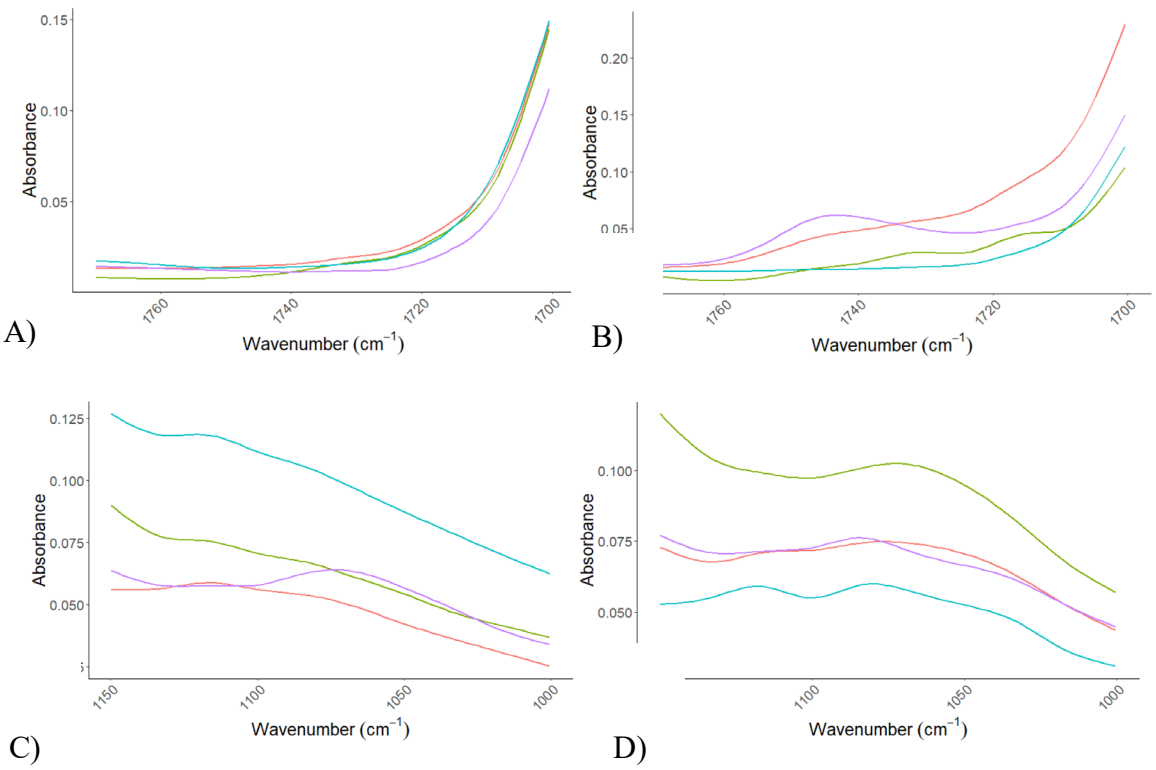


Figure 2.7. Lipid and carbohydrate regions for all protein standards. (A) MIR spectra of the whey proteins = β-lactoglobulin, = α-lactalbumin, = BSA, = IgG, comparing the lipid peak spectral region (≈1743 cm^{-1}), (B) MIR spectra of = Brown Rice, = Casein, = Egg Albumin, = Pea, comparing the lipid peak spectral region (≈1743 cm^{-1}), (C) MIR spectra of the whey proteins, = β-lactoglobulin, = α-lactalbumin, = BSA, = IgG, comparing the carbohydrate peak spectral region (≈1080 cm^{-1}), and (D) MIR spectra of = Brown Rice, = Casein, = Egg Albumin, = Pea, comparing the carbohydrate peak spectral region (≈1080 cm^{-1}).

Table 2.1. MIR data for proteins in the amide I/II, lipid, and carbohydrate spectral regions.

Protein Standard	Amide I (cm^{-1})	Amide II (cm^{-1})	Lipid (cm^{-1})	Carbohydrate (cm^{-1})
β-lactoglobulin	1635 ± 1	1537 ± 2	N/A	N/A
α-lactalbumin	1657 ± 5	1541 ± 2	N/A	N/A
BSA	1651 ± 1	1528 ± 4	N/A	N/A
IgG	1642 ± 4	1540 ± 1	N/A	1075 ± 1 (w)*
Casein	1627 ± 1	1516 ± 0	N/A	1074 ± 0 (w)*
Egg Albumin	1652 ± 0	1539 ± 0	N/A	1079 ± 0 (w)*
Brown Rice	1653 ± 0	1539 ± 0	N/A	1080 ± 0 (w)*
Pea	1653 ± 0	1541 ± 1	17 43 ± 0 (w)	1082 ± 0 (m)*

*Lipid/carbohydrate peak abbreviations: m = medium & w = weak absorbance.

The MIR spectra for β-lactoglobulin, α-lactalbumin, and a 1:1 mixture of the two whey proteins was acquired, and the amide I region is shown in **Figure A.1 A**. The amide I absorbance maximum for β-lactoglobulin is 1635 cm^{-1}, α-lactalbumin is 1654 cm^{-1}, and the mixture of the two proteins is 1653 cm^{-1}. In the 1:1 mixture, the influence of β-lactoglobulin appears to dominate the maximum peak wavenumber. To explore the influence of a protein mixture further, **Figure 2.5 B** shows the amide I absorbance maxima for β-lactoglobulin (green), α-lactalbumin (red), BSA (cyan), and a 1:1:1 mixture of the three (magenta). In this latter case, the absorbance wavenumber of the mixture at 1645 cm^{-1} is easily differentiated from the three component proteins (1635 cm^{-1}, 1654 cm^{-1}, and 1652 cm^{-1}, respectively). In the case of the proteins in **Figure A.1 A**, the absorbance of α-lactalbumin dominates the absorbance maximum for the mixture, whereas **Figure A.1 B** shows that BSA influences the absorbance maximum comparable to α-lactalbumin, while

β-lactoglobulin has little influence on either mixture. The amide II region for the proteins and mixtures in **Figure A.1** can be viewed in **Figure A.2**. The exact wavenumbers for the amide I and amide II peak maxima for the proteins and mixtures are summarized in **Table 2.2.**

Table 2.2. MIR peak maxima for whey proteins and protein mixtures in the amide I and II spectral regions.

