Detection of Whey Protein in a Hot Dog Using Immunomagnetic Separation Coupled with Surface Enhanced Raman Spectroscopy

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Dedication

This paper is dedicated to my friends and family. Your love and support have allowed me to achieve great things and will never be forgotten.

Abstract

With the passing of recent legislation, most notably the Food Allergen Labeling and Consumer Protection Act in 2006 and the Food Safety Modernization Act of 2011, the focus on allergens in the food supply is a top priority for the food industry. With the consideration of unintentional allergens now being considered an adulteration, companies are trying to find detection methods that can accurately identify an unintentional allergen, but that are also rapid enough to use so as not to interrupt the production line. Immunomagnetic Separation (IMS) coupled with Surface Enhanced Raman Spectroscopy (SERS) was investigated in this research as one possible detection method.

We decided to test and compare two types of IMS methods, antibody and aptamer, to see if one or the other would produce better results. The methods were based off of previous work by Dr. Lili He and were adapted to detect whey in a hot dog. During initial testing in a pure solution, both of the IMS methods appeared to show similar results, both being able to detect whey at levels of at least 125μg/mL of solution. But once we switched over testing whey in a hot dog, the antibody based IMS method proved to be the better IMS method. With a detection limit of 600μg of whey protein isolate/g of hot dog, the antibody based IMS method proved to be the more effective method. The aptamer IMS method ran into trouble with non-specific binding to the magnetic beads and was unable to detect any whey protein isolate in the hot dogs during the experiment. It is therefore concluded by the results of this experiment that the antibody based

IMS-SERS method is a better method to detect whey protein in a hot dog versus the aptamer method.

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1. Introduction

1.1 Food Allergens

With the increase in the food industry's ability to deliver their products to consumers comes an increase in the variety of different foods that the general population has access to. No longer are we restricted to only what we grow ourselves or what is available in the local region, but instead we now have access to foods from around the world. With this increase in dietary variety also comes an increase in the likelihood someone will have an allergic reaction to a new food. With over 15 million Americans suffering from some sort of food allergen and the percentage of children with food allergies increasing by 50% since 1997, food allergens have moved to the foreground of the food industry's concerns (CDC, 2013). The other issue with food allergies is the broad range of reaction dosages that can cause a food allergy to act up. This means that while one person will react to a few milligrams of an allergen, it's possible that only a fraction of that could kill another person. This makes setting tolerable limits in food a difficult task, since how can you justify having an amount of an allergen in food that could potentially kill someone. This is what groups like Dr. Steven Taylor at the University of Nebraska are working on. Dr. Taylor runs the Food Allergen Research and Resource Program, which is a laboratory that does assessments of food allergens in products and the risk that those allergens pose to the general population. The results will then be able to tell us at "x" level of allergen in a product, there is "x" percent chance that someone from the general

population will react to it. These types of assessments will be key in the future to help the industry set standards for tolerable allowances.

1.2 Food Allergen Laws

Since 1938 when the Food, Drug, and Cosmetic Act was put into place, the food industry has been trying to create safer food for the population. But with the rise in allergen attacks over the last 30 years, new laws were needed to adapt to the new concerns from the general public. The 2006 passing of the Food Allergen Labeling and Consumer Protection (FALCPA) was the first major piece of legislature that was specifically targeted at allergens and the need to protect the public from them. FALCPA had three main pieces to it, the first being that if a company's product contains one of the Big 8 allergens, then the label of that product must declare that that allergen is present (Sec. 203(c)(1)). This meant that the company had to have on their label the words "Contains" or "May Contain" if there was or was a chance that an allergen was present. This also included products that were manufactured or processed in facilities that also manufactured or processed known allergens, though this doesn't give companies a pass on preventing cross contamination. This was important since the consumer was alerted that there could be a possibility of cross contamination and to be cautious while eating this product. The second piece that FALCPA did was to define and categorize allergens in a meaningful way. FALCPA created what we know today as the "Big 8" or 8 grouping of major allergens that are responsible for over 90% of food allergens in the US (Sec. 202(2)(A)). And

finally, the last piece that FALCPA did was to require the industry to identify the source of an ingredient while listing the ingredients on the label. This meant that if a food manufacturer added sodium caseinate to their product, on the label after sodium caseinate (Milk) had to appear after it. This was to further identify that it was from a major allergen group and to better help consumers identify allergens in their food.

The other major law directed at food safety in the last 20 years is the Food Safety and Modernization Act (FSMA). Passed in 2011 as a response to a massive outbreak of Salmonella, this food safety law gave the FDA new, broader powers to enforce food safety and redefined the rules for a product containing an allergen. As mentioned, FSMA has given the FDA more power to enforce the food safety laws, such as mandatory recalls (Sec. 403(a)(1)). Before, the FDA could ask a company to recall a product if it was found to be contaminated with an allergen. The company at this point didn't have to recall their product, and if a recall was performed, it was voluntary and most likely in the best interest of the company performing the recall. With the passing of FSMA, if the FDA discovers a contaminated product, it can go to a company and demand a recall with the threat of forced seizure of the contaminated product behind the demand. The other major portion that relates to food safety is how a contamination by an allergen is labeled. Before, an undeclared allergen was labeled as misbranding by the FDA, since it wasn't included on the label of the product. But after the passing of FSMA, an undeclared allergen is now considered an adulterant under FDC 402(a)(1). This redefining of undeclared allergens in products shows that

allergens are now considered a much larger concern to the FDA and they will go after companies with more intensity than before.

1.3 IMS

Immunomagnetic Separation, IMS, is a technique that is commonly used to separate a target molecule or protein from a solution, typically for concentrating the target of interest or to help separate the target for use later in the method. The way IMS does this is by attaching some type of capture agent on the surface of magnetic beads, typically antibodies or aptamers, mixing the bead/capture agent complex with the sample, and then separating the beads from the sample via a strong magnet. IMS has an especially useful place when dealing with food since most food samples can be considered complex in nature (Dwivedi et al, 2010). IMS offers a technique that allows for the separation of the targeted molecule or protein from solution, where it can be analyzed away from interfering components of the original sample. The uses for IMS are numerous, including separating the target component for detection of a pathogen (Cudjoe et al 1991 and 1997), purification and identification of proteins or peptides (Safarik and Safarikova, 2004), and even with q-PCR (Wright, Chapman, and Siddons, 1994). While there are some limitations, such as sporadic non-specific binding or beads being entrapped by sample (BioRad and Thermo Scientific, 2016), IMS is a very versatile separation technique and will continue to be improved upon for various different methods.

1.4 SERS

Surface Enhanced Raman Spectroscopy, SERS, is a variation of Raman spectroscopy that uses a metal surface to help boost the Raman signal from a sample. The most agreed upon reasoning behind this signal amplification is that the light source, typically a laser, excites the surface of the material and creates localized surface plasmons that, when close enough to a Raman active sample, oscillate at the same frequency as the light source and assist in amplifying the signal from the sample. First described in literature back in the mid-1970's by Dr. Fleischmann and his team to detect pyridine on chemically roughed silver (Fleischmann et al, 1974), it has since grown to detecting numerous types of samples that were once thought to be undetectable by Raman spectroscopy, such as protein or organic materials that are not very Raman active (He et al, 2009 and Blackie et al, 2009). The advantages of SERS is that the actual test itself can be relatively quick and only requires minimum training for new users (He et al, 2011). This rapid turnaround time for testing would be beneficial to the food industry. Instead of having product wait around for the results of a test, waiting to be shipped, SERS testing would allow for shorten testing time and allow for the products to be shipped earlier, saving time and money for a company. Also, SERS testing typically requires less material to run a sample versus other methods, such as ELISA, meaning over time SERS will help save money on testing materials.

1.5 Null Hypotheses

The null hypothesis for this research were as follows:

1. We won't be able to develop a method to detect whey in a hot dog using antibody-based IMS coupled with SERS.
2. We won't be able to develop a method to detect whey in a hot dog using aptamer-based IMS coupled with SERS.

1.6 Objectives

The objectives to investigate the hypotheses for this research were as follows:

1. Develop a method to adequately extract whey from hot dogs to be used with IMS-SERS methods.
2. Adapt previous described method to be able to detect whey in a hot dog using antibody IMS coupled with SERS.
3. Adapt previous described method to be able to detect whey in a hot dog using aptamer IMS coupled with SERS.
4. Using TQ Analyst software, analyze spectra from IMS-SERS to determine if the procedure is adequate as a detection method for whey in a hot dog.

2. Literature Review

2.1 Whey

Made up of primarily water, lactose, water soluble proteins, and minerals, liquid whey has primarily been a byproduct of cheese making for the greater part of history. This most likely had to do with cheese whey being mostly water, as seen in Table 2.1.

Table 2.1- Table of approximate composition of whey after cheese processing (Swaisgood, 1982).

Approximate composition of Cheese whey	
Constituent	%
Water	94.00
Total solids	6.00
Lactose	4.50
True protein	0.60
Ash (minerals)	0.50
NPN (non-protein nitrogen)	0.20
Potassium	0.14
Chloride	0.09
Lactic acid	0.05
Fat	0.05
Sodium	0.045
Phosphorus	0.040
Calcium	0.035

2.1.1 Physical Properties of Whey Proteins

Whey consists of four main groupings of proteins, beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, and immunoglobulins (Swaisgood, 1982). Each of these proteins are considered soluble in water and differ slightly with respect to individual properties. Whey proteins in general make up about

20% of the total protein that is found in cow's milk, the rest being primarily casein proteins.

Beta-lactoglobulin, β-LG, is the most abundant of the whey proteins making up approximately 65% of all whey proteins (Swaisgood, 1982). β-LG is a 18.3kD molecular weight protein consisting of 162 amino acids, which come together to form 9 beta-sheets and 1 alpha helix, as seen below in Figure 2.1 (Swaisgood, 1982). Like most water soluble proteins, the outer surface contains more hydrophilic regions, whereas the center contains the hydrophobic core. While fairly homologous, there are 2 different variants of the protein, an A and a B strand, that differ only with a substitution of an aspartic acid in B for a glycine in variant A (Papis et al, 1986). It is also worth mentioning that, unlike its casein counterparts, β-LG contains a free sulfhydryl group, allowing it to create sulfide bonds under the right conditions (Papis et al, 1986). Under most conditions, including the native pH of milk, β-LG is present as a dimer between a pH of 3.1-5.1, but at pH below 3.0 it is a octamer and at pH above 8.0 it is a monomer (Whitney, 1977).

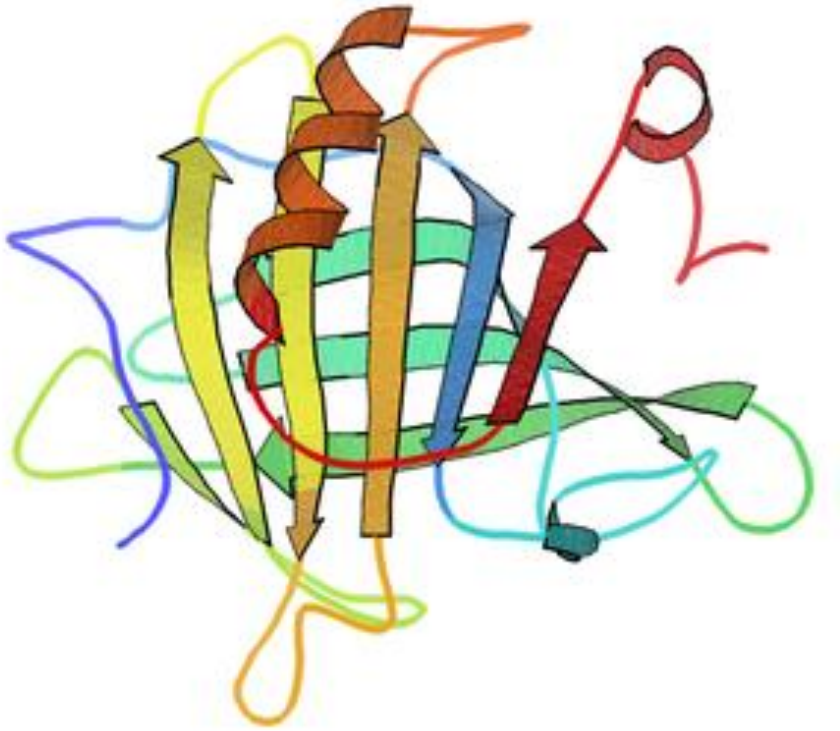

Figure 2.1: Image of β-LG with its secondary amino acid structures (RCSB Protein Data Bank, 2016).

Alpha-lactalbumin, α-LA, is the next most prevalent protein found in whey, making up about 25% of all whey proteins. It is 123 amino acids long and weighs about 14.1kD. Unlike B-LG, α-LA's structure is more dominated by the alpha-helix structure versus the beta-sheets in β-LG, as seen below in Figure 2.2 (Brew and Grobler, 1990). Also, α-LA lacks the free thiol group that β-LG has, meaning it won't form covalent bonds needed to form gels when it's denatured or acidified (Brew and Grobler, 1990). The main function of α-LA has been found to be as a catalyst for galactosyl transferase by reducing the K_m of the addition reaction of

UDP-galactose to glucose to make lactose by over 250x, thus linking the presence of lactose in milk with the presence of α-LA (Brew and Grobler, 1990). It is also worth noting that α-LA is more heat stable than the other whey proteins, especially with regards to the presence of calcium ions. Normally calcium serves to facilitate the aggregation of proteins, but with α-LA, it has been known to stay soluble at temperatures up to 100° (Swaisgood, 1996).

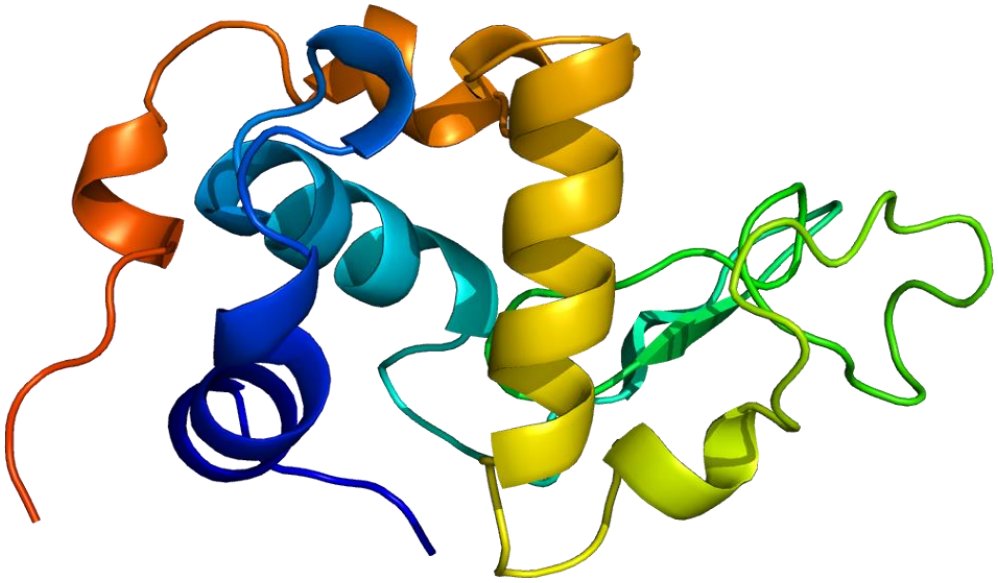

Figure 2.2: Image of alpha-lactalbumin and its secondary structure (RCSB Protein Data Bank, 2016).

Bovine serum albumin, BSA, is the third most prevalent protein in whey. Unlike β-LG and α-LA, BSA does not originate from the mammary gland, rather it comes from the bloodstream where it passively flows into the mammary gland and then onto the milk (Whitney, 1977). With a molecular weight of 69.0kD, it is

much larger than β-LG and α-LA, as can be seen below in Figure 2.3. The main purpose of BSA in the blood is to bind to free fatty acids through use of its hydrophobic binding sites, and assist in their transportation (Whitney, 1977). It has also been theorized that it may bind some of the free fatty acids in milk as well (Whitney, 1977). BSA is typically associated with protein standards in labs or as a blocking agent for such experiments as ELISA or Western Blots as it blocks potential binding sites from other proteins that would interfere with the final signal or result.

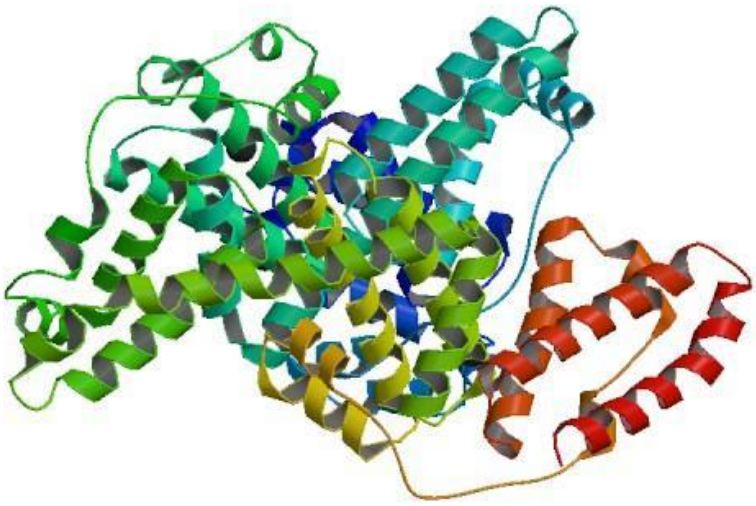

Figure 2.3: Image of bovine serum albumin and its secondary structure (RCSB Protein Data Bank, 2016).

2.1.2 Production Methods

While there are varieties of different uses for whey powder, from demineralized whey powder to permeate powder, this section on whey protein powders. For commercial whey protein powders, there are essentially three forms which differ in protein concentration. From the lowest concentration there is whey protein concentrate 35, (WPC 35), WPC 50-80, and whey protein isolate, (WPI), which has a protein concentration of at least 90% (TetraPak, 2016). The remaining constituents that make up whey powders differ in percent composition based on which type of whey powder concentration and method of drying, but typically consist of moisture, non-whey protein, lactose, fat, and excess salts.

WPC 35 is the most basic whey powder available and contains the least amount of protein per volume. It is made by first putting the whey through ultrafiltration to remove excess minerals and lactose. From there the whey is then concentrated by spray drying or drum drying to its WPC 35 powdered form.

WPC 50-80 is essentially the same as WPC 35, the difference being the amount of whey protein concentration ranging from 50%-80%. These differ in the amount of diafiltration necessary to achieve the proper protein content (TetraPak, 2016). The production of WPC 50-80 is the same as WPC 35, except that after the ultrafiltration step, a diafiltration step is added to further concentrate the whey protein. Depending on how many times it is washed and ultrafiltered, the protein content of the final product will increase with the amount of times washed.

Finally, WPI is the most concentrated form of whey protein with at least 90% protein content by volume. For WPI, there are two methods most commonly used, ion-exchange chromatography and extensive membrane filtration. For the ion-exchange, there are two different methods, cation and anion; both depend on the pH mobile phase, the stationary phase, and the eluent used (TetraPak, 2016). For cation exchange, the mobile phase typically has an acidic pH, around pH 3, and the stationary phase of the column uses a material with an overall negative net charge (TetraPak, 2016). The eluent used to separate the whey from the column is then a liquid with a pH that is neutral or slightly alkaline. For anion exchange, everything is basically the opposite of cation exchange, the mobile phase is closer to neutral in pH. The stationary phase has a positive net charge, and the eluent is a liquid with an acidic pH. The other method for WPI resembles the method to create WPC, except it is much more extensive. First, the whey is microfiltered to remove any fat that is still in the whey(TetraPak, 2016). Then it is ultrafiltered and extensively diafiltered before spray drying to achieve a product that can be as concentrated as 97% protein by volume.

2.1.3 Allergenicity of Whey Proteins

Allergenicity is the term that refers to the body's reaction to a certain foreign material, whether protein or toxin, by rejecting it and causing some type of negative bodily reaction to occur. With regards to food intolerences, we are concerned with two primary types of reactions, as seen below in Figure 2.4; immune response, which would include an IgE response or hypersensitivity, the

other is a non-immunological mediated response, which would include intolerances to a specific component of milk such as lactose (Ciara et al, 2012). The most common form of an allergy would be the immunological response. This response typically involves the absorption of the allergen either through the digestive tract or by physical contact. From there the allergen is eventually bound to an IgE through the highly affinitive Fc receptor (Janeway et al, 2001). Once this bond is formed between the antigen and antibody, inflammatory mediators and other reactions associated with allergic reactions start to take place eventually leading to an allergic response (Janeway et al, 2001). The other type of reaction, the non-immunological response, has multiple different pathways from which a response can be induced. These can either be enzymatic, some type of pharmacologic, or a combination of both that can't be defined (Ciara et al, 2012).

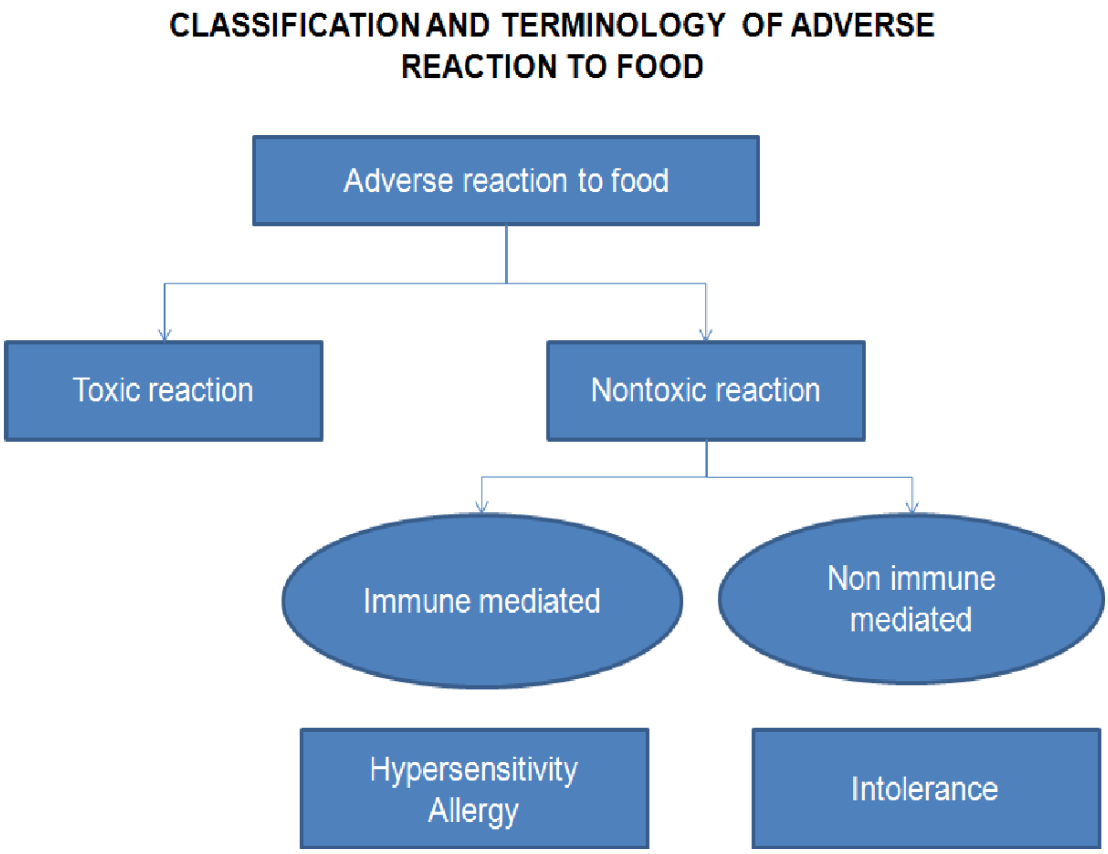

Figure 2.4: Diagram of different classifications of adverse reactions to food.

For whey proteins, the type of reaction that is most prevalent is the immune IgE response to one of the proteins. With milk allergens, there isn't a specific protein that is responsible for the anaphylactic response, where most patients tend to have IgE responses from multiple proteins, as seen below in Figure 2.5. Also complicating the reaction, is that on each of the proteins, multiple epitopes, sequences of amino acids that elicit the allergic response, can exist that allow for multiple IgE binding sites to occur (Ciara et al, 2012). This means that certain processing techniques aren't able to reduce the allergenicity of the proteins (Ciara et al, 2012), though hydrolyzing the protein has been

shown to reduce the allergenicity of milk proteins significantly (Kaminogawa, S. and Totsuka, M., 2003). While pasteurization at lower temperatures, between 50-60°, does not significantly reduce the allergenicity of milk proteins, a study by Ehn and his colleagues showed that extreme heating, between 80-90°C, significantly reduces the allergenicity of milk proteins (Ehn et al, 2004).

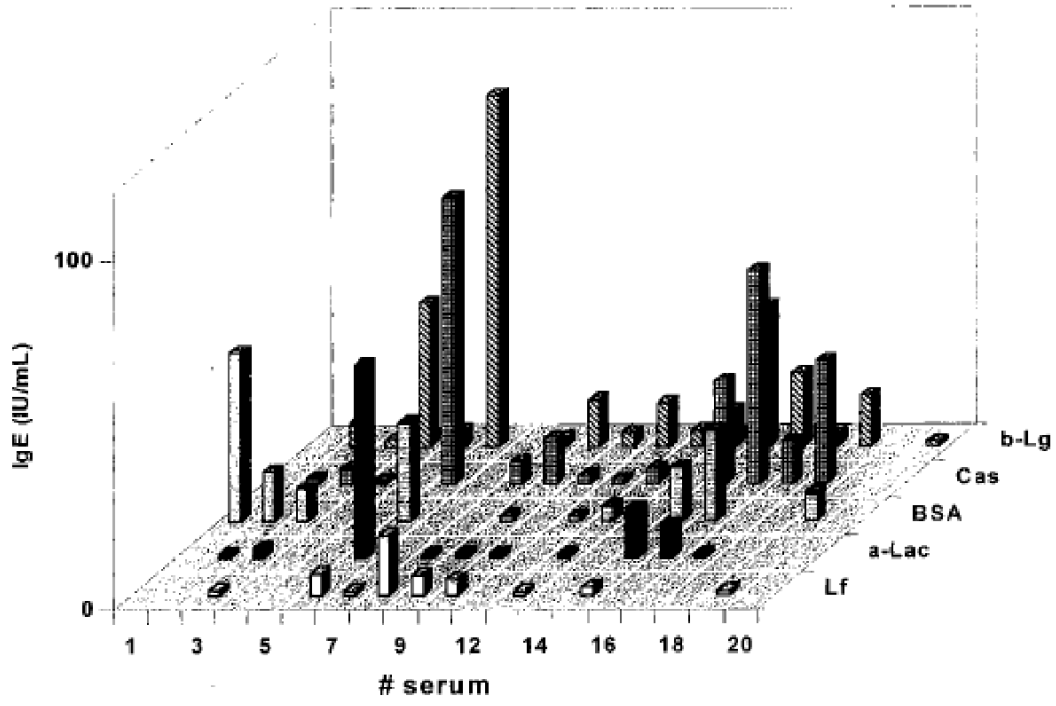

Figure 2.5: IgE response from various serum samples taken from different patients to five different milk proteins. On the right from the top down are beta-lactoglobulin, casein, bovine serum albumin, alpha-lactalbumin, and lactoferrin (Wal, 2002). It should be noted the different responses in the different patients, such as the large responses from patients 6, 15, and 16, while 19 and 20 show barely any response to any of the proteins.

The prevalence of milk protein allergies is relatively low compared to the other “Big 8” food allergies, but the allergic reactions are just as bad, if not worse (Hill et al, 1997). Allergies to milk proteins typically are found in young children, although it still can be diagnosed as an adult. The average prevalence in small children has been found to be about 2-6% of the general population, (Hill et al, 1997), with the commonality decreasing as age increases. It has also been found that if symptoms of the allergy first appear before the age of three that the chances of decreased severity or complete disappearance of symptoms is likely, whereas if the symptoms appear after the age of three it is unlikely that they will outgrow their reaction to milk proteins (Ciara et al, 2012). Onset of adult allergic reaction is much rarer with it occurring in only 0.1-1.0% of the adult population. It has also been reported that while the occurrence of milk protein allergies in adults is rarer than in children, adults have been shown to experience more severe reactions than children (Lam et al, 2008). Again, this most likely has to do with most adults having developed an immunity to the proteins, while only the adults who are susceptible have the strongest reactions to milk proteins start to elicit symptoms.

2.2 Modern Laws and Allergens

All material and information in this section was retrieved from the Food Drug Administration’s website unless specifically sourced differently.

2.2.1 Food Allergen Labeling and Consumer Protection Act

The Food Allergen Labeling and Consumer Protection Act, FALCPA (Public Law 108-282, Title II), was the first major piece of federal legislature to focus entirely on protecting the general public from allergens in food. First going into effect on 1/1/06 as an amendment to the Food, Drug, and Cosmetic Act, FALCPA "requires that the label of a food that contains an ingredient that is or contains protein from a major food allergen declare the presence of the allergen in the manner described by the law" (Sec 203(c)(1)). In other words, if a company's product contains an ingredient from a known allergen source, no matter how small, the company must report it on their labels. FALCPA also defined what is a "major food allergen" by creating 8 major categories for which 90% of all documented food allergies in the USA represent (Sec 202(2)(A)). The 8 major allergens that FALCPA lists are: milk, eggs, fish (bass, flounder, cod, salmon, fona, etc.), crustacean shellfish (shrimp, crab, lobster, oysters, etc.), tree nuts (cashews, macadamia, walnuts, etc.), peanuts, wheat, and soybeans. The labeling requirements that FALCPA enacted also changed the way manufacturers listed ingredients. Now, companies had to use one of the two alternative. They can include the source of the food in parenthesis after the ingredient if the usual or common name of the food didn't already contain it, such as whey (Milk) or enriched flour (wheat flour….) (Sec 203(a)(1)(B)). The other option was for companies to put the phrase "Contains" and then follow that with any and all categories of the major 8 allergens that their product contains (Sec

203(a)(1)(A)). Listing the specific category of allergen that was included eliminated companies from putting a blanket statement of simply “Contains Allergens” on their label in an attempt to cover all their bases. While the term “Contain” must be used if an allergen is used, the term “May Contain” is not required since it is an advisory statement and not a stipulation of the law (Sec 204 (3)(A)). This means if a company uses a factory that also produces another product with a known allergy or if there is a slight chance of cross-contamination from similar ingredients, the company is not required to warn consumers of the chance their product contains an allergen. Another part of the law that helped create more transparency on labels that under FALCPA, flavors, colors, and food additives were now required to put any of the aforementioned ingredients on a label if they contain or are derived from a major food allergen (Sec 203 (a)(4)).

While the law did a good job of shoring up some of the issues that allergens caused, it was in no way complete. Along with not requiring the statement “May Contain”, FALCPA has certain food groups that are exempt from the law, raw agricultural commodities, and highly refined oils, even if the oil is derived from one of the major food allergens (Sec 203(a)(1) and Sec 203(c)(1)). And in the event that an allergen does find its way into a product and isn’t labeled, its deemed misbranding and only subjected to seizure and removal from the marketplace.

2.2.2 The Food Safety and Modernization Act

The Food Safety and Modernization Act, FSMA, that was signed into law by President Obama on January 4th, 2011 as a response to the large food related illnesses, recalls, and mishaps that occurred during the late 2000's. This far sweeping, and much needed bill granted the FDA new powers to help control and regulate the industry while also implementing new rules and regulations to help bring the food industry into the 21st century. Considered the largest and most far reaching law to be applied on the food industry since the Food, Drug, and Cosmetic Act of 1938, this piece of legislation also puts additional focus on allergens in the food supply by requiring that companies create measures to prevent cross-contamination of the food supply by allergenic proteins.