Protein	Amide I (cm^{-1})	Amide II (cm^{-1})
β-lactoglobulin	1635 ± 1	1537 ± 2
α-lactalbumin	1657 ± 5	1541 ± 2
BSA	1651 ± 1	1528 ± 4
Mixture (β:α) (1:1)	1652 ± 1	1539 ± 1
Mixture (β:α:BSA) (1:1:1)	1647 ± 4	1534 ± 3

Discussion

The KM can provide a measurement of percent nitrogen in a sample, but it is an approximation, and cannot account for the amount of each protein in a protein powder that may contain several proteins, nor does the KM differentiate between other nitrogen-containing compounds that may be in the powder, like creatine or amino acids. The protein content results are susceptible to error because the KM calculates the percent protein of a food product based on percent nitrogen approximation using predetermined conversion factors assuming all nitrogen comes from protein. The KM analysis of a sample takes on the order of 4-6 hours to complete. Many of the limitations of the KM may be addressed by MIR spectroscopy, which can provide qualitative protein analysis and detection of product tampering by doping with proteins or amino acids within a matter of minutes.

The amide I and amide II regions of a MIR spectrum can be used when identifying the individual proteins that make-up a protein powder. The amide I peak of the four most

abundant whey proteins and casein are distinct and can be distinguished from one another, the amide II region of brown rice and pea protein also vary enough to be distinguished. In addition to the amide I/II regions, there are two other sections of a MIR spectrum that can be used to monitor protein purity, those associated with lipids and carbohydrates. In the case of brown rice, egg albumin, and pea protein the lipid and carbohydrate portions of the MIR spectrum can differentiate these proteins from whey proteins, in part because the whey proteins have no notable absorbance in these regions.

CHAPTER THREE: USE OF MID-INFRARED SPECTROSCOPY FOR QUALITY ASSURANCE TESTING

Introduction

The Kjeldahl method (KM) is the industry standard to quantitate protein in food and food products and has been for over 100 years. The KM measurement of percent nitrogen in a sample that serves as the basis for calculated protein content can be deceived by product doping with lower value protein (wheat, brown rice, etc.) or amino acids. Chapter two detailed how proteins can be differentiated from one another based on the amide I/II, lipid, and carbohydrate regions of their MIR spectrum, whereas this chapter describes protein powder analysis, and the utility of MIR spectroscopy to detect product tampering.

Protein Powder Evaluation

In 2016, studies conducted on protein powders reported that four out of ten products sold in the United States, and seven out of ten supplements surveyed from products sold in Brazil, had lower protein content than what was reported on their nutritional labels, as determined by SDS-PAGE and statistical analysis.[92] Spiking a protein powder with amino acids is done for profit; amino acids are far less expensive than whey or pea protein. In 2017, a class action lawsuit was filed against the MusclePharm Corporation in which the defendant claimed that the supplement “Arnold Schwarzenegger Series Iron Mass” had product labeling that was misleading regarding protein composition.[93] The label stated 40 g of protein per serving, and the consumer was led to believe the 40 g of protein were solely

from the protein sources specified on the label. The suit goes-on to state that the label also mentions performance growth ingredients that are added in the form of amino acids, which are then used in the protein calculation, leading to confusion for the consumer. Because product adulteration can lead to misinformation and confusion for the consumer, I wanted to test whether IR could be used to differentiate protein powders based on different protein contributors, MIR spectroscopy was used to study five commercially available protein powders; four whey-based (ISO100, JYM, NitroTech, and Signature) and one plant-based (Vega) product. Surveys of whey protein products, with labels that specifically state whey as being the sole source of protein, have identified the presence of lower cost proteins.[94] The study by Garrido et al., used proteomic analysis to evaluate several whey protein products and identified the presence of high concentrations of soybean, wheat, and rice proteins. Consumer purchasing habits are highly impacted by marketing and packaging tactics employed to promote food or beverage products.[95] I wanted to test whether product tampering could be studied by MIR spectroscopy. To do this, the protein powder NitroTech was spiked with increasing amounts of the whey protein BSA. While NitroTech and BSA have very similar amide I peaks, their amide II peaks are notably different. NitroTech has an amide II peak of 1540 cm^{-1} and BSA has an amide II peak of 1532 cm^{-1}. The use of inexpensive amino acids like glycine and glutamic acid to increase the nitrogen content of protein products is a practice that has made the news in recent years. In 2014, a class action lawsuit was filed against the makers of “Body Fortress Super Advanced Whey Protein”, claiming that free amino acids including glycine were used to increase the perceived protein content of this product.[96] The ingredient label stated that each serving contained 30 g of protein, but independent lab testing identified only 21.5 g of whey protein per serving,

a 28.3% difference between reported protein and actual protein. It has been shown that whey protein concentrate (WPC) adulteration can be visualized by FTIR-ATR, in a study done by Andrade et al., as whey powder was substituted for WPC, protein content decreased.[97] I wanted to test whether product tampering, with amino acids could be visualized by MIR. To do this, the protein powder ISO100 was spiked with the amino acids (glutamic acid, lysine, and glycine) at four concentrations (10%, 25%, 50% and 75%) and MIR spectra were monitored.

Materials and Methods

Equipment

Protein powder weights were taken on a Torbal AGZN200 top loading balance to the nearest 0.0001 g. Infrared (IR) spectra were recorded using a Nicolet^{TM} iS20 FT-IR spectrometer equipped with a Nicolet^{TM} iZ10 module and OMNIC 9 Software Suite. The IR spectrometer was used in conjunction with an attenuated total reflectance (ATR) diamond plate that was cleaned with isopropanol, allowed to dry, and a background spectrum recorded prior to sample runs. In each case, the background spectrum was subtracted from the protein powder spectrum, to generate a true sample spectrum. Protein powder samples were loaded on the surface of the ATR accessory and a force probe was tightened to ensure adequate contact with the crystal; a total of three spectra were collected for each protein powder, after every sample analysis the crystal was cleaned with isopropanol and a new sample was analyzed. Collection parameters included 512 scans at a resolution of 4 cm^{-1}, with data spacing at 0.482 cm^{-1}, using a DTGS KBr detector and KBr beam splitter. Spectra were collected using Blackman-Harris apodization and Mertz phase correction. After data collection, the advanced ATR-correction and auto

optimization features of Thermo Scientific™ OMNIC™ software were applied to all spectra. The Blackman-Harris apodization increases the signal:noise ratio and the Mertz phase correction ensures that a true sample spectrum is generated. The advanced ATR-correction feature includes correcting for variations in the depth of penetration and absorption band shifts between samples and the auto optimization feature includes baseline correction, blanking the saturated peaks, and smoothing and normalizing each spectrum. To plot data into graphs, CVS files were downloaded from OMNIC™, consolidated into one file, and saved as Excel files. The Excel files were imported into RStudio where figures were constructed.

Materials, Samples, and Standards

Commercially available whey protein powders were purchased from BodyBuilding.com and pea protein powder was purchased from a local grocery store. The protein standards β-lactoglobulin (≥90%, Catalog #L3908-5G), α-lactalbumin (≥85%, Catalog #50-176-5110), and IgG (≥95%, Catalog #I5506-10MG) were purchased from Sigma Aldrich. The protein standard BSA was purchased from Fisher Scientific (Catalog #BP9700100). The pea (80%) and brown rice (80%) proteins were purchased from Amazon.com and both were sourced from Terrasoul Superfoods. The amino acid glycine at 99% purity was purchased from Leco.com (Part #502-211). The L-lysine monohydrochloride (98.5-100.5%, Catalog #BP386-100) and L-glutamic acid (≥99%, Catalog #A125-100) were purchased from Fisher Scientific.