While FSMA was passed in 2011, the events leading up to it were a long time in the making. When the FDCA was passed in 1938, the US food industry looked a lot different than today. Being more rural and homegrown focused, the commercial food markets were still in their infancy and definitely not as far reaching as today. With the expansion of consumerism, also aided by various processing techniques that allowed us to ship and store foods over greater distances and time periods, we are able to enjoy a larger variety of food products. But with this new expansion, comes new risks involved. The tipping point came in the form of the massive Salmonella outbreak in 2009 from Peanut Corporation of America's peanut paste and peanut butter. The outbreak, which sickened 714 people, killed 9, and effected over 3,900 SKUs and over 350

companies, is considered the largest foodborne illness outbreak to hit the US, as illustrated in Figure 2.6 below which depicts the number of cases state by state (CDC, 2009). It is also considered the necessary catalyst that got FSMA introduced and eventually passed, with a lot of FSMAs new legal clauses being directly tied to issues with the PCA case.

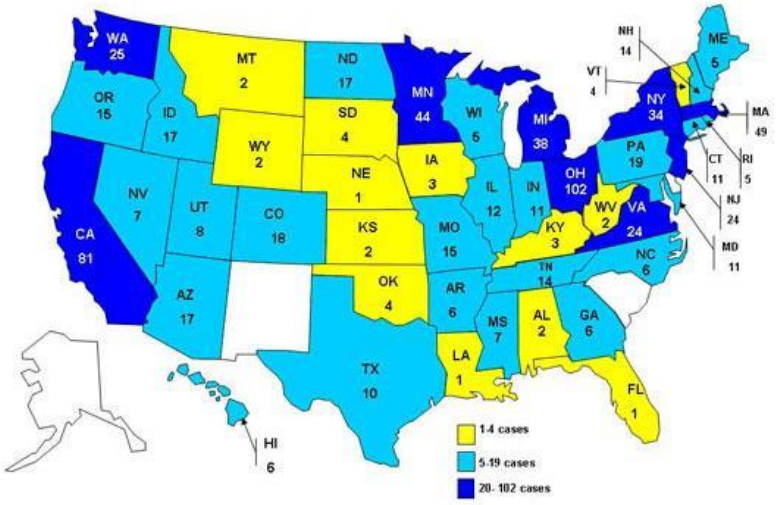

Figure 2.6: State by state cases of *Salmonella* Typhimurium from the PCA outbreak in 2009 (CDC, 2009).

While FSMA has extensive sections on various food safety laws, the focus here on will mainly be on allergens and what FSMA has decided to do with them. Prior to FSMA, if a company had a product that had an unintended allergenic ingredient that caused an illness, whether from cross-contamination or from negligence by the company whom did not list the allergenic ingredient, the FDA would request that the company do a voluntary recall, as the FDA didn't have the

power to force a company to do a mandatory recall. Also, as noted above, it wasn't considered adulteration to have an unintended allergen in a product. What the FDA would ask the company to do a recall on their product for was a misbranding of ingredients, as the allergen wasn't known to be in the product and thus wasn't printed on the label. This was the FDA's way of working around the excuse by companies that said they didn't know or didn't care to double check their products. With the introduction of FSMA, this all had changed. Now the FDA was given the explicit power of mandatory recall authority, which allowed the forced recall of any contaminated product, as stated by FSMS section 423 (a)(1). It also reclassified allergens that weren't labeled as adulterants, thus giving the FDA more power to force recalls and help prevent injuries or deaths from cross contamination of allergens. This reclassification of allergens from a misbranding to adulteration shows the concern of what an undeclared allergen could do to the susceptible population. This can be further seen as now the cGMPs listed by the FDA also explicitly list allergen control in HACCP, section 418(n)(3)(D). The law also requires that federal and state officials be given the proper training, and/or experience necessary to properly carry out all forms of testing for food safety, as seen in section 1011(a). This measure focuses more on the government side and making sure all employees are trained and can properly carry out their duties in food safety testing.

2.3 Nutritional Value of Whey

Whey proteins contain a variety of nutritional functionalities that are highly sought after. For starters, whey proteins are considered an excellent source of essential amino acids. They contain a great variety of necessary amino acids needed by the body to function properly. They also are considered one of the best sources of protein for the promotion of muscle synthesis and for the use in a healthy diet. As seen by the table below, taken from Korhonen et al and Maduriera et al, 1998 and 2007 respectively, each of the individual constituents of whey have their own unique nutritional value that they add for wanting to use whey proteins in a final product (Ha and Zemel, 2003).

Table 2.2: Shown above is the list of whey proteins and a brief description of the nutritional functions that each of the proteins offers (Korhonen et al, 1998 & Maduriera et al, 2007).

Protein	Concentration (g/l)	Function
β-lactoglobulin	1.3	Retinol carrier, fatty acids binding, possible antioxidant
α-Lactalbumin	1.2	Lactose synthesis in mammary gland, Ca carrier, immunomodulation, anticarcinogenic
Immunoglobulins A, M and G	0.7	Immune protection
Bovine serum albumin	0.4	Fatty acid binding, anti-mutagenic function, prevention of cancer, disease protection through passive immunity
Lactoferrin (LF)	0.1	Antimicrobial, antioxidative, immunomodulation, iron absorption, anticarcinogenic

Starting with β-LG, it is a great fat soluble vitamin binder, especially vitamin A. Having a higher concentration of a protein that binds to fat soluble vitamins will allow more of the vitamins to be absorbed into the bloodstream and

dispersed where needed. Since it binds fat soluble vitamins, it is also able to find free fatty acids. As with the fat soluble vitamins, this allows the absorption of fatty acids to increase. Now whether this is a positive or negative effect depends on the diet of the individual, with those who consume more saturated fatty acids or trans fats being at a greater disadvantage over those who consume healthier fats. There is also a possible antioxidant trait that has been linked to β-LG as well, but the mechanism of this isn't as well understood as the other functions of β-LG (Marshall and Keri, 2004 & Maduriera et al, 2007).

α-LA is present in all mammalian milk as it is a catalyst for the production of lactose in the mammary glands. It has been reported to speed up the production of lactose by up to 250x by lowering the K_m of the reaction to make it more favorable (Brew and Grobler, 1990). It is also a great carrier of calcium, which is essential for proper bone health and upkeep. This could be used by those experiencing bone deficiencies or for the elderly trying to stave off osteoporosis. It could also be used as a supplement in infant formulas or for younger children to help with their growing bones and to make sure the calcium they are ingesting isn't just being passed through their systems and excreted. Also, another use would be for women who are currently going through or have gone through menopause, as they are more likely to develop osteoporosis than those who haven't experienced menopause yet (Marshall and Keri, 2004 & Krissansen, 2007).

BSA, which enters into the mammary glands from the bloodstream, is another great fatty acid binder. As with β-LG, this could be used to help the body better absorb fatty acids that would normally pass through the body. Also like β-LG, depending on the diet of the individual or what product BSA is a part of, this could be seen as a negative or positive depending on the type of fatty acid it binds too. Another interesting quality of BSA is that this protein has the ability to bind with certain pro-oxidant transition metals (Ramesh et al, 2007). This ability has a number of positive uses in regards to one's health. First, it helps by binding to and eliminating certain metals that help to oxidize other nutrients or parts of the body. In this way, it acts like an antioxidant, at least indirectly. Secondly, since it has been known that radicals can cause mutations to DNA and RNA and that these certain transitional metals play a role in creating said radicals, by taking the pro-oxidant metals out of the equation BSA acts as an anti-mutagenic and anti-cancer compound, thereby helping to prevent mutations in DNA or RNA that could possibly lead to cancerous promoters in the sequences (Ramesh et al, 2007).

2.4 Detection of Whey in Food Systems

2.4.1. Capture Methods

2.4.1.1 Antibodies

Antibodies are Y-shaped Immunoglobulin proteins, Ig, produced by the immune system and are most commonly found in blood plasma. Each antibody

that is created is specifically designed by the immune system to attach itself to its target, or antigen, as seen below in figure 2.7. Once attached, the antibody will then either disable the antigen, or act as a signal for other immune cells to destroy or remove the antigen from the host's system (Janeway et al, 2001).

The immune system creates antibodies in response to foreign matter that is in the body. B-cells secrete the antibodies in response to the foreign matter in one of two forms; a soluble form and a membrane-bound antibody that is referred to as a B-cell receptor. The soluble form floats around the body until it finds its target antigen, thus signaling the body's immune system to remove the foreign material. The B-cell receptor is located on the surface of the B-cell and is responsible for the B-cell creating plasma cells, cells that create specific antibodies, or memory B-cells, which are used to help the immune system respond to future infections faster by remembering the target antigen (Janeway et al, 2004).

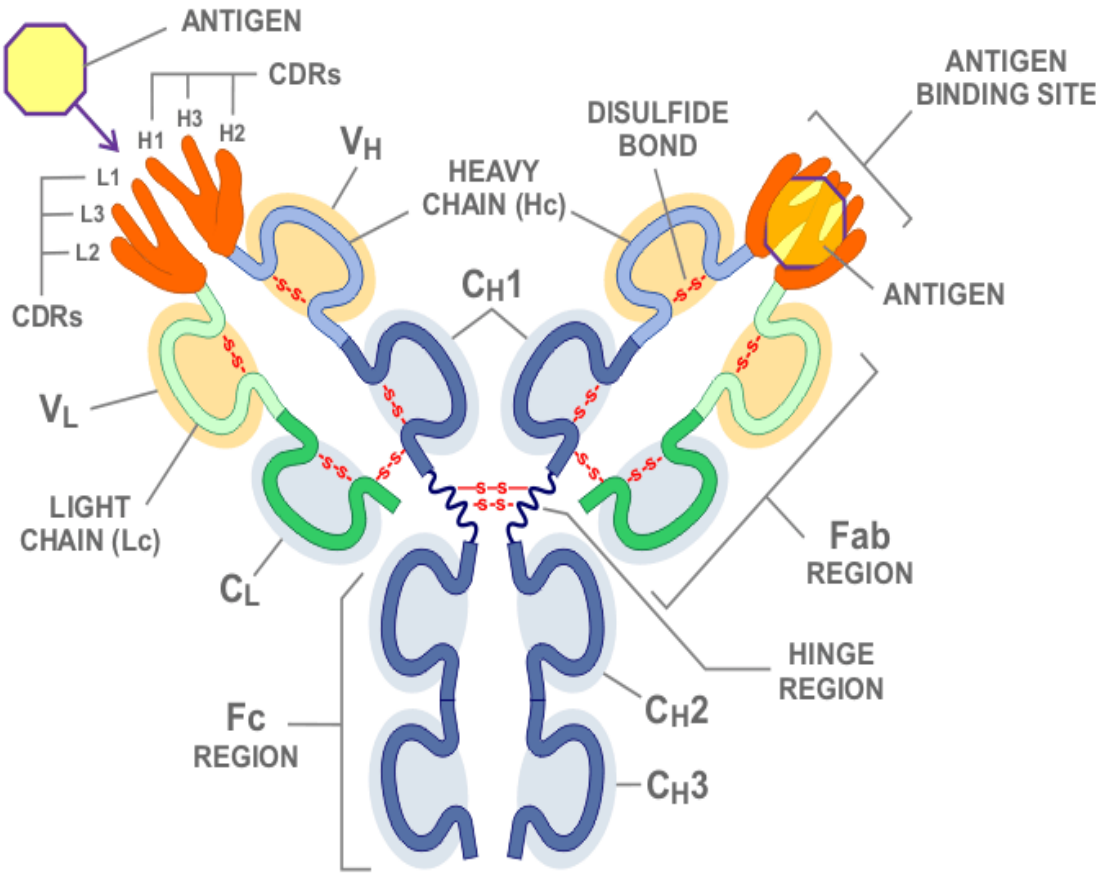

Figure 2.7: Illustration of the typical structure of an antibody. Consisting of heavy and light chain sections, the Y-shaped section of the antibody is responsible for the capture of antigens that are specific to the individual antibody (Novimmune, 2016).

2.4.1.2 Aptamers

Aptamers are single stranded nucleotides that are synthetically created to attach themselves to a target antigen by covalent or hydrophilic/hydrophobic interaction (Mosing, 2004). These aptamers are typically created through a process called Systematic Evolution of Ligands through Exponential Enrichment, or SELEX. This SELEX process can vary based on certain tools or methods used, but in general it involves mixing the target antigen with a random single stranded library of oligo nucleotides that are 10-30 base pairs long. From there the ligands that are able to attach themselves to the antigen are separated, again this is where some methods vary, and the successful ligands are enriched through PCR amplification. After the amplification, this process is repeated from the beginning for a number of rounds until an acceptable ligand with a good binding capacity is found. This whole process has been known to take as little as a few days to achieve a working aptamer, whereas antibodies can take weeks for new ones to be created and harvested from their host animals (Mosing et al, 2009).

Like antibodies, aptamers in detection methods, are either allowed to incubate directly with the antigen or are fixed onto a surface and then have the sample with the possible antigen incubated or passed over it. While both are used to capture or attach to an antigen, an aptamer focuses in on a target based on its conformation, size, or shape, whereas an antibody typically has a certain sequence that it attaches to on the antigen theoretically in the epitope region.

This allows aptamers to hone in on antigens with more specificity, since the odds of proteins being of similar shape and size is far less likely than having similar amino acid sequences. It also allows the aptamers to distinguish between native and denatured proteins, such as the aptamer used in the paper by Janardhanan et al (Janardhanan et al, 2013). This would mean a company could better differentiate better between toxins that are in their native and harmful forms and those that are denatured and pose no threat to the general public. This would also lead to fewer recalls or false-positives and save the company a lot of money on unnecessary testing and bad publicity.

2.4.1.3 Immunomagnetic Separation

Immunomagnetic separation, IMS, uses a magnetic bead, most typically a ferrous blend, to coat small beads. These beads are then coated with a compound, either protein G or A for antibody based IMS or streptavidin for aptamer IMS. This allows a capturing agent, antibodies or aptamers, to stick to the bead. The magnetic beads are then allowed to be coated with the capture agent at which time the bead/capture agent is then allowed to mix with the food sample or surface swab. Once incubated for an appropriate amount of time, the beads are separated from the solution by using a strong magnet to pull the beads away from the rest of the solution.

IMS is ideal for complex samples that would either be impossible or time consuming to separate the target. In food science, this would be most samples.

Whether it's carbs, lipids, or proteins, IMS allows the rapid capture and separation of a target from a complex sample. IMS has been used extensively to detect microorganisms for some time now, such as *E. coli* and *Salmonella* (Wright et al, 1994 and Cudjoe et al, 1997), but also has been used more recently to capture harmful proteins such as ricin (He et al, 2011), adulterants such as melamine (Li et al, 2015), or illegal pesticides (Pang et al, 2014).

2.4.2 ELISA

The Enzyme Linked Immunosorbent Assay, or ELISA, is a detection technique that combines an enzyme with some type of substrate that then causes a detection signal, typically color a change, to be produced and detected by an outside source. Typically, antibodies have been used to attach themselves to their antigen, which has been already adhered onto some type of surface. Once attached, an enzyme is then mixed into solution that attaches itself to the antibody. From there, a substrate is then mixed with the solution that reacts with the enzyme and gives off a reporter signal that is measured and possibly quantified. The amount of color change and how it is measured is based off of what type of ELISA method is used. There are essentially four types of ELISA methods; direct, indirect, sandwich, and competitive ELISA. Direct ELISA is when an antigen is allowed to coat itself to the wall of a well and a blocking solution is added to make sure there are no open spots left on the wall of the well. Then an antibody with the enzyme linked to it is added to the well and allowed to attach itself to the antigen, if present. Then a substrate is added and the color change in

the well is measured, typically by some type of plate reader. This was the first method that was developed that was labeled as ELISA (Engvall and Perlmann, 1971). The direct method has some disadvantages, such as the need to create the linkage between the enzyme and the antibody for each antigen, adding extra time and money to the method. Indirect ELISA is similar to direct, except that the antibody that attaches to the antigen isn't labeled, but a secondary antibody, which is enzyme labeled, is used to attach itself to the primary antibody and then react with the substrate to give a signal. This deals with the issue of having to have that extra step of labeling the antibodies and can be a problem if the secondary antibody isn't properly washed away, thus giving a false-positive signal. Both direct and indirect ELISA also suffer from the issue of detecting the antigen in complex matrices. The antibodies can attach themselves to non-antigen targets or fail to find the antigen altogether. Sandwich ELISAs help to solve the issue with complex matrices by having a primary antibody coat the plate instead of the antigen complex. This way the antibody on the plate captures the antigen from solution, allowing the separation from the matrix that would normally interfere with direct and indirect ELISA. From there, the procedure is similar to the other two ELISA methods, where an antibody and enzyme are attached to the antigen and a color change is measured. The final method, competitive, uses the lack of color change as the basis for the presence of the antigen. First, a known amount of the primary antibody is mixed with the sample It assumes that any antigen that is in the sample will bind to the antibody. Next, the whole complex is then mixed into a well that has been coated with the

antigen. This way, if there was no antigen in the sample, the antibodies that were mix in before will bind to the antigen on the coated plate. From there, again, the direct or indirect method can be used to get a signal. A positive sample for competitive ELISA will yield less of a signal than a negative, since the antibodies attach to the antigen in the sample versus attaching to the antigen in the well.