Results

Protein Powder Analysis

The MIR spectral overlays of the amide I/II regions of the five protein powders are shown in **Figure 3.1**. The amide I region of the five protein powders, show a common peak maximum at ≈1650 cm^{-1} (**Figure 3.1 A**), but the magnitude of that absorbance maximum is lowest for the protein powder JYM (yellow) and highest for the protein powders ISO100 (red) and Signature (blue). The amide II region (**Figure 3.1 B**) shows the same pattern as observed for the amide I, with the absorbance maxima being consistently at ≈1540 cm^{-1}. The exact peak absorbance wavenumbers for each protein powder in the amide I/II regions are summarized in **Table 3.1**.

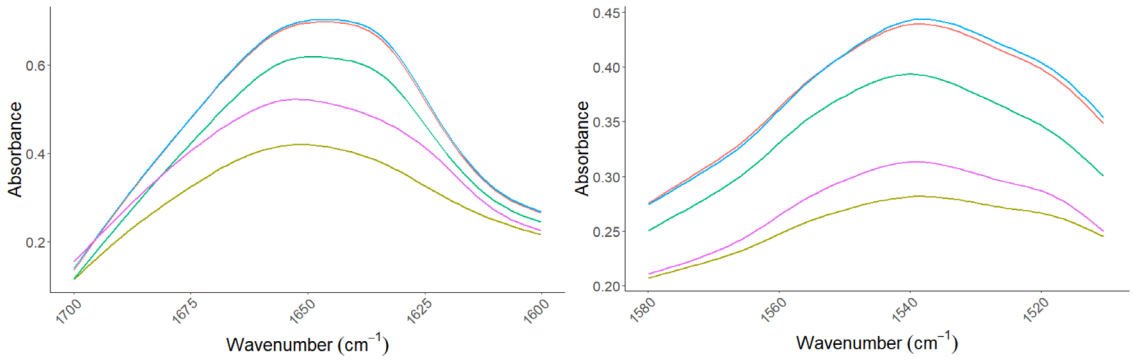


Figure 3.1. Amide I and amide II regions of protein powders. (A) MIR spectra of four whey protein powders and one pea protein powder in the amide I spectral region (1700-1600 cm^{-1}), and (B) the amide II spectral region (1580-1510 cm^{-1}), where = ISO100, = JYM, = NitroTech, = Signature, = Vega.

Given the similarity in the amide I and amide II peak regions across the protein powders, analysis of the lipid and carbohydrate spectral regions was reviewed. When the lipid and carbohydrate regions were reviewed, the lipid peak showed the most discernable distinction, **Figure 3.2 A** shows the lipid peak region from 1770-1700 cm^{-1} for the five protein powders. The most noticeable peak observed, with the highest absorbance

maximum, was that of JYM protein powder at 1745 cm^{-1}. Absorbance for three other products were seen in this region (NitroTech, Signature, and Vega), but to a much lesser extent, having smaller absorbance maxima than the JYM protein powder. In the case of the ISO100 protein powder, no lipid peak was observed. When looking at the carbohydrate region for the five protein powders from 1150-1000 cm^{-1} (**Figure 3.2 B**), all show low levels of absorbance around 1080 cm^{-1}, but they are not distinct enough from one another for this wavenumber to be useful for differentiating these products. Tabulation of these result is summarized in **Table 3.1**.

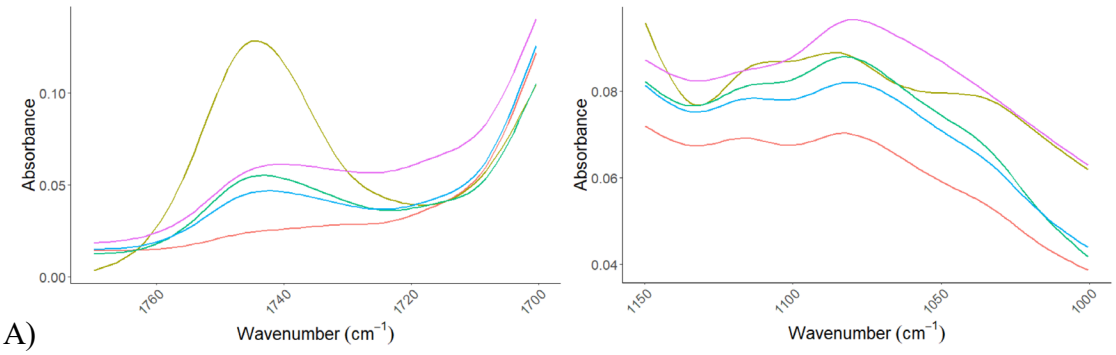


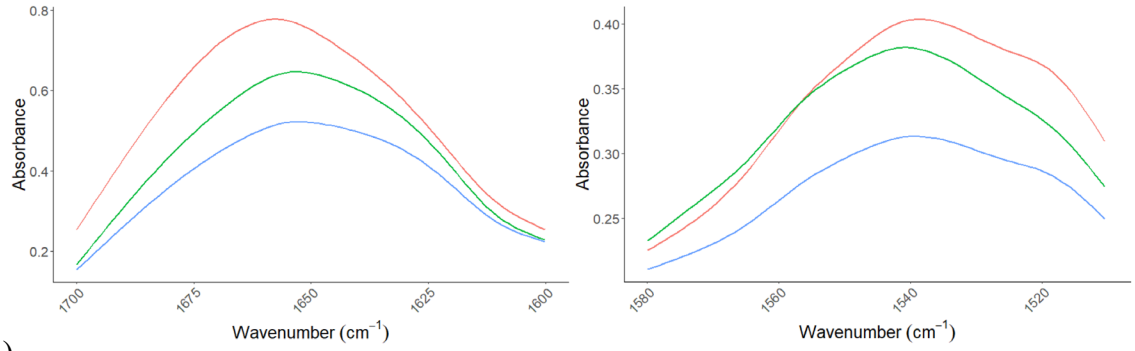
Figure 3.2. Lipid and carbohydrate region of protein powders. (A) the lipid spectral region (≈1743 cm^{-1}), and (B) the carbohydrate spectral region (≈1080 cm^{-1}) for = ISO100, = JYM, = NitroTech, = Signature, = Vega.

Table 3.1. MIR data of protein powders in the amide I/II, lipid, and carbohydrate spectral regions.

Protein Powder	Amide I (cm^{-1})	Amide II (cm^{-1})	Lipid (cm^{-1})	Carbohydrate (cm^{-1})
ISO100	1646 ± 0	1539 ± 0	N/A	1079 ± 0
JYM	1652 ± 0	1539 ± 0	1745 ± 1 (m)*	1080 ± 0
NitroTech	1652 ± 0	1540 ± 0	1743 ± 0 (w)*	1079 ± 0
Signature	1645 ± 0	1539 ± 0	1742 ± 0 (w)*	1078 ± 0
Vega	1652 ± 0	1539 ± 0	1741 ± 0 (w)*	1079 ± 1

*Lipid peak abbreviations: N/A= Not Applicable, m =medium and w = weak absorbance.

To verify that the plant-based protein powder (Vega) was made-up of the two proteins listed on the product label, pea and brown rice, the amide I/II regions of the Vega protein powder were compared to the brown rice and pea protein standards (**Figure 3.3**). When comparing the amide I absorbance for all proteins to Vega; the amide I absorbance of the Vega protein powder closely resembles that of pea protein standard consistent with pea protein being the major protein constituent of Vega (**Figure 3.3 A**). Inspection of the amide II spectral overlay (**Figure 3.3 B**) shows that the Vega protein powder contains absorbance characteristics consistent with the brown rice protein standard. When both the amide I and amide II regions of the Vega protein powder are considered, the results are consistent with pea and brown rice being the two protein contributors. The wavenumber for the amide I peak for the Vega protein powder and the brown rice and pea protein standards were all around 1652 cm^{-1}, while the amide II peak for all three was 1540 cm^{-1}, as seen in **Table 3.2**.



A) B)

Figure 3.3. Amide I and amide II regions comparing Vega protein powder to protein standards. MIR spectra of plant-based protein product (— = Vega) and protein standards (— = Brown Rice and — = Pea, looking at (A) the amide I spectral region (1700-1600 cm^{-1}), and (B) the amide II spectral region (1580-1510 cm^{-1}).

Table 3.2. IR data comparing brown rice and pea protein standards to Vega protein powder.

Protein	Amide I (cm^{-1})	Amide II (cm^{-1})
Brown Rice	1653 ± 0	1539 ± 0
Pea	1653 ± 0	1541 ± 1
Vega protein powder	1652 ± 0	1539 ± 0

The MIR spectra for whey protein standards (β-lactoglobulin, α-lactalbumin, BSA, and IgG) were then compared to ISO100. The protein powder ISO100 was selected for this comparison because it did not show absorbance in the lipid or carbohydrate regions, indicating that the composition of the mixture may be entirely whey proteins. The amide I/II regions are shown in **Figure 3.4**. The amide I absorbance maximum for ISO100 at 1646 cm^{-1} does not match perfectly to any one of the whey protein standards (**Figure 3.4 A**), which was to be expected considering the product label lists the protein ingredients as consisting of two contributors, hydrolyzed whey protein isolate and whey protein isolate. The amide II absorbance maximum for ISO100 at 1539 cm^{-1} again does not match to any one protein, but is the result of a mixture (**Figure 3.4 B**). When both the amide I/II peaks

are analyzed, the spectrum of the protein powder ISO100 is consistent with a combination of the whey protein components (β-lactoglobulin, α-lactalbumin, BSA, and IgG), which are expected to be in the product. The amide I/II wavenumbers of maximum amplitude absorbance (1646 cm^{-1} and 1539 cm^{-1}) are unique from the four whey proteins, but in the middle of the grouping. The wavenumbers for the amide I/II peaks for the whey protein standards and the protein powder ISO100 are summarized in **Table 3.3**.

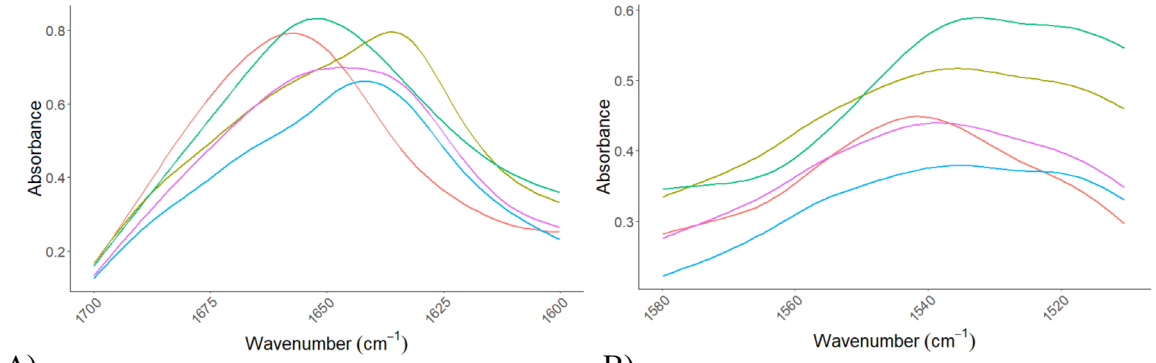


Figure 3.4. Amide I and amide II regions comparing ISO100 protein powder to protein standards. MIR spectrum of the four whey protein standards (— = β-lactoglobulin, — = α-lactalbumin, — = BSA, — = IgG) and whey protein product — =ISO100 in (A) the amide I spectral region (1700-1600 cm^{-1}), and (B) the amide II spectral region (1580-1510 cm^{-1}).

Table 3.3. IR data comparing whey protein standards to ISO100 protein powder.

Protein	Amide I (cm^{-1})	Amide II (cm^{-1})
β-lactoglobulin	1635 ± 1	1537 ± 2
α-lactalbumin	1657 ± 5	1541 ± 2
BSA	1651 ± 1	1528 ± 4
IgG	1642 ± 4	1540 ± 1
ISO100 protein powder	1646 ± 0	1539 ± 0

The whey protein standards were then mixed in a 1:1 (mass/mass) ratio and compared to the ISO100 protein powder. The MIR spectra of the mixtures are seen in **Figure 3.5**, specifically looking at the amide I region, comparing the ISO100 protein powder to three different protein mixtures. As each protein is added, the spectra of the mixture and that of the protein powder line-up better; **Figure 3.5 A** compares the ISO100 protein powder to a mixture of β-lactoglobulin and α-lactalbumin, **Figure 3.5 B** compares the ISO100 protein powder to a mixture of β-lactoglobulin, α-lactalbumin, and BSA, and finally **Figure 3.5 C** compares the ISO100 protein powder to a mixture of β-lactoglobulin, α-lactalbumin, BSA, and IgG. When comparing the ISO100 protein powder to the protein standard mixtures, the one that most closely resembles the protein powder is the mixture that contains all four of the protein standards; **Figure 3.5 C**.