While there is no official method for detecting whey, both as a protein or as an adulterated allergen, ELISA is viewed as the go-to confirmatory method in the food industry. This most likely has to do with the fact that the reagents are readily available from a number of different commercial sources, it being fairly versatile in what matrices that it can work in and the fact that it has been established to work as a detection method for whey for quite some time (Rodriquez et al, 1990 and Abramowski et al, 1991). The biggest issue that ELISA has is the time required to do it. Even if one was to buy the pre-coated plates for a sandwich ELISA for a specific antigen, the incubation and blocking steps typically add at least 4-5 hours before you are able to get a signal from the test. In industry, especially where time is done daily, the time needed to do a test is crucial. For this reason, as of now, ELISA methods have been mostly used for confirming of an adulteration. Yet there are new developments of fully automated ELISA instruments being tested right now. These instruments are capable of running multiple samples in an hour and are fully automated to run everything that a normal ELISA would require (Morier et al, 2016 and Pearson et al, 2016). While these are being tested on pure solution using human Ig, the possibility for

conversion over to whey proteins in a complex matrix such as hot dogs and other industry methods looks promising.

2.4.3 Other Methods

While ELISA might be the standard for detection of whey proteins in industry, there has been a fair amount of research into other areas. Besides ELISA, the next method for whey detection would be liquid chromatography, or LC. Whether it's normal or reverse phase, ion-exchange, or affinity, LC offers a variety of different pathways for detection, depending on the matrix of the sample (Hurley and Rejman, 1988, Elgar and Palmano, 2002, Safirik and Safirikova, 2004, and Tolkach and Kulozik, 2005). LC also shows its versatility when combined with the different detectors, such as UV-vis or photodiode array detectors, that allow improved detection. LC can also be used with mass spectrometry to further increase the level of detection, even down to deciphering the individual amino acid sequence for a protein (Fauquant et al, 1997 and Salemi et al 2002). Capillary electrophoresis is another niche tool that has been investigated as a detection tool for whey (Benito et al, 1999). Similar to LC in that it helps to separate out the proteins in the sample to better detect the target, the difference being instead of having the sample travel through the large column, it uses a small capillary that can be only micrometers in diameter. It also differs in that the primary driving force for the samples to move through the capillary is

charge differential, similar to gel electrophoresis. A technique that has been around for a little longer and is fairly well established is blotting. These techniques use some type of stain on either a gel or membrane to show a band or spot where the protein is and then compare that spot or band to a predetermined protein size ladder to to calculate the relative size (Janssen et al, 1987). The issue with this method is that there isn't a lot of specificity, such as there is in LC or other methods, and that one needs to run a standard simultaneously to determine protein size. This can be improved by the addition of antibodies, such as in western blots, but this will increase the time needed to run the method and further lengthen the time until detection. Another technique that is more rapid and whose technology has gained more use in recent years, especially in food safety (Alocilja and Muhammad-Tahir, 2003 and Rauch et al, 2009), is lateral flow. Lateral flow devices, or more commonly known as test strips, are used by adding the sample to the test strip and then allowing the sample to migrate to the detection method, whether it's an electrical sensor or some type of immuno color changing method (Duary et al, 2009). Once it reaches the detection region, the target, if present, will interact with the detection location and give off a signal. This method is great since most of the time the strips are relatively easy to transport and store and can be used on-site in the plant. The tradeoff for that ease of use is that they are sensitive to some properties of the sample matrix, such as lipids, which can render the test useless.

2.5. Raman Spectroscopy

2.5.1 History of Raman Spectroscopy

Raman spectroscopy is an analytical tool that uses monochromatic light to detect the scattering of light. Typically, a laser is focused on a sample at a specific wavelength. This allows the excitation of the molecules through the absorption of the energy from the laser. This excitation also alters the refractionation of the incoming light that is proportional to the type of bond that it is hitting, such as a N-O bond will alter the light at a different wavelength than say a O-H or a C-O bond. C.V. Raman first discovered this phenomenon back in 1928 while working at the Indian Association for the Cultivation of Science, IACS. He observed that some light was able to pass through a cross filter after already having passed through a monochromatic filter, thus showing that the light was changing ever so slightly (Raman, 1928). This work would eventually win him the Nobel Prize in Physics in 1930, making him the first Indian scientist to win the Nobel Prize in a science category and only the 2nd to win in any category. Raman would use this research to show proof of the quantum nature of light (Raman and Bhagavantam, 1931).

There are two types of raman scattering that are most commonly recorded; Stokes and anti-Stokes scattering. Stokes scattering occurs when the energy from the light source is transferred to the sample resulting in a light source of lower energy reading. Anti-Stokes, as the name implies, is the opposite

where energy from the sample is transferred to the light source resulting in a higher energy reading. These two differences in energy and wavelength are what makes up Raman spectroscopy and allows the detection of different bond types, as shown below in figure 2.8. The overall spectra, which combines the signals from a wide range of wavelengths, is what gives each sample a unique fingerprint that allows the detection of individual substances and elements. While this technique does give detailed information about the sample, the biggest issue with Raman is that only a small fraction of the light that reaches the sample gives a signal.

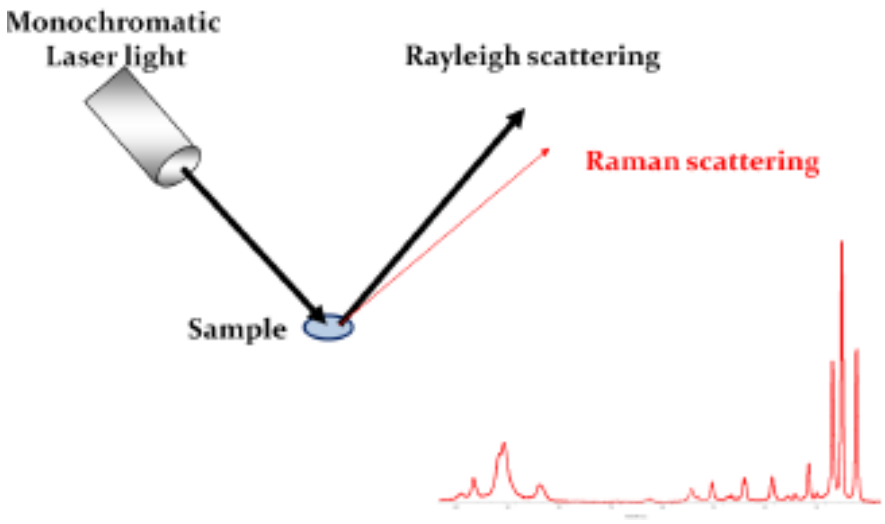

Figure 2.8: Diagram that demonstrates how Raman spectroscopy works by detecting the difference between the light source and the Raman scattering and then correlating it to a spectrum.

2.5.2 Surface Enhanced Raman Spectroscopy

First observed by Dr. Fleischmann in his 1974 paper on the detection of pyridine on roughed silver, this form of Raman helps to overcome the limitations of simple Raman spectroscopy by amplifying the signal exponentially (Fleischmann et al, 1974). This signal amplification, which can be 10^6-10^{11} in magnitude depending on the sample, has been shown to reveal signals on spectra that otherwise wouldn't be detectable otherwise (Blackie et al, 2007 and Ru et al, 2009). It has also been shown that not only does the signal become amplified, it also reveals signals that were not shown under normal Raman conditions, as demonstrated below in figure 2.9. This most likely has to do with the surface portion of the method and the amplifying ability that it brings. Most scientists who work with surface enhanced Raman spectroscopy, SERS, agree that the mechanism behind the enhanced signal is from one of two methods; a chemical method or an electromagnetic one. The theory behind the chemical mechanism is that through some type of chemisorption of the sample with the surface metal, a charge transfer occurs between the two that allows the amplification of the signal to occur. It also contributes to the chemical bonding between the sample and the surface metal as helping to keep the sample and the metal at an ideal distance, since this theory suggests that there is a finite distance from the surface metal that the enhancement can occur within (Lombardi et al, 1986). The other theory, the electromagnetic theory, states that the light source, when striking the surface metal, excites localized surface

plasmons around the contact point creating a small field of enhanced signal (Campion et al, 1998). Surface plasmons, which are the key to the amplification of the Raman signal, are localized, excited electric fields that, when oscillating at the same frequency as the light source give off this enhanced signal. This is theorized to happen because the surface plasmons first amplify the incoming light source, which will thus increase the Raman scattering from the sample. The signal is further amplified from there by the surface plasmons, which after the Raman scattering occurs, the plasmons amplify that signal as well. The key difference between the two theories is that the chemical theory states there must be some type of bond between the two surfaces, suggesting that the sharing of electrons is responsible for the enhancement, while the electromagnetic theory states that the creation of excited surface plasmons and the electric field that accompanies them is what is responsible for the signal boost. As of today, there appears to be more evidence for the electromagnetic theory as researchers have demonstrated that they are able to still demonstrate the signal enhancement even when the sample is farther apart from the surface metal (Kukushkin et al, 2013). This research shows that the chemical bonding from the chemical theory isn’t necessary, and more likely involves the chemical bond and the electromagnetic field around the sample to achieve the signal enhancement.

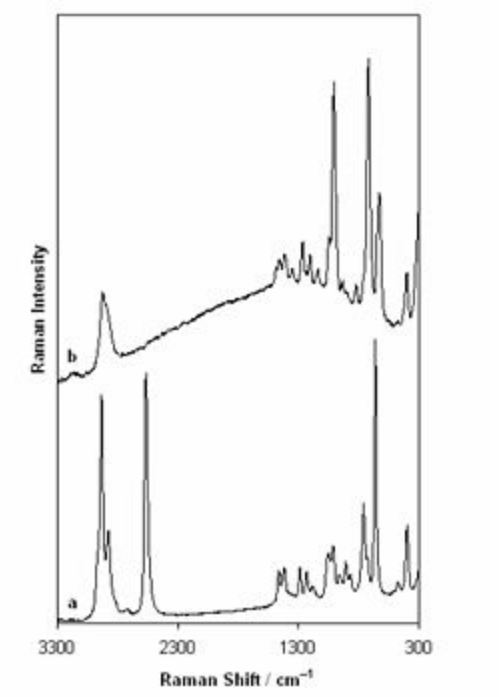

Figure 2.9: Two spectras showing the difference between normal Raman, top, and SERS, bottom. The spectra are of 2-Mercaptoethanol, and as you can see, there are peaks that are more clear in the SERS graph or even that weren't seen before in the normal Raman spectra.

2.5.3 SERS Substrates

What allows the extra sensitivity of SERS, and is the key to the entire procedure, is the substrate that the sample is placed upon. Since the first observation of the SERS effect back in 1974 where they used chemically roughened silver, there has been an explosion of different materials that have been used to enhance the Raman signal. Today, the most common materials that are being used are noble metals, such as gold and silver, or those that

incorporate silica into the matrix (Ru et al, 2007). Since the 1970's there has also been advances in the use of other metals as well, such as copper or aluminum for certain sample types (Creighton et al, 1991), and there has even been breakthroughs, more recently, in liquid SERS with the development of a technique called Slippery Liquid Infused Porous SERS, SLIPSERS, where a liquid is used in conjunction with a porous material to further enhance the signal (Yang et al, 2016). While there seems to be more and more creative and new ways to take advantage of the surface enhancement, there are some issues with them that will need to addressed before these techniques can be reasonably used in industry. While there are plenty of techniques that are quite sensitive, they are either costly to mass produce or take considerable time to create or implement with the sample, such as incubation or mixing. There are some substrates that have demonstrated their ability to be quick, cheap, and sensitive enough for a broad range of sample types. One example would be silver dendrites. Silver dendrites, AgD, have been referenced and used since the 1980's, but more so as a novel approach for a surface metal and weren't extensively used until about the mid 2000's as an economically viable method for SERS in industry (Seki, 1982 and Jing and Fang, 2007). The method described by Jing and Fang uses a simple replacement reaction between zinc plates and silver nitrate. A cleaned zinc plate is submerged into an aqueous solution of silver nitrate and allowed to react for about one minute. During that minute, the silver in solution will replace the zinc on the surface creating silver dendrites, seen below. These silver dendrites are then carefully scraped off the surface of

the unreacted zinc and can be stored at RT in DI water for at least 6 months. It also has the added benefit of having many locations on the silver dendrite surface that the sample could find a suitable place to be read by SERS, more specifically by being the appropriate distance away from the silver dendrites that the surface plasmons will create the enhancement effect needed, as seen below in figure 2.10. This technique uses materials that are cheap while also being easy to create and use, thus showing great potential for industry use in the future.

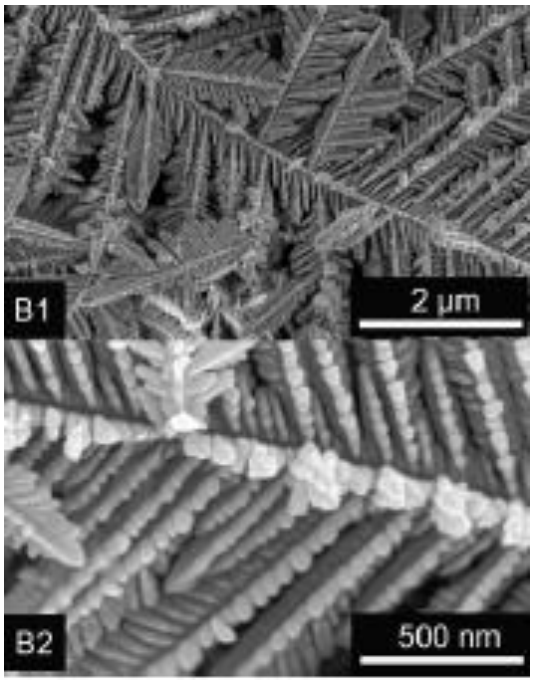

Figure 2.10: SEM of silver dendrites created using Jing and Fang's replacement method. (Picture from Dr. He's PhD dissertation, Massachusetts University, 2009).

2.5.4 SERS in the Food Industry

SERS hasn't taken off in the food industry until recent years with advancement in the use of different substrates that better allow the magnification of the Raman signal. Also the advancement in sample preparation that allows for the target in the sample to be removed from solution. These two advancements in the methodology have allowed for the detection of certain target molecules in certain food matrices.

Ranging from illegal food additives and antibiotics (Gao et al, 2013 and Xie et al 2011) to more recently certain allergenic and toxic proteins (He et al, 2011) the uses of SERS in recent years have exploded, as seen in the extensive table below. As mentioned before, while SERS can be an incredibly sensitive instrument, that sensitivity cuts both ways, meaning that certain contaminants and other ingredients in the food matrix can interfere or block the target molecule's signal. Proteins also have the hurdle of being larger molecules when it comes to weak signals. Since they have a larger physical structure than say a pyridine ring, they are typically too far away from the surface to receive the plasmonic enhancement that other molecules benefit from. Typically, proteins that contain an abundance of Raman active peptides, such as phenylalanine, can overcome this since these peptides are better able to give off a Raman signal.