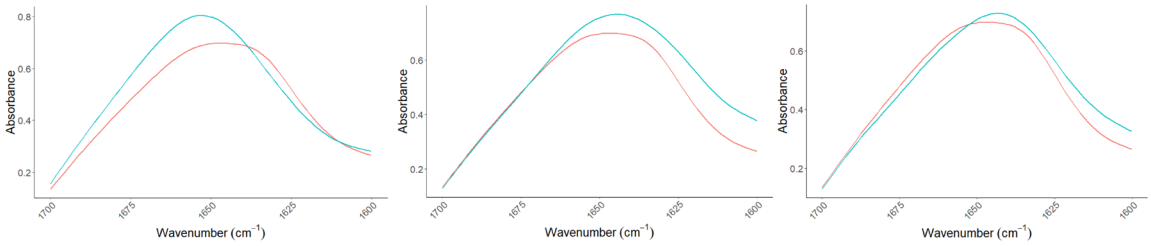


Figure 3.5. Amide I region (1700-1600 cm^{-1}), comparing ISO100 protein powder to known mixtures of protein standards. (A) MIR spectrum comparing ISO100 protein powder to a (1:1) mixture of α-lactalbumin:β-lactoglobulin. (B) MIR spectrum comparing ISO100 protein powder to a (1:1:1) mixture of α-lactalbumin:β-lactoglobulin:BSA. (C) MIR spectrum comparing ISO100 protein powder to a (1:1:1:1) mixture of α-lactalbumin:β-lactoglobulin:BSA:IgG. In all cases — = ISO100 protein powder and — = mixture of proteins.

The whey protein product JYM, which is a "protein blend" consisting of whey protein isolate, micellar casein, milk protein isolate, and egg protein, was compared to the MIR spectra for each of the constituents. The product label states that 50% of the 24 g of

protein per serving is derived from casein protein, 40% whey protein, and 10% egg protein. To begin, the MIR spectrum of JYM was compared directly to the main protein constituent, casein. The MIR spectra for the amide I peak of JYM and casein are shown in **Figure A.3 A**, JYM has an absorbance maximum at 1652 cm^{-1}, and casein at 1627 cm^{-1}. The absorbance peak shape was also distinctly different between the two, with JYM being more uniform, and casein being broader and more intense. Next, the amide I peak of JYM was compared to that of the four whey protein standards (see **Figure A.3 B**). From the amide I peak amplitude and signal broadness, it may be predicted that the primary protein that makes-up the JYM protein powder is not one or a combination of the four whey proteins. The amide I peak of JYM is broad and shallow, while the four whey protein peaks are distinct in peak amplitude, absorbance wavenumber, and more intense with respect to magnitude of absorbance. The ingredient label listed egg protein as a component of the protein blend, so the MIR spectrum for egg albumin was compared to JYM. The overlay of MIR spectra for JYM and the egg albumin protein standard are shown in **Figure A.3 C**. While, the amide I peak of JYM is broad and shallow and the amide I peak of the egg albumin standard has a distinct peak amplitude, both have a maximum absorbance at 1652 cm^{-1}. The lipid peak was also evaluated, comparing JYM to that of the casein, whey, and egg albumin protein standards (see **Figure A.3 D**). The figure shows that while the JYM protein powder has a very distinct peak in this region, none of the protein standards have a peak in this region. Inspection of the JYM protein powder label listed coconut oil as an ingredient, and it is thought that the observed lipid peak is due to the coconut oil.

Protein Spiking Analysis

NitroTech was spiked with increasing amounts of BSA, using a percent mass/mass ratio, and the amide II peak was monitored as it shifted from 1540 cm^{-1} to 1532 cm^{-1} at a final ratio of 1:10 NitroTech:BSA. The exact amide II peak absorbance wavenumbers for NitroTech, BSA, and subsequent spiked samples are summarized in **Table 3.4**. The amide I peak of NitroTech (brown; bottom), BSA (red; top), and the BSA-spiked samples (cyan-green) are shown in **Figure 3.6 A**. While NitroTech and BSA have amide I peaks consistently around 1650 cm^{-1}, a general trend could be seen; as the protein powder NitroTech was spike with increasing amounts of BSA, the peak shape changes from the broader peak of NitroTech to the more pronounced peak of BSA. The amide II peak of NitroTech, BSA, and the subsequent spiked samples of NitroTech with BSA are shown in **Figure 3.6 B**. The amide II peak of NitroTech can be seen to shift to a lower wavenumber and align with the shape of the BSA with each successive addition of BSA.

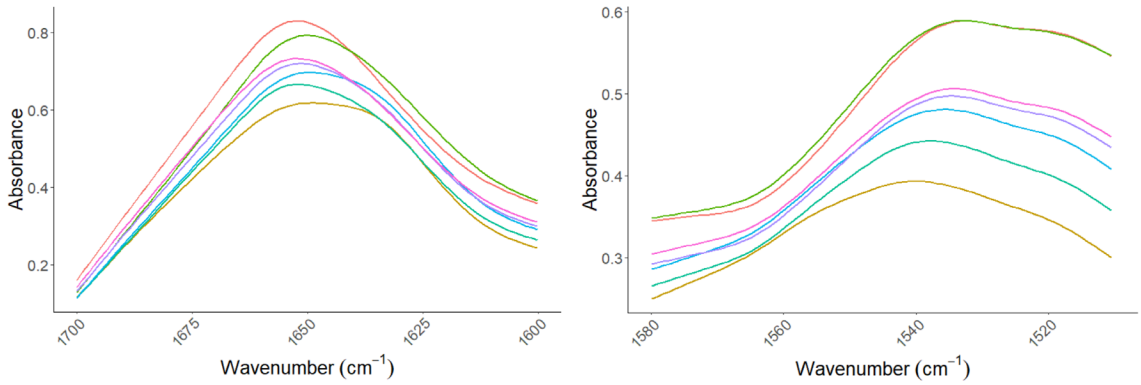


Figure 3.6. Amide I and amide II regions of NitroTech protein powder, BSA, and spikes. MIR spectra showing the doping of NitroTech PP with BSA to a ratio of 1:10 (%m/m), where (A) is the amide I spectral region (1700-1600 cm-1), and (B) is the amide II spectral region (1580-1510 cm-1). = NitroTech, = NitroTech:BSA(1:2), = NitroTech:BSA(1:4), = NitroTech:BSA(1:6), = NitroTech:BSA(1:8), = NitroTech:BSA(1:10), = BSA.

Given an observable trend for the amide II absorbance for NitroTech, with increasing amounts of BSA, the lipid and carbohydrate regions of the MIR spectra were reviewed. NitroTech has a lipid peak at about 1747 cm^{-1}, which gradually disappears as the ratio of BSA increases from 1:1 to 1:10 (see **Figure A.4 A**). The carbohydrate region (1100-1050 cm^{-1}) showed little discernable variation upon product doping (**Figure A.4 B**). The absorbance wavenumbers for NitroTech, BSA, and subsequent ratios of the two are summarized in **Table 3.4**.

Table 3.4. IR data of whey protein powder NitroTech, spiked with a known amount of a single whey protein, Bovine Serum Albumin (BSA).

Spike Ratio	Amide I (cm^{-1})	Amide II (cm^{-1})	Lipid (cm^{-1})	Carbohydrate (cm^{-1})
NitroTech PP	1652 ± 0	1540 ± 0	1743 ± 0	1079 ± 0
NitroTech/BSA(1:2)	1652 ± 0	1539 ± 0	1742 ± 0	1080 ± 1
NitroTech/BSA(1:4)	1652 ± 1	1537 ± 3	1743 ± 1	1081 ± 2
NitroTech/BSA(1:6)	1651 ± 1	1532 ± 1	1743 ± 1	1082 ± 0
NitroTech/BSA(1:8)	1650 ± 3	1531 ± 4	1743 ± 4	1082 ± 2
NitroTech/BSA(1:10)	1651 ± 1	1532 ± 1	1743 ± 0	N/A*
BSA Protein	1651 ± 1	1528 ± 4	N/A*	N/A*

*Lipid peak abbreviations: N/A= Not Applicable

To test how product tampering, with a known protein would affect the protein percent calculation, the whey-protein powder NitroTech was spiked with known amounts of BSA and analyzed using the KM. BSA made-up 25% (0.5 g), 50% (1.0 g) and 75% (1.5 g) of a 2.0 g sample, the other 75% (1.5 g), 50% (1.0 g) and 25% (0.5 g) was that of the NitroTech protein powder. When comparing the protein percentages of each; the NitroTech

protein powder alone, the incremental spikes (25%, 50% and 75%), and the BSA protein standard alone, the total protein content increases (**Figure 3.7**). The nitrogen content of a 2.0 g sample was calculated by multiplying the total nitrogen content by the respective amount of NitroTech and BSA used in the NitroTech:BSA spiked samples (25%, 50% and 75%), resulting in two nitrogen calculations. Each nitrogen calculation was then multiplied by the appropriate conversion factor; 6.38 for the NitroTech protein powder and 6.07 for BSA, resulting in two protein totals. The two protein totals were added together, resulting in a final total protein content for a 2.0 g sample. The protein powder at 70.6% protein to 100% BSA, which was measured to be 92.8% protein. The BSA result is within the manufacturer specification of 90-100%.

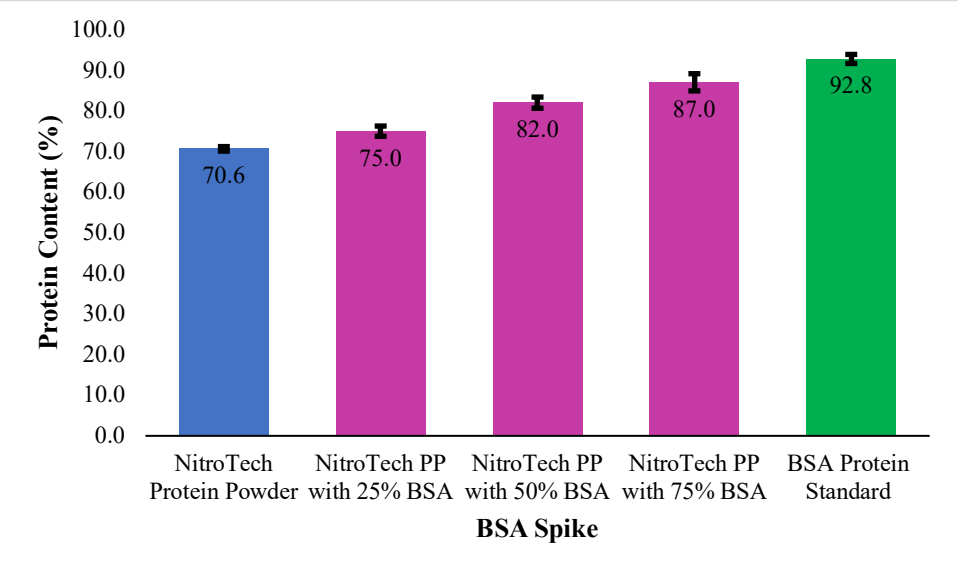
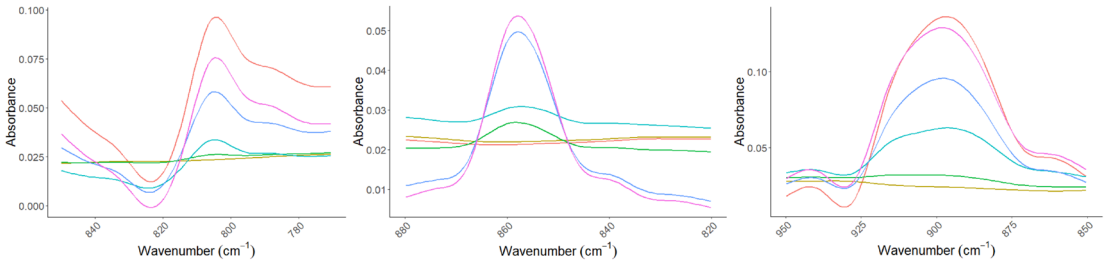


Figure 3.7. Bar graph showing percent protein values for NitroTech Protein Powder and each subsequent BSA spike as well as the BSA protein standard. Standard deviation is shown for each, error bar based on a sampling size of 5 measurements (means with SEM) of a 2.0 g sample.

Amino Acid Spiking Analysis

Spiking of a protein powder with the protein BSA was able to be visualized by MIR spectroscopy, leading to an investigation as to whether MIR spectra may be used to identify amino acid doping of commercial protein powder products. In the case of amino acid spiked protein powders, the fingerprint region (1200-700 cm^{-1}) of the MIR spectrum was studied. When ISO100 was spiked with increasing amounts of glutamic acid, a discernable peak appears at 806 cm^{-1}, where there was none in the protein powder (**Figure 3.8 A**). The same trend was seen with lysine (**Figure 3.8 B**), that as ISO100 was spiked with increasing amounts of the amino acid lysine, the appearance of a distinct peak could be observed at 857 cm^{-1}. The protein powder, ISO100 has no peak at 857 cm^{-1}, while lysine has a very distinctive peak. In **Figure 3.8 C**, the MIR spectral overlays from 950-850 cm^{-1} for ISO100 and ISO100 with added glycine, show a peak attributable to glycine at 909 cm^{-1}, where there was none in the protein powder. The amino acids glutamic acid and glycine begin to be observed with as little as 10% (ISO100/amino acid) concentration, with a discernable peak visible at the 25% (ISO100/amino acid) concentration. The amino acid lysine was observed at doping levels of 25% (ISO100/amino acid) concentration.