Table 2.3: List of different SERS methods for different food contaminants and what the limit of detection for them is. (Taken from Zheng and He, 2014)

Analytes	Substrates	LOD*	References
Food additives:			
Benzoic acid	Au colloids	–	(Gao and others 2013)
Phthalic acid	Au colloids	–	(Gao and others 2013)
Sodium benzoate	Ag colloids	–	(Pelca and others 2007a)
Butylated hydroxyanisole	Au colloids	10 ppm	(Yao and others 2011)
Aspartame	Ag films	–	(Pelca 2009)
Monosodium glutamate	Ag colloids	10^{-5} M	(Pelca and others 2007b)
I-	Rh_6G-adsorbed Au colloids	30 ppt	(Dasary and others 2013)
4-Arsanilic acid	Ag/polydimethylsiloxane	–	(Olavarria-Fullerton and others 2011)
Roxarsone	Ag/polydimethylsiloxane	–	(Olavarria-Fullerton and others 2011)
Acetarsone	Ag/polydimethylsiloxane	–	(Olavarria-Fullerton and others 2011)
HMB	Ag colloids	–	(Podstawka and others 2007)
L-Carnitine	Ag colloids	–	(Podstawka and others 2007)
Creatine	Ag colloids	–	(Podstawka and others 2007)
Pesticides:			
Methamidophos	Ag colloids	100 ppb	(Xie and others 2012a)
Chlorpyrifos	Ag-coated Au NPs	70~700 ng/cm^2 (fruit)	(Liu and others 2012a)
	Metal-doped sol-gel coated capillaries	~10 ppb	(Shende and others 2004a, b)
4-Methyl parathion	Ag-coated Au NPs	25~100 ng/cm^2 (fruit)	(Liu and others 2012a)
	Cyclodextrin decorated 1 D Au NPs	ppt level	(Wang and others 2010)
	Metal-doped sol-gel coated capillaries	~10 ppb	(Shende and others 2004a, b)
Azinphosmethyl	Q-SERS^{TM} G1 Au coated substrates	7 ppm (apple) 3 ppm (tomato)	(Liu and others 2013a)
Phosmet	Q-SERS^{TM} G1 Au coated substrates	7 ppm (apple) 3 ppm (tomato)	(Liu and others 2013a)
Malathion	Ag colloids loaded filter membrane	62 ppb	(Yu and White 2012)
	Metal-doped sol-gel coated capillaries	–	(Shende and others 2004a)
Dimethoate	Ag colloids	2 ppm	(Guerrini and others 2011)
	Metal-doped sol-gel coated capillaries	–	(Shende and others 2004a)
Fonofos	Metal-doped sol-gel coated capillaries	~10 ppb	(Shende and others 2004a, b)
Thiram	Ag-coated Au NPs	1~7 ng/cm^2 (fruit)	(Liu and others 2012a)
	Single clusters of self-assembled hydrophobic Ag NPs	24 ppb	(Yuan and others 2011)
	Sandwich nanostructure composed of GO nanosheets and Au/Ag NPs	30 ppb (grape juice)	(Zhang and others 2013)
Tricyclazol	Ag colloids	2 ppb	(Tang and others 2012)
Carbaryl	Q-SERS^{TM} G1 Au coated substrates	5 ppm (apple) 5 ppm (tomato)	(Liu and others 2013a)
	Metal-doped sol-gel coated capillaries	~10 ppb	(Shende and others 2004b)
Antibiotics and illegal drugs:			
Furadantin	Au colloids	~5 ppm	(Xie and others 2012b)
Furaltadone	Au colloids	~5 ppm	(Xie and others 2012b)
Tetracycline	Ni/Au core-shell microparticles	100 ppt	(Li and others 2011c)
Ciprofloxacin	Ag dendrites	20 ppb	(He and others 2010)
Enrofloxacin	Klarite^{TM} Au substrate	–	(Zhang and others 2012a)
Chloramphenicol	Klarite^{TM} Au substrate	50 ppb	(Lai and others 2011)
Brilliant green	Ag films over nanospheres	10^{-6} M	(Stropp and others 2003)
Malachite green	Q-SERS Au substrate	200 ng/g (tilapia fillets)	(Zhang and others 2012a, b)
	PdI_2 nanomaterials	10^{-7} M	(Wang and others 2011)
Crystal violet	Au colloids	~200 ppt	(He and others 2008a)
	Q-SERS Au substrate	20 ppb	(Lai and others 2011)
	ZnO/Ag nanoarrays	10^{-12} M	(Hu and others 2011)
Furazolidone	Q-SERS Au substrate	800 ppb	(Zhang and others 2012a)
Melamine:			
Melamine	Klarite^{TM} Au substrate	3×10^{-7} M	(He and others 2008b)
	Klarite^{TM} Au substrate	100 μg/g (wheat gluten) 50 μg/g (chicken feed) 50 μg/g (cakes) 70 μg/g (noodle)	(Lin and others 2008b)
	Klarite^{TM} Au substrate	1 μg/g (albumen) 2 μg/g (yolk)	(Cheng and Dong 2011)
	Au colloid agglomerates	100~200 ppb (various food matrix)	(Mecker and others 2012)
	Ag colloids	500 ppb (in liquid milk)	(Zhang and others 2010)
	ZnO/Au nano-needle arrays	10^{-8} M	(Chen and others 2010)
	Ag-coated Fe_3O_4 @SiO_2 three-ply composite microspheres	<< 10^{-6} M	(Hu and others 2010)
	Ag NP-decorated Ag/C nanospheres	3×10^{-7} M	(Chen and Liu 2011)
	Ag NP-coated poly(styrene-co-acrylic acid)	10^{-7} M	(Li and others 2011b)
	Ag NP-coated PS-NH_2 microspheres	2×10^{-8} M	(Zhao and others 2013)
	Vertically aligned CTAB-coated Au nanorods	10^{-15} M	(Peng and others 2013)

	Spherical aggregates from Ag NPs	10^{-7} M	(Liu and others 2013b)
Illegal food dyes:			
Sudan I	Au colloids	48 ng/g (chilli powder)	(Cheung and others 2010)
	Electropolished Al	10^{-7} M	(Di Anibal and others 2012; Lopez and others 2013)
Sudan II	ZnO/Ag nanoarrays	10^{-12} M	(Hu and others 2011)
Sudan IV	ZnO/Ag nanoarrays	10^{-12} M	(Hu and others 2011)
Ponceau 4R	Au colloids	5 ppm	(Xie and others 2012a)
Tartrazine	Ag colloids	10^{-10} M	(Peica and others 2005)
Mycotoxins and other small-molecule toxins:			
Aflatoxin B1	Ag nanorod array	5×10^{-5} M	(Wu and others 2012)
Aflatoxin B2	Ag nanorod array	1×10^{-4} M	(Wu and others 2012)
Aflatoxin G1	Ag nanorod array	5×10^{-6} M	(Wu and others 2012)
Aflatoxin G2	Ag nanorod array	5×10^{-6} M	(Wu and others 2012)
Saxitoxin	Ag colloids	2×10^{-9} M	(Olson and others 2011; Huai and others 2013)
Tetrodotoxin	Triangulated Ag nanoparticle arrays	900 ppt	(Lin and others 2009)
Microcystin	End-to-end assembly of Au nanorods	5 ppt	(Zhu and others 2012)

What makes SERS a great candidate for use in the food industry is that, besides the initial startup cost of the machine, it is a relatively cheap method. Once the machine is bought, the only expenses come from the separation method in the preparation step and the substrate cost, which typically can be either reused or can be made in a batch that can last up to 6 months (He et al, 2008). Another great feature of SERS is that it is relatively quick and can even have portions of the scanning process be automated, such as the repetitions per sample which in turn will better help with accuracy to prevent false positives and false negatives. With FSMA being now implemented and companies are being held responsible for more and more safety of their products, they will be looking for cheap and effective methods that can prevent bad product from reaching the market. With SERS having shown that it can be cheap, effective, and quick, it shows great potential to be used in the food industry. There are also advancements in the miniaturization of Raman spectrometers so that they can be implemented in a variety of different settings and don't need to be tied down in a certain lab setting. These machines, which can typically fit in a briefcase and

weigh roughly 25 pounds, open up possibilities for SERS to be used in the field and on production lines directly, saving even more time of having to transport samples from production time to the lab to get tested.

3 Materials and Methods

In this section, the materials and methods used to detect whey protein in a hot dog are discussed. With the help of men and women in the Andrew Boss Laboratory of Meat Science, model hot dogs were created to test the accuracy and effectiveness of the different testing conditions used in the methodology. These hot dogs were created to simulate real life conditions that this test could one day be used for.

3.1 Hot Dog Ingredients

The hot dogs were created at the Andrew Boss Meat Science Laboratory at the University of Minnesota Twin Cities. The hot dogs were cooked for an hour and a half at 150°F. The recipe for the hot dogs is as follows and is all in pounds:

1. Meat - 25
2. Salt - .50
3. Seasoning - .30
4. paprika - .04
5. white sugar - .10
6. modern cure - .06
7. sodium tripolyphosphate - .10
8. sodium erythorbate - .03
9. water - 2.5

The seasoning recipe for used for the hot dogs is as follows:

1. 2 parts white pepper
2. 2 parts black pepper
3. 2 parts ground coriander
4. 2 parts ground nutmeg
5. 1 part ground mustard
6. 1 part paprika

3.2 Whey Protein Isolate

The whey protein isolate (WPI, BiPRO™) that was used throughout all the experiments was donated by Davisco Food International, Inc. (Eden Prairie, MN). Its protein content was 97.2% by weight (dry basis) according to its Certificate of Analysis that was provided by the company.

3.3 Silver Dendrites Preparation

The silver dendrites used throughout the project were created using a replacement reaction between zinc metal and aqueous silver, as seen below. This method was adopted from a similar experiment and procedure developed by Dr. He (He et al, 2011).

$$Zn_{(s)} + 2\ AgNO_{3(aq)} \rightarrow 2Ag_{(s)} + Zn(NO_3)_{2(aq)}$$

A 200mM aqueous solution of $AgNO_3$ was created by dissolving $AgNO_3$ (Sigma Aldrich, St. Louis, MO) in distilled, deionized water (DDI). A sheet of zinc

metal (Fisher Scientific, Rochester, NY) was cut into a 1”x3” piece and cleaned by scrubbing it in dilute HCl (~0.02M) to remove any contaminants or oxidants from the surface for approximately 1 minute. The zinc strip was then rinsed with DDI, dried, and dipped into the 200mM $AgNO_3$ solution for 2 minutes. As the replacement reaction was taking place, the surface of the zinc became coated with silver metal, becoming black, and then silver as the reaction progressed. After 2 minutes, the strip was carefully removed with tweezers and the silver metal on the surface was carefully scrapped off into a 50mL conical tube filled with 15mL of DDI water. The silver dendrites were washed by letting all the silver settle to the bottom of the tube and then the supernatant was carefully decanted and replaced with new DDI water. This was done 5 times to remove any excess Zn^{2+} or NO_3^- ions that remained. After washing, the silver dendrites, AgD, were allowed to equilibrate at room temperature in the DDI water for 2 weeks before use. The equilibrated AgD could be stored at RT in DDI water for at least 6 months until ready to use.

3.4 Hot Dog Sample Preparation

Using the method described below, hot dog samples were prepared for IMS and then for analysis using SERS:

1.) 25g of hot dog were weighed out, cut into 1cm pieces, and placed into 250mL blender cups.

2.) 100mL of extraction liquid was then added (10mM PBS for antibody IMS and 10mM NaCl for aptamer IMS) and the lid was screwed on.
3.) The hot dog was then blended in a Osterizer Blender (Oster Manufacturing, Boca Raton, Florida) on low liquefy setting for 2 minutes.
4.) The blended hot dog was then transferred to a 500mL beaker and allowed to shake for a minimum of 8 hours on an orbital shaker (Fisher Scientific, Rochester, NY) at 200rpm in a 4°C cooler. This allowed the whey to be extracted from the solid material and dissolve into the extraction liquid.
5.) The blended hot dog was then taken from the cooler and strained through a steel-mesh strainer (Target, Roseville, MN) for 5 minutes to allow the liquid to separate from the solids.
6.) The extracted hot dog liquid was then further strained through 0.5g of glass wool (Sigma-Aldrich, St. Louis, MO) using a forced air, vacuum filter. This whole straining process yielded on average ~50-55mL of extracted liquid
7.) 50mL of the extracted liquid was then transferred to a 100mL beaker and 25µL of Tween 20™ was added and mixed with a magnetic stir bar on a Fisher magnetic mixer (Fisher Scientific, Rochester, NY) for 5 minutes on high.
8.) The extracted liquid was then placed in a 4°C cooler until ready to use.

3.5 Preparation of Antibody-based Magnetic Beads

SureBeads™, protein G coated magnetic beads, were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). The beads, as obtained, came in vials of 3mL at a concentration of 10mg/mL magnetic beads. The beads were suspended in 10mM TBS with 0.05% Tween 20™ and 0.09% sodium azide as preservatives. The beads were 2.4–3.4 µm in diameter with an average antibody binding capacity of ≥6 µg antibody/mg of magnetic bead.

A polyclonal anti-whey protein was purchased from Sigma-Aldrich (St. Louis, MO) in the form of whole antiserum. The protein concentration of the antiserum was 63.9mg/mL and the antiserum was preserved using sodium azide at a concentration of 15mM. The antiserum was aliquoted and stored at -20°C for future use. Antibodies were conjugated to the beads according to the manual, illustrated below in figure 3.1, steps 1 and 2:

1.) The beads were first thoroughly resuspended by vortexing them for 1 min.
2.) 100µL of the resuspended beads were placed into a 2mL centrifuge tube and was allowed to separate from the supernatant for 2 minutes, on a magnetic rack, (Thermo Fisher, Rochester, NY) and afterwards the supernatant was discarded.
3.) The tube with the beads was then taken off the magnetic rack and washed with 1mL of PBS-T (10mM PBS + 0.1% Tween 20™).

4.) The beads were then vortexed for 5 seconds and centrifuged for 5 seconds so as to remove any excess beads from underneath the cap.
5.) The tube was then placed on the magnetic rack again for 2 minutes. This washing step was repeated two more times for a total of three washes.
6.) After the final wash step, the beads were taken off the rack and mixed with 10µg of antibody and then the total volume of the tube was brought up to 200µL using 10mM PBS-T (0.1% Tween 20™) and the beads were resuspended.
7.) The bead/antibody complex was then allowed to incubate on a tube rotator (Fisher Scientific 05-450-200, Rochester, NY) for 15 minutes at 35rpm at room temperature.
8.) After the incubation, the tubes were removed and centrifuged down at 3000rpm for 5 seconds to prevent loss on the cap of the tube (Fisher Scientific 05-090-100, Rochester, NY). Then the complex was washed three times in the same fashion as steps 2-5 above.
9.) The beads were then resuspended in 200µL of PBS-T and stored at 4°C until ready to use.

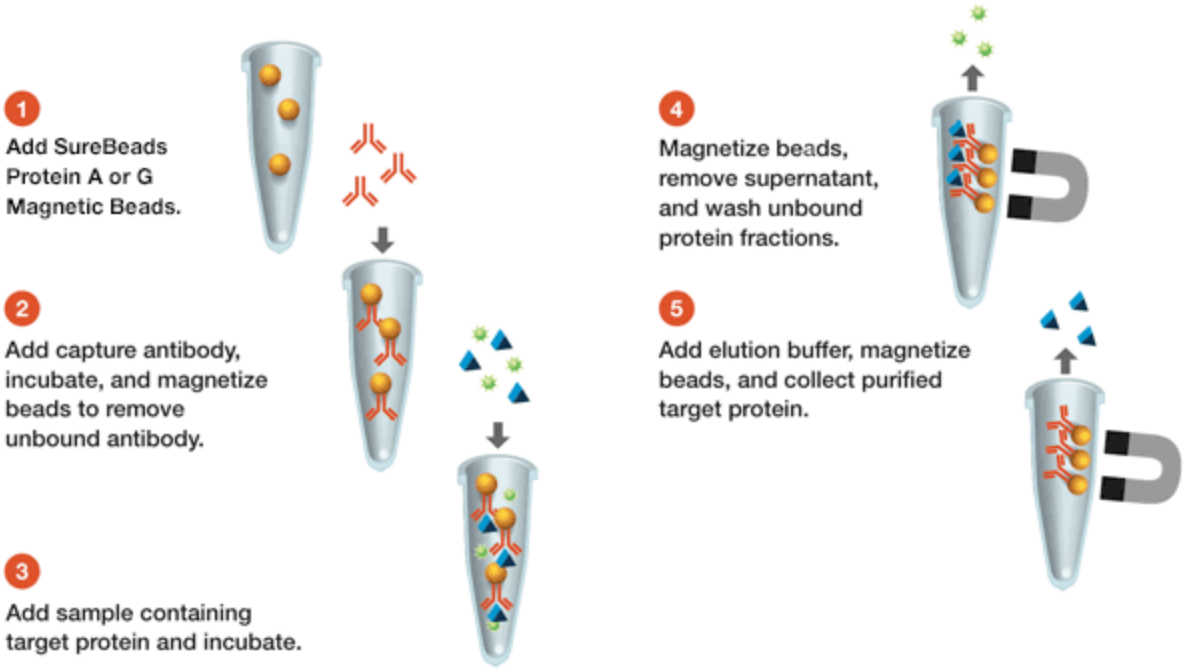

Figure 3.1: Illustration of the process of Immunomagnetic Separation.