A) B) C)

Figure 3.8. MIR spectral overlays for ISO100 with increasing amount of amino acid to a ratio of 1:3. (A) is doping with the amino acid glutamic acid spike in the range of 850-770 cm^{-1} (— = ISO100, — = ISO100:GA (10:1), — = ISO100:GA (4:1), — = ISO100:GA (1:1), — = ISO100:GA (1:3), — = GA), (B) is doping with the amino acid lysine over the range from 880-820 cm^{-1} (— = ISO100, — = ISO100:Lysine (10:1), — = ISO100/Lysine (4:1), — = ISO100:Lysine (1:1), — = ISO100:Lysine (1:3), — = Lysine), (C) is doping with the amino acid glycine over the range from 950-850 cm^{-1} (— = ISO100, — = ISO100:Glycine (10:1), — = ISO100:Glycine (4:1), — = ISO100/Glycine (1:1), — = ISO100/Glycine (1:3), — = Glycine).

Discussion

MIR analysis of the five commercial protein powder products tested, indicated that four of them were consistent with the protein sources listed on their nutritional labels. Three of the whey-protein powders primary sources of protein come from whey proteins as noted. Amide I MIR spectral comparison of protein standards to protein powder revealed that one of the whey-protein powders main source of protein was not that of casein or whey, but may be a mixture of these proteins. When comparing the prices of the four whey protein powders tested, the most expensive (JYM @ $34.99) was found, with MIR using qualitative analysis, to not have the expected protein profile. Amide I/II MIR spectral comparison reveled that the one plant-based protein powder analyzed was found to have the proteins that were stated on the label, pea and brown rice. By looking at the peak shape, qualitatively it was found that the amide I/II regions can be used to identify the individual whey proteins in a mixture, each protein has a distinct signature at a specific wavenumber and peak shape in these regions and these characteristics can be used to identify the protein.

MIR results demonstrate that both protein and amino acid spiking of products can be detected, supporting the theory that quality assurance evaluation of products by MIR may be a valuable complement to protein quantitation by the KM. Through the KM, protein spiking of the protein powder NitroTech with increasing amounts of BSA resulted in an increase in total protein content. Amide I/II MIR spectral comparisons of the NitroTech protein powder and NitroTech:BSA spiked samples showed that as BSA amounts were increased, a change in peak shape was visualized; from the broad NitroTech peak to the more narrow BSA peak. Amino acid spiking experiments revealed that MIR can be used to detect and visualize amino acid spiking in protein powders. For the three amino acids used in this study (glutamic acid, lysine, and glycine), the fingerprint region (wavenumbers 1200-700 cm^{-1}) was used for structural confirmation and was able to differentiate between the amino acids. As product doping with amino acids has been documented, the threshold of detection for MIR was estimated to be between 10-25% for the three amino acids studied. In conclusion, the KM permits quantitative protein estimation based on nitrogen content, while MIR can be used as a quality assurance check of protein composition compared to a product label, so consumers can be confident they are getting what they are paying for.

CHAPTER FOUR: CONCLUSION AND FUTURE DIRECTIONS

The work detailed in this thesis demonstrates the potential to use MIR spectroscopy for quality control and quality assurance when combined with the KM in the testing of dietary supplements such as whey protein powders. Evidence was presented that MIR spectroscopy is useful for qualitative protein analysis because this method can differentiate individual protein components commonly used in protein powders, and MIR can also be used to detect product adulteration by either proteins or amino acids. The current study provides a framework for the development of a quantitative approach when looking at amino acid product tampering. A future goal is to create a calibration curve for the detection of lysine, glycine, and glutamic acid, which could be used to determine the relative concentration of these amino acids in a protein powder mixture. Once sufficient data points are recorded at a range of amino acid concentrations, the calibration can be evaluated using control samples of intentionally doped protein powders. The next study beyond amino acid calibration will be the quantification of the whey proteins β-lactoglobulin, α-lactalbumin, BSA, and IgG.

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APPENDIX

Supplemental Material

Table A.1. Protein content of protein powders, comparing the label to testing by Kjeldahl Method.

Protein Powder	Servings per Container (#)	Label-Protein per Serving (g)	KM-Protein per Serving (g)	Protein per Container (g)	Cost of Container ($)	Value of Protein per Container ($)
ISO 100	24.0	25.0	24.1	578.4	$32.99/1.6 pounds	$0.06
JYM	23.0	24.0	22.8	524.4	$34.99/2.0 pounds	$0.07
NitroTech	31.0	24.0	23.3	722.3	$32.99/2.2 pounds	$0.05
Signature	27.0	25.0	23.4	631.8	$20.24/2.0 pounds	$0.03
Vega	18.0	20.0	18.1	325.8	$29.99/1.3 pounds	$0.09

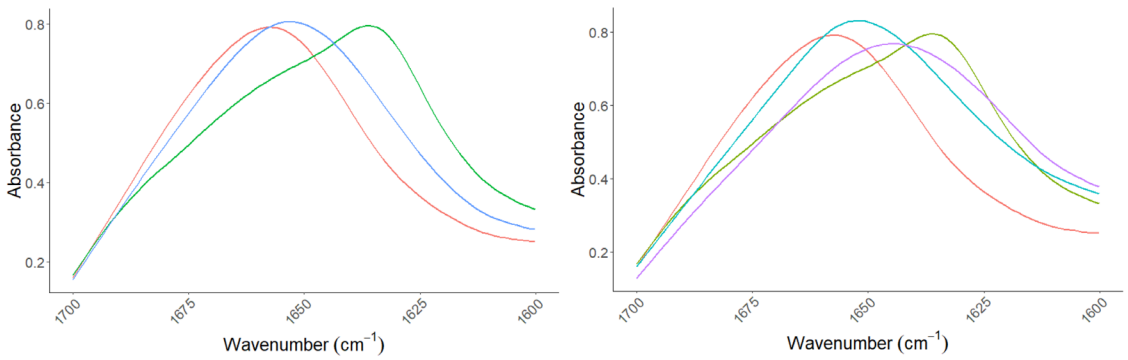

Figure A.1. Amide I region comparing protein standards to known mixture of protein standards. (A) MIR spectra of = β-lactoglobulin, = α-lactalbumin, = Mixture (1:1), comparing the amide I spectral region (1700-1600 cm^{-1}), and (B) MIR spectra of = β-lactoglobulin, = α-lactalbumin, = BSA, = Mixture (1:1:1), comparing the amide I spectral region (1700-1600 cm^{-1}).

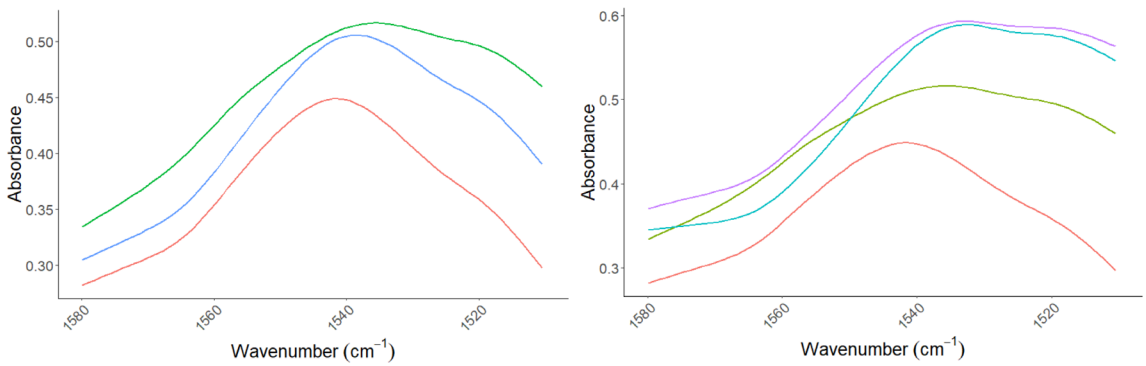

Figure A.2. Amide II region comparing protein standards to known mixture of protein standards. (A) MIR spectra of = β-lactoglobulin, = α-lactalbumin, = Mixture (1:1), comparing the amide II spectral region (1580-1510 cm^{-1}), comparing the amide II spectral region (1580-1510 cm^{-1}), and (B) MIR spectra of = β-lactoglobulin, = α-lactalbumin, = BSA, = Mixture (1:1:1), comparing the amide II spectral region (1580-1510 cm^{-1}).

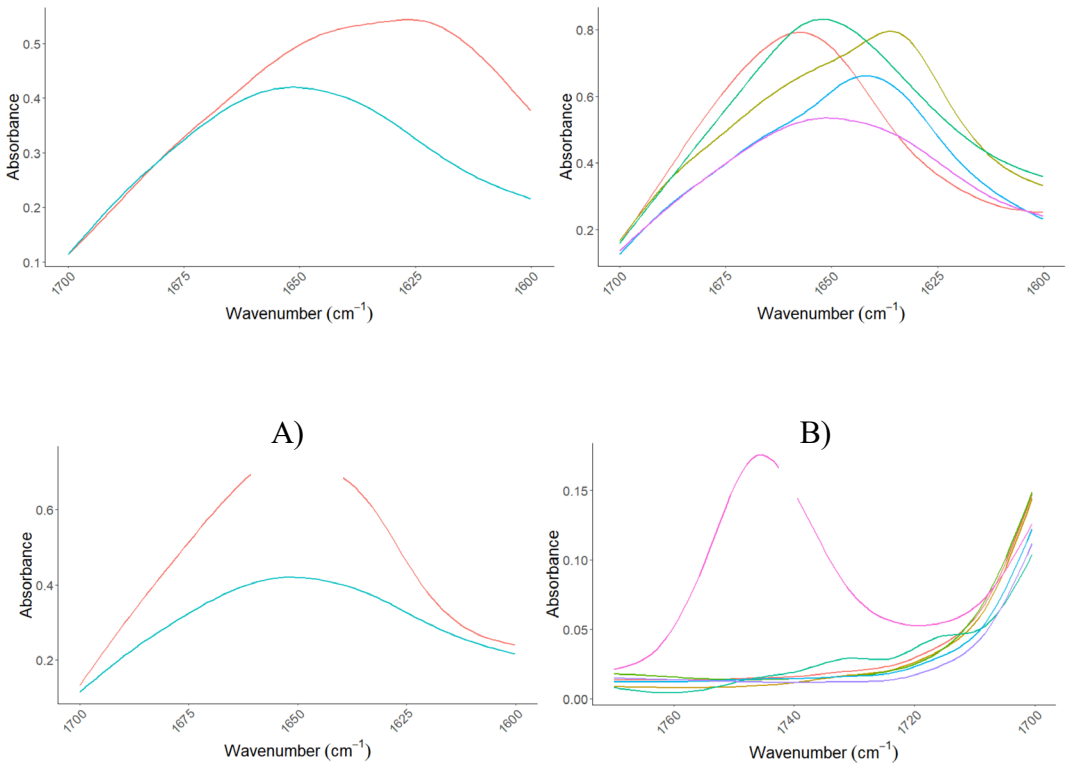

Figure A.3. Amide I and lipid regions comparing JYM protein powder to protein standards. MIR spectra of protein product JYM, where (a) is the amide I spectral region (1700-1600 cm^{-1}) of = JYM and = casein, (b) is the amide I spectral region of the four whey protein standards = β-lactoglobulin, = α-lactalbumin, = BSA, = IgG, and = JYM, (c) is the amide I spectral region comparing = egg albumin and = JYM, and (D) is the lipid spectral region (≈1740 cm^{-1}) for = β-lactoglobulin, = α-lactalbumin, = BSA, = IgG, = casein, = egg albumin, and = JYM.

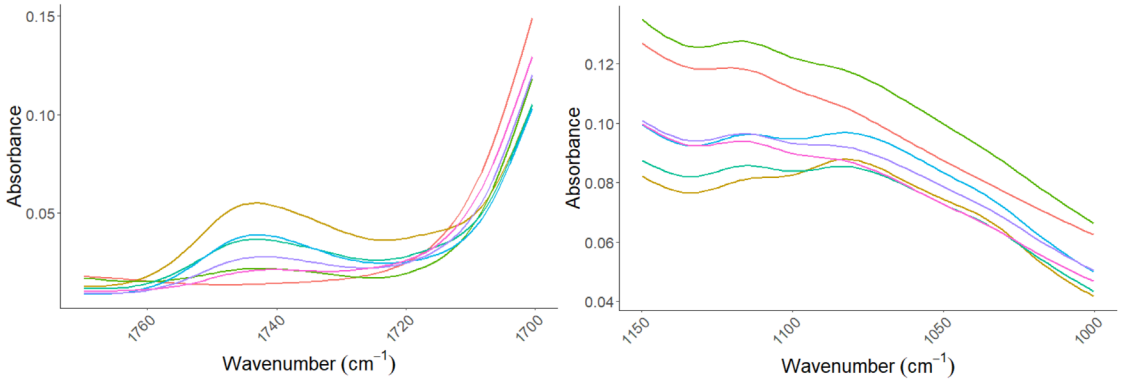

Figure A.4. Lipid and carbohydrate regions of NitroTech protein powder, BSA, and spikes. MIR spectra of NitroTech PP spiked with increasing levels of BSA from 1:1 to 1:10 in the (A) lipid spectral region (≈1743 cm^{-1}) and (B) carbohydrate spectral region (≈1080 cm^{-1}), where = NitroTech, = NitroTech:BSA(1:2), = NitroTech:BSA(1:4), = NitroTech:BSA(1:6), = NitroTech:BSA(1:8), = NitroTech:BSA(1:10), = BSA.