3.6 Antibody-based IMS Procedure

Whey was captured from solution using the magnetic bead/antibody complex that was created in section 3.4 above and by following the manual provided by the company. An illustration of this procedure can be seen in steps 3-5 in figure 3.1 above:

1.) 200µL of magnetic bead/antibody complex was placed in a 2mL Eppendorf centrifuge tube and placed on a magnetic rack and allowed to separate for 2 minutes. While still on the magnetic rack, the supernatant was then discarded so as to retain the magnetic beads.

2.) Next, 1mL of possible whey containing sample was added to the bead/antibody complex and was again allowed to incubate on an orbital rotator (Fisher Scientific, Rochester, NY) for 30 minutes at 35rpm at room temperature.

3.) After that, the tube was removed from the rotator, centrifuged down for 5 seconds and magnetized for 2 minutes. The supernatant was discarded and the complex was washed with PBS-T, as described in steps 2-5 above in section 3.4.

4.) The beads were then resuspended by vortexing for 5 seconds. Then the solution was pipetted out and transferred to a new 2mL Eppendorf centrifuged tube and washed three more times, in the same way as above in step 3.

5.) After the final washing step, the tube was again placed on the rack and allowed to separate for 2 minutes. The supernatant was then removed and the tube was removed from the rack.

6.) The complex was then centrifuged down for 5 seconds at 3000rpm in a Fisher Scientific mini centrifuge and placed back on the rack and any residual supernatant is removed by decanting.

7.) The tube was again removed from the rack and centrifuged down for 5 seconds. Then, 20µL of 20mM glycine pH 2.75 (adjusted with concentrated HCl) was added to the tube and agitated by hand for 10-15 seconds. The glycine acts as an elution buffer, allowing the antibody and whey to be separated from the magnetic beads.

8.) The elution buffer, still in the centrifuge tube, was then placed on an orbital shaker (Fisher Scientific, Rochester, NY) for ten minutes at room temperature.

9.) After incubation with the elution buffer, the tube was then transferred back to the magnetic rack and the beads were allowed to separate out of solution for 2 minutes. Afterwards, the supernatant was removed and transferred to a separate, 200µL centrifuge tube until it was ready to be analyzed by the Raman Spectrometer.

3.7 Preparation of Aptamer-based Magnetic Beads

Similar to the preparation of the antibody-based method, the main difference being that the capture agent was a biotinylated aptamer and the beads were coated with streptavidin, instead of protein G, to bind the aptamer to the magnetic bead. Dynabeads™ M-280 streptavidin coated beads were purchased from Thermo Fisher Scientific (Waltham, MA). The beads came in vials of 2mL at a concentration of 10mg/mL magnetic beads. The beads were suspended in 10mM PBS pH 7.4 with 0.1% BSA and 0.02% sodium azide as preservatives. The beads were 2.8µm in diameter with an average ss-oligonucleotide binding capacity of 200pmol/mg of magnetic bead.

An 23bp ss-oligonucleotide was ordered through Integrated DNA Technologies (Coralville, IA) with a sequence of 5’- GGGGGTTGGGGTGTGGGGTTGGGG-3’. The 5’ end was biotinylated to allow the interaction with the streptavidin beads. 3.11mg of dried ss-oligo was

obtained and resuspended in 4mL of nuclease-free H_2O for a concentration of 100μM. From there, it was diluted down to 10μM, aliquoted, and stored in a -20°C for future use. The aptamer was conjugated to the magnetic beads according to the manual:

1.) The beads were first thoroughly resuspended by gently shaking the vial of beads for 30 seconds to resuspend the Dynabeads (Thermo Fisher).
2.) 100μL of the resuspended beads were placed into a 2mL centrifuge tube, and mixed with 100μL of 2x Binding and Washing buffer (B&W buffer- 10mM Tris-HCl (pH 7.5) + 1mM EDTA + 2M NaCl + 0.2% Tween 20™).
3.) The 200μL of the resuspended beads in B&W buffer were placed onto a magnetic rack (Thermo Fisher)and allowed to separate for 2 minutes. Afterwards the supernatant was removed and discarded.
4.) The tube with the beads was then taken off the magnetic rack and washed with 1mL of Binding and Washing buffer.
5.) The beads were then vortexed for 5 seconds and centrifuged for 5 seconds as to remove any excess beads from underneath the cap.
6.) The tube was then placed on the magnetic rack again for 2 minutes. This washing step was repeated two more times for a total of three washes.
7.) After the final wash step, the beads were taken off the rack and mixed with 600pmol of the biotinylated aptamer. Then the total volume of the tube was brought up to 200μL using binding and washing buffer and the beads were resuspended.

8.) The bead/aptamer complex was then allowed to incubate on an Fisher Scientific mini tube rotator (Fisher Scientific 05-450-200) for 30 minutes at 35rpm at room temperature.

9.) After the incubation, the tubes were removed and centrifuged down for 5 seconds to prevent loss on the cap of the tube. Then the complex was washed three times in the same fashion as steps 3-6 above.

10.) The beads were then resuspended in 200µL of 1x B&W buffer and stored at 4°C until ready to use.

3.8 Aptamer-based IMS Procedure

Whey was captured from samples using the magnetic bead/antibody complex that was created in section 3.6 above and by following the manual provided by the company:

1.) 200µL of magnetic bead/antibody complex was placed on a magnetic rack (Thermo Fisher) and allowed to separate for 2 minutes. The supernatant was then decanted.

2.) Next, 1mL of possible whey containing sample was added to the bead/antibody complex and was again allowed to incubate on an orbital rotator (Fisher Scientific, Rochester, NY) for 30 minutes at 35rpm at room temperature.

3.) After that, the tube was removed from the rotator, centrifuged down at 3000rpm for 5 seconds in a Fisher Scientific mini centrifuge, followed by

being magnetized in the same rack for 2 minutes. The supernatant was discarded and the complex was washed with binding and washing buffer, as described in steps 3-6 above in section 3.6.

4.) The beads were then resuspended by vortexing for 5 seconds. Then the solution was pipetted out and transferred to a new 2mL centrifuged tube and washed three more times, in the same way as above in step 3.

5.) After the final washing step, the tube was again placed on the magnetic rack (Thermo Fisher) and allowed to separate for 2 minutes. The supernatant was then removed and the tube was removed from the rack.

6.) The complex was then centrifuged down for 5 seconds at 3000rpm and placed back on the magnetic rack and any residual supernatant is removed.

7.) The tube was again removed from the rack and centrifuged down for an additional 5 seconds at 3000rpm. Then, 20μL of PBS pH 7.4 was added to the tube and agitated by hand for 10-15 seconds. Different than the antibody IMS, elution was done by heating the complex to 80°C in PBS. This heating step is enough to break the streptavidin-biotin bond and separate the ss-oligo and any sample from the beads.

8.) To reach 80°C, the tube was placed into an incubator for 15 minutes at 80°C. After 15 minutes, the tube was placed back on the magnet and placed back inside the incubator for another 3 minutes to allow separation of the beads and the supernatant.

9.) After the 3 minutes on the rack, the rack was taken out of the incubator and the supernatant was transferred to a 200µL centrifuge tube until ready to be analyzed by the Raman Spectrometer.

3.9 Raman Instrumentation Preparation and Analysis

The method described below was used to analyze the IMS eluents from both antibody and aptamer IMS for the presence of whey. The Raman Spectrometer that was used for this experiment was a Nicolet Almega XR Raman Spectrometer (Thermo Scientific, Waltham, MA) and the software the spectrometer used was OMNIC. The Raman spectrometer used a 780nm laser excitation frequency along with a 10x objective. The resulting laser spot was ~3µm in diameter and had a specific resolution of $5cm^{-1}$. For this experiment, the aperture of the laser was set to 25µm slit setting. Raman scattering was collected for 5 seconds in quadruplicate for each reading and each sample was read 10 times at 10 different spots on the AgD to minimize variance and to ensure a well-represented spectrum. All spectrums were collected in the range of 500-$3600cm^{-1}$.

1.) 1µL of AgD, described in section 3.2, was placed onto a glass, gold-coated slide (Thermo Scientific, Waltham, MA).
2.) Next, 1µL of eluent was carefully deposited on top of the 1µL of AgD. The slide was then allowed to dry completely for 10 minutes in a 35°C incubator.

3.) Once dried, the slide was taken to the Raman spectrometer and placed inside the sample chamber under the microscope. Using the microscope, the laser was focused on the sample.

4.) Using the OMNIC software, a spectrum was taken. After the 9 other spectrums were taken, each spectra were saved individually before an average spectra were created from all 10. A 2nd derivative of the spectrums was also created and saved individually. These spectra were the basis to analyze the data using TQ analyst.

3.10 Data Analysis Using TQ Analyst

Spectra created by the Raman spectrometer were analyzed for differences or similarities using the TQ Analyst (v8.0.2.97) software package (Thermo Scientific, Waltham, MA). The two main tools that were used to analyze the data were the partial difference feature and the partial component analysis (PCA). The software could analyze the spectra or the 2nd derivative of the spectra to show the patterns between the samples spectra. The partial difference tool took two spectra and compared them, essentially showing if the two spectra were similar or not. The closer the spectra plot points were to each other on a graph, the more that they had in common. Two spectra that were similar would appear with their points over lapping, while two different spectra had points that were far way. This was tool was used primarily to compare spectra to the negative control, a yes or no method put simply.

PCA was used when there were multiple different variables to compare, such as a negative control, a sample with very little whey in it, and a sample that has quite a bit of whey in it. It allowed you to see not only if spectra were similar or different from each other, but also how the spectra compared with others as well at the same time. This spectra, with a large enough spectra library collected, was used to estimate the rough concentration of whey in each of the different samples.

For either method, the region that the software focuses on similarities and differences can be changed. By either narrowing or expanding the comparison region, the software is better able to make accurate predictions.

4 Results and Discussion

4.1 Spectra of Procedure Reagents

To make sure that none of the reagents that were used would interfere with the detection of whey, a spectra was obtained of each of the reagents commonly used throughout the experiment.

Below is figure 4.1 which shows the spectra of the silver dendrites, AgDs, which was used throughout the experiment as the surface enhancing metal in SERS. The peak at roughly 1050-1060cm^{-1} was most likely from the residual (NO_3^{-1}) that was left over from when the AgD were originally created. This sharp peak has been seen in other works that have used $AgNO_3$ replacement reactions (Song et al, 2006) and was found not to overlap with the distinguishing peak from whey that was used.

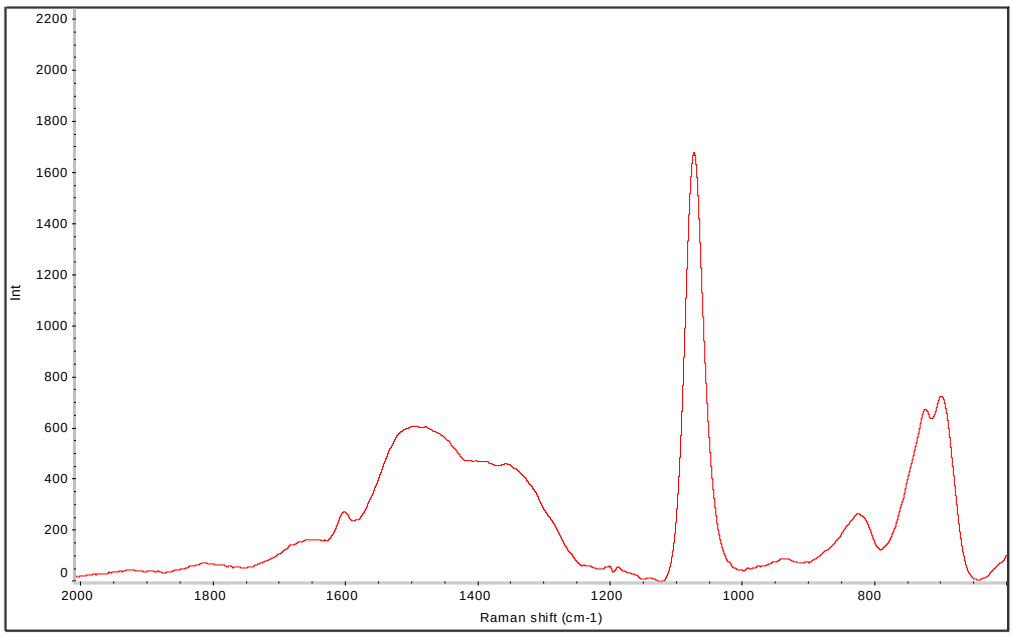

Figure 4.1: Average spectra of the AgDs.

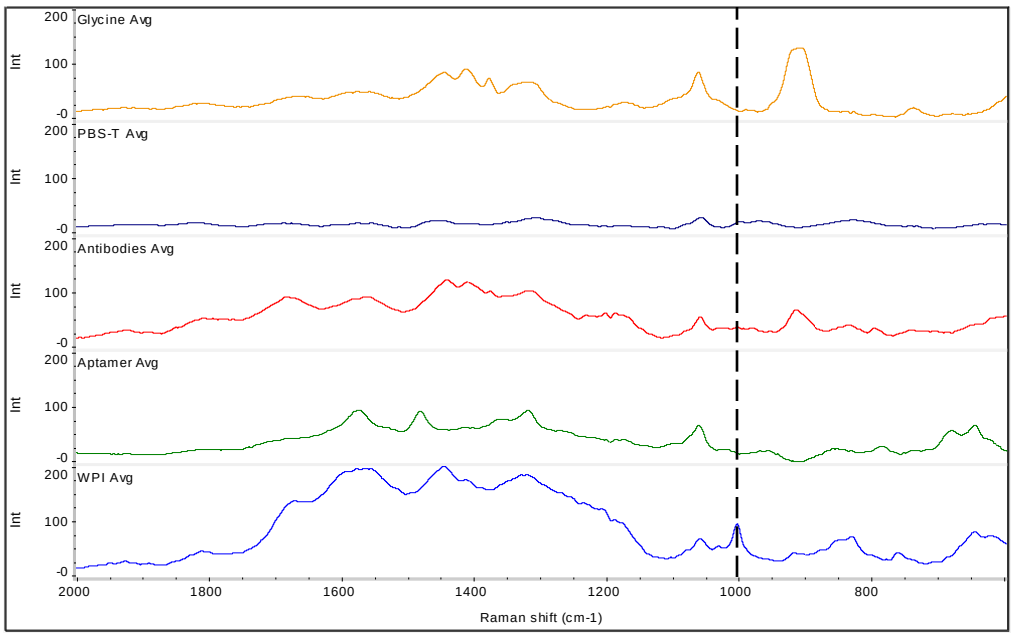

Figure 4.2: Average Spectra that were taken from the common reagents used throughout the experiment. A vertical line has been drawn at 1004cm^{-1}, the location of the peak that was used to distinguish the presence or absence of whey in a sample.

Figure 4.2 above shows the common reagents that were used throughout the experiment; glycine, which was used as an elution buffer, the antibodies used during antibody IMS, the aptamer used during aptamer IMS, and PBS-T which was used during the washing step throughout the experiments. Luckily, none of these reagents contained peaks that overlapped with the distinguishing peaks of whey. To make sure of this, a 2nd derivative and PCA plot were created as seen below in figure 4.3 and figure 4.4.

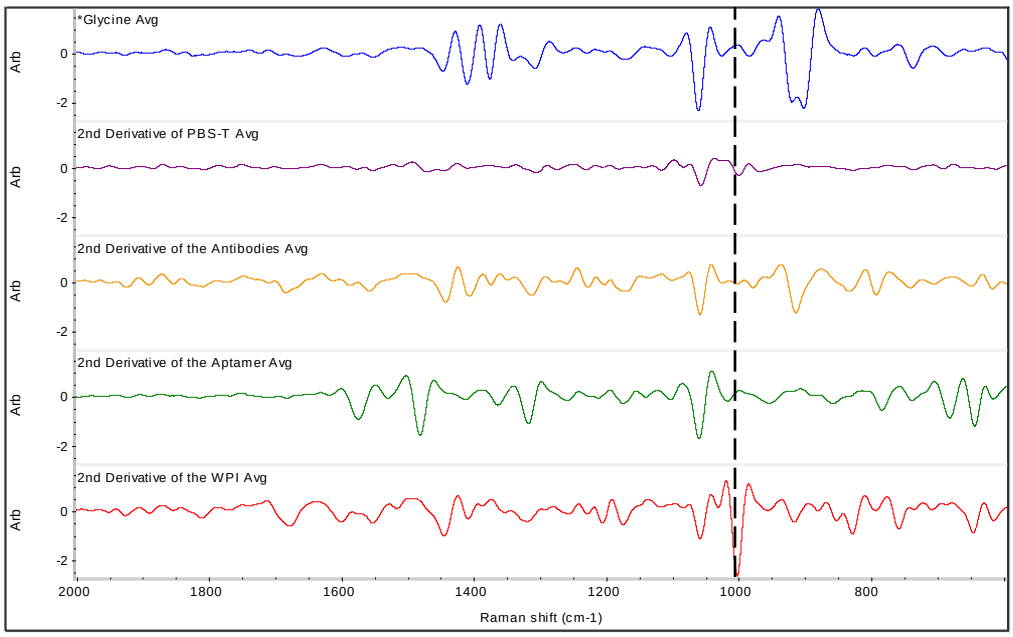

Figure 4.3: 2nd derivative of the average spectra of common reagents compared to the 2nd derivative of WPI in PBS.

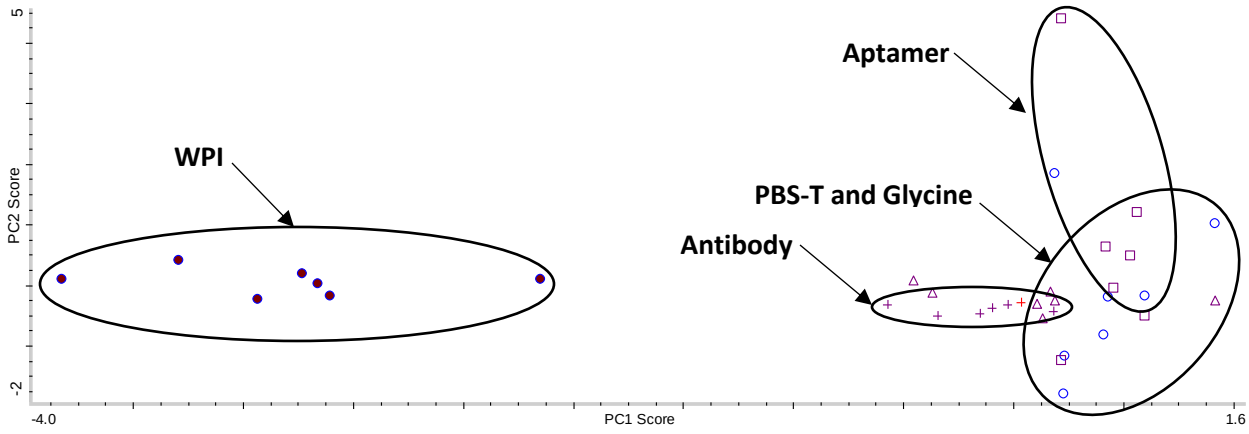

Figure 4.4: PCA graph, in the range of 975-1025cm^{-1}, of the spectra from the common reagents and WPI. Notice how WPI is clearly separated from the common reagents, which are more clustered together and indicates that they are more closely related.

The 2nd derivative spectra in figure 4.3 gives a clearer picture of how the common reagents are different from the WPI spectra and don't have any peaks that overlap with WPI. This process of using the 2nd derivative of a spectra acts as a baseline correction that allows the comparison of two spectra even when their baseline intensities are different (He et al, 2011). The PCA chart in figure 4.4 focuses on the spectra range of 975-1025cm^{-1}, which further shows the difference between WPI and the common reagents. The chart shows WPI is clearly separated from the rest of the group, while glycine and PBS-T appear so similar that their positions on the PCA chart overlap. The antibody and aptamer spectra, while slightly different from the glycine/PBS-T grouping, still are easily differentiated from the WPI grouping.

4.2.1 Detection of Whey Using Antibody IMS Coupled with SERS

The first step that was done to detect whey in a hot dog, was to get a spectrum of whey that could be used throughout the experiment to compare results to. It was decided that a 1mg/mL of WPI solution would be used as our comparative spectra. 1g of WPI was dissolved in 10mM PBS and then brought up to 1L to create the stock solution. For this experiment, we decided to use the large peak found at 1004cm^{-1}, as seen below in figure 4.5, since it was unique and didn't appear in the spectra of any of the other reagents. This peak is

associated with a benzene ring, highlighted in figure 4.6, and is most likely found in WPI as phenylalanine.

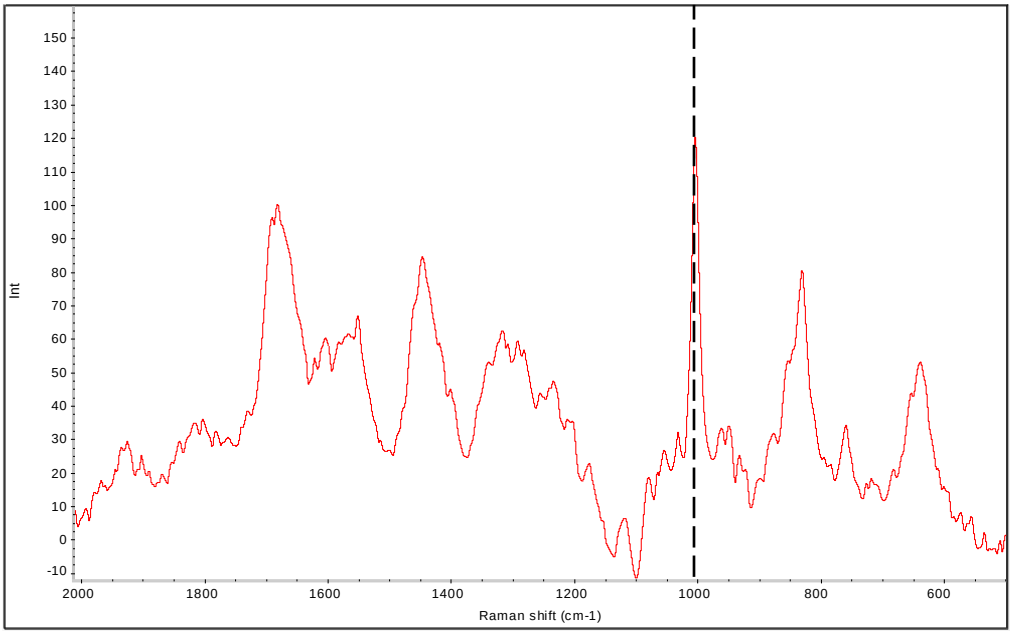

Figure 4.5: Average spectra of 1mg/mL WPI in 10mM PBS. This spectrum was compared to other spectras during the experiment to determine if WPI was present in the sample or not.

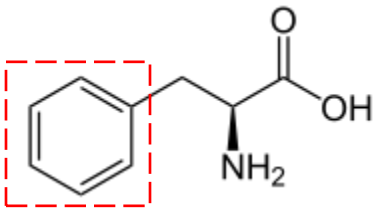

Figure 4.6: The benzene ring of phenylalanine was the Raman active compound that allowed for WPI to be tracked using SERS.

Once we had a standard spectrum that we were able to track, the next step was to use the antibody IMS procedure, outlined in section 3.5 and 3.6 of

the Methods and Materials, to try and detect WPI in a pure solution. For the experiment, we created concentrations of WPI in 10mM PBS by diluting the 1mg/mL stock solution with PBS. 100, 50, 25, and 12.5µg/mL were created as working standards to test the IMS procedure in pure solution. A negative control using 10mM PBS was used to get a baseline spectrum to compare the others to.

Figure 4.7 below shows the average Raman Spectra of all the dilutions and the negative control, 10mM PBS.

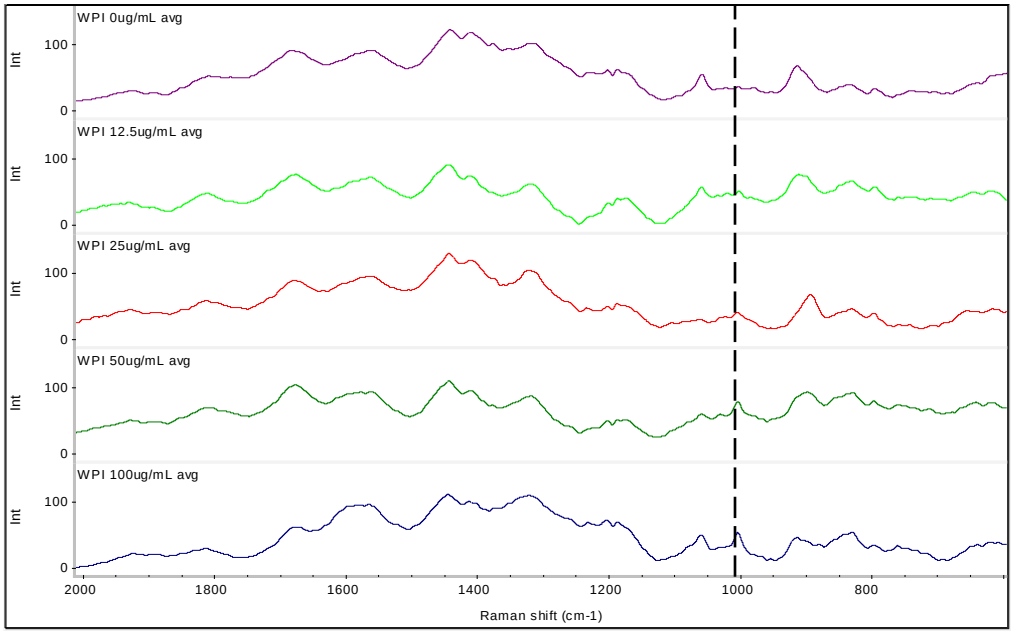

Figure 4.7: Raman spectra of the dilutions used to test the feasibility of IMS to detect whey in a 10mM PBS solution.

As seen in figure 4.7, the IMS method was able to capture enough whey for the Raman spectrometer to detect it. The 100 and 50µg/mL dilutions can be differentiated by simply looking at the figure, but a 2nd derivative spectra, seen in

figure 4.8, was created to see if the method could differentiate from the 25 and 12.5µg/mL dilutions from the negative control.

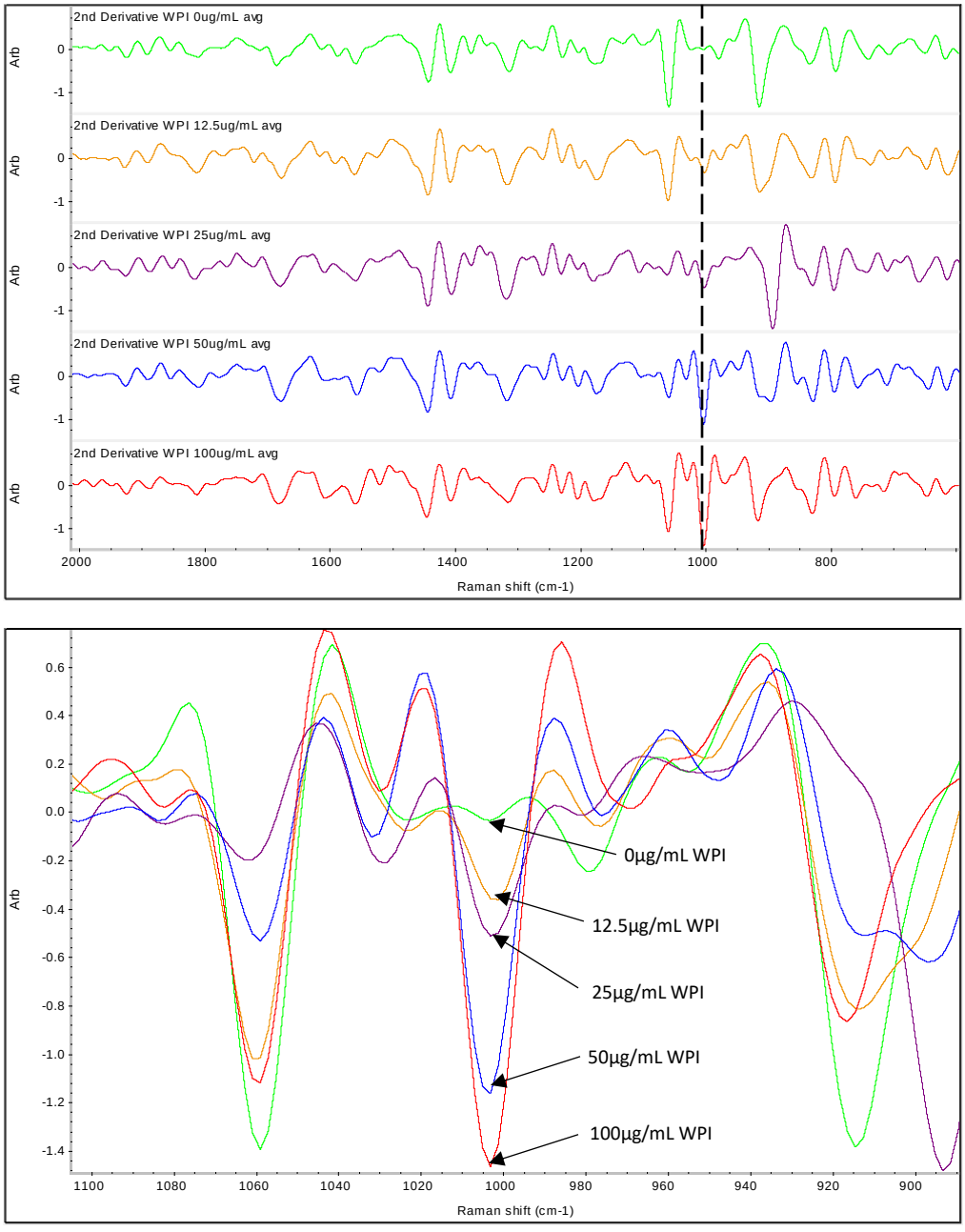

Figure 4.8: 2nd derivative spectra of the IMS/SERS detection experiment. The top figure shows the spectra individually, while the bottom figure focuses in on the 1004cm^{-1} peak with the spectra overlaying each other.

Figure 4.8 gives a baseline corrected view of at the spectra, which shows a decreasing peak height with a decrease in WPI concentration. Looking at this figure, we can see that that the 25 and 12.5µg/mL concentration can be differentiated slightly from the negative control.

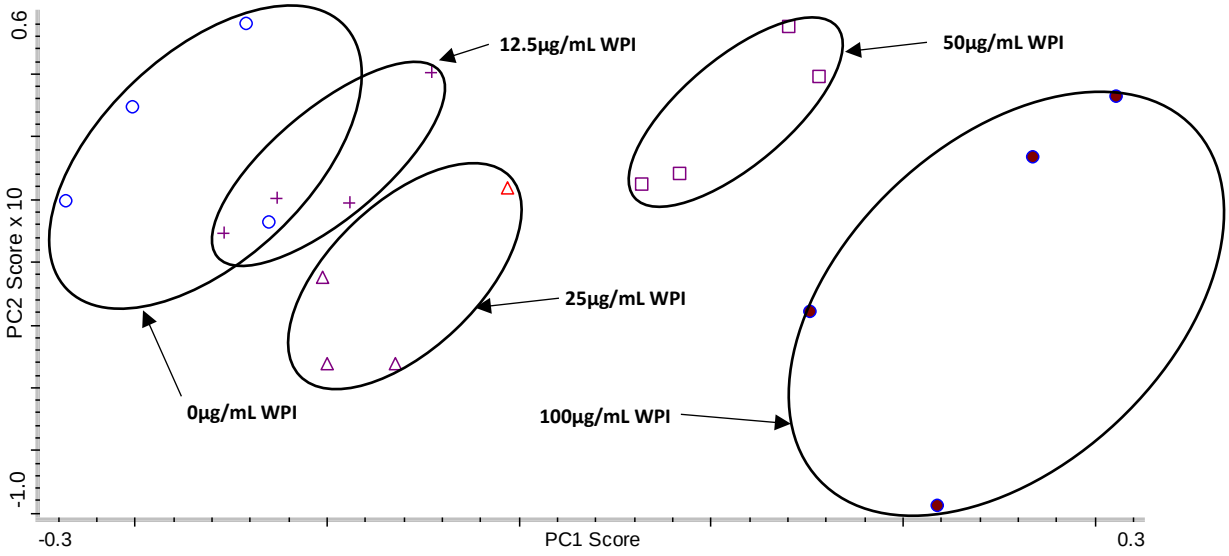

Figure 4.9: PCA graph of IMS/SERS detection in 10mM PBS. The 100, 50, and 25µg/mL samples are clearly separated from the other concentrations, but the 12.5 and 0µg/mL concentrations overlap.

The PCA graph shows the upper three concentrations of 100, 50, and 25µg/mL as being separated from each other, but the 12.5 and 0µg/mL concentrations overlap on the graph. This is different than what figure 4.8 shows, where all the concentrations appear separated from each other. This is most likely due to the fact that the spectra in figure 4.8 are made up of the average of

all the samples at that concentration, whereas the PCA graph in figure 4.9 shows each sample as its own point on the graph. Most likely the broad range of readings from the 12.5µg/mL sample, when averaged out, make it appear that it was different from the 0µg/mL readings, when it really wasn't. This shows that while taking the average spectra of a concentration helps to get a general idea of the samples, a PCA graph is the best method to show differentiation between concentration spectra (He et al, 2009, He et al, 2011, and Li et al, 2015), thus the limit of detection is 25µg WPI/mL.

4.2.2 Detection of Whey in Spiked Hot Dogs Using Antibody IMS/SERS

The next step, after we were able to determine that the antibody IMS method worked in a simple PBS solution, was to try and use the method with a real world sample. We wanted to test to see if the hot dog matrix would have any negative effects on the method. To test this, we had the staff in the Andrew Boss Meat Science Laboratory create hot dogs for us using the recipe found in section 3.1 of the Methods and Materials. Once we had the hot dogs with no whey in them, WPI was added to the extraction volume, 100mL of 10mM PBS, to create a concentration of 1000µg/mL of WPI. The spiked extraction volume was then added to the hot dog in a 250mL blender cup and blended for 2 minutes and allowed to rock on an orbital shaker (Fisher Scientific, Rochester, NY) for a minimum of 8 hours in a 4°C cooler. The samples were then strained through a wire mesh strainer to remove the larger particulates and then vacuum filtered

through glass wool to remove the smaller ones. The solution was then used with the antibody IMS method to detect for whey. The spectra in figure 4.10 are the average spectra from the 1000µg/mL spiked hot dog and 0µg/mL hot dog.

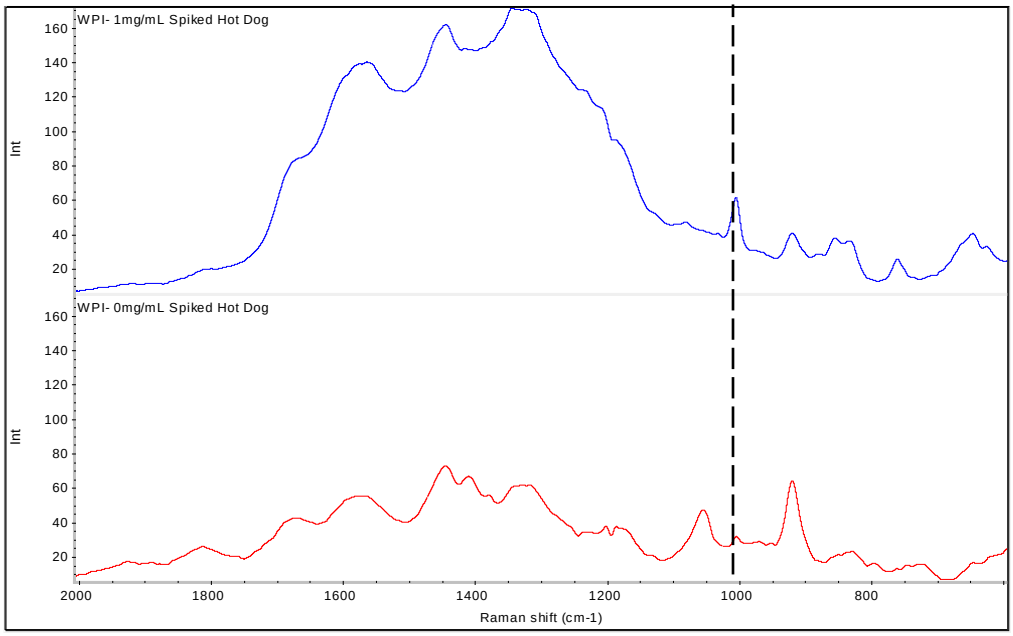

Figure 4.10: Average spectra of the 1mg WPI/mL spiked hot dog and the 0mg WPI/mL spiked hot dog.

The 1mg/mL spiked hot dog spectra appears exactly as expected, with a peak at 1004cm^{-1} to indicate a positive for whey, the negative control hot dog with 0mg/mL of whey also shows a small peak at 1004cm^{-1}. The source of this small peak was most likely from some form of non-specific binding (Safirik et al, 2004). While the magnetic beads, in most cases, have low non-specific binding, there are a few cases where some peptides or proteins can non-specifically bind to the magnetic beads. The 2nd derivative spectra, shown below in figure 4.11,

indicates that there is a real difference between the two, even with the small peak present in the negative control.

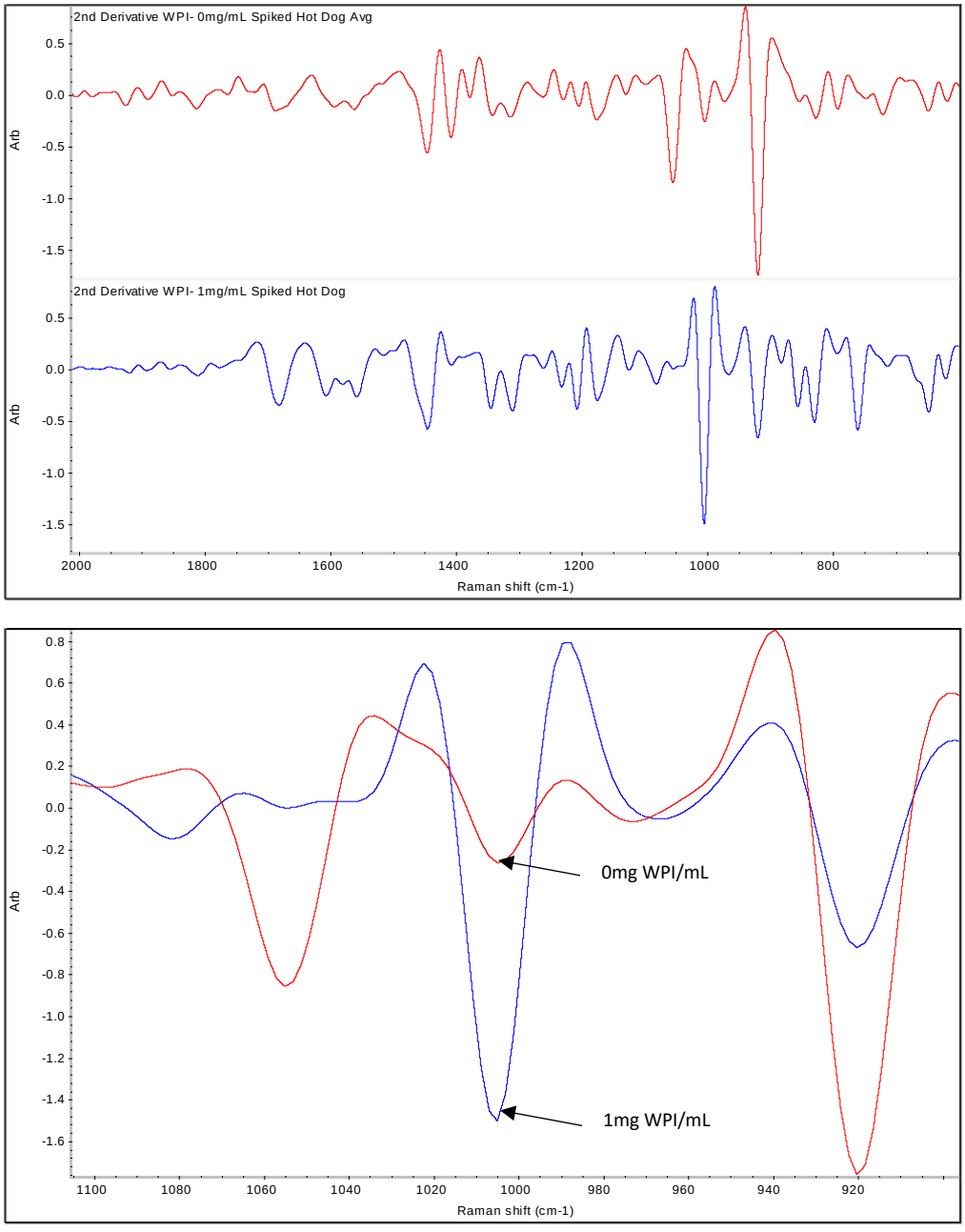

Figure 4.11: 2nd derivative spectra of the spike hot dogs. The top figure shows the spectra individually, while the bottom figure focuses in on the 1004cm^{-1} peak with the spectra overlaying each other.

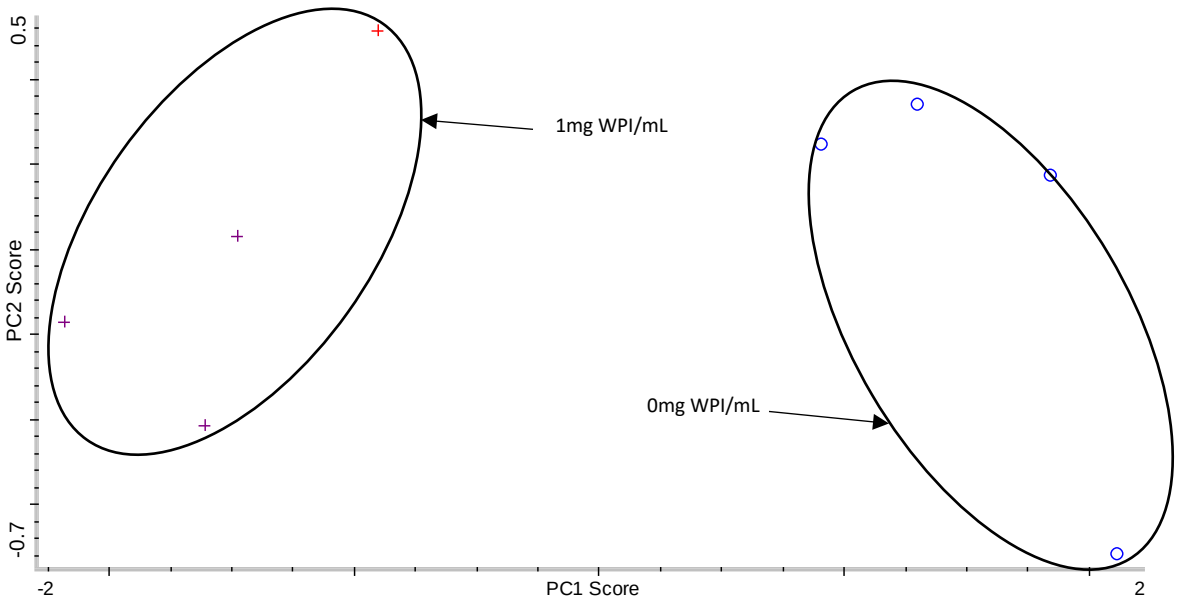

Figure 4.12: The PCA graph shows the difference between the spiked hot dog matrix, 1mg WPI/mL, and the negative control.

The PCA graph in figure 4.12 shows the extent of the difference between the spiked hot dog sample and the negative control. With such a large distance between the groupings, this graph shows that the hot dog matrix doesn't seem to interfere with the IMS/SERS detection method, assuming the extraction method is at 100% (Rhode et al, 2011).

Next, hot dogs with WPI cooked into them were created by the Andrew Boss Meat Science Laboratory staff with concentrations of 30mg and 15mg per 50g hot dog, Methods and Materials Section 3.1. We decided that 30mg per hot dog as the highest concentration was a good starting point since that concentration was roughly the level of protein detected in the Oscar Meyer hot dogs in the New England Journal of Medicine article that was used as a

reference (Gern et al, 1991), so this was used a reference point for this experiment. As with the spiked hot dogs, the preparation method in section 3.4 was used to prepare the hot dogs for the IMS/SERS procedure, except with no whey added to the extraction volume. The spectra taken from the 30mg WPI hot dogs are shown below in figure 4.13.

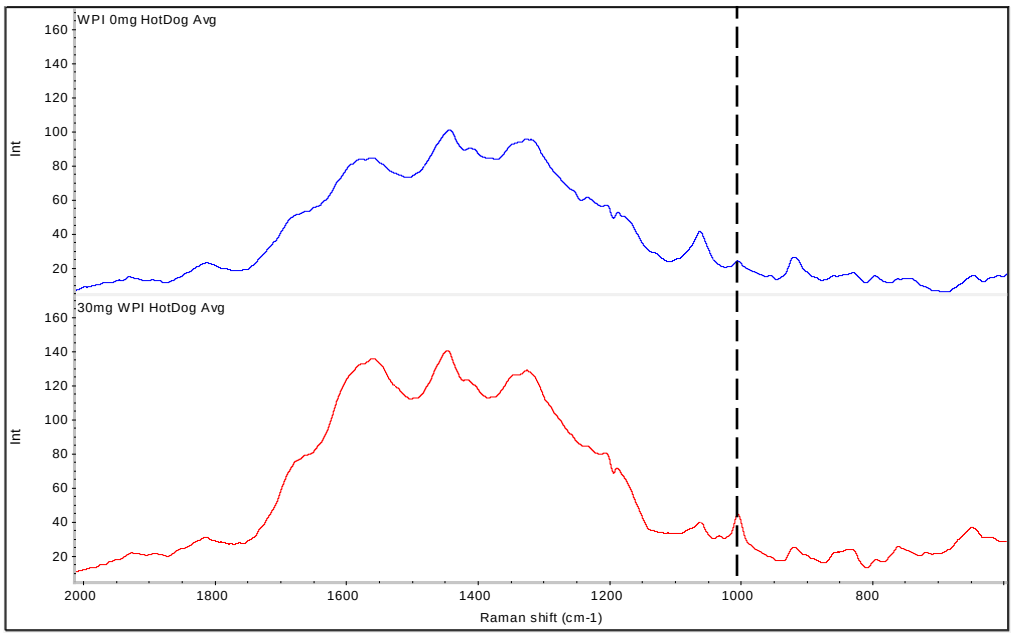

Figure 4.13: Spectra of the 30mg WPI hot dog and the negative control. While weaker in strength, the peak at 1004cm^{-1} is still present.

The peak of from phenylalanine is still present, showing that the whey present in the spiked hot dog was still able to interact with the antibodies of IMS. This makes sense since the cooking temperature of the hot dogs only reached a peak of 150°F, which is still not quite high enough to irreversibly denature all of the whey proteins (Swaisgood, 1982). Figures 4.14 and 4.15, below, show the

2nd derivative of the Raman spectra and the PCA graph of the 30mg WPI/ hot dog run.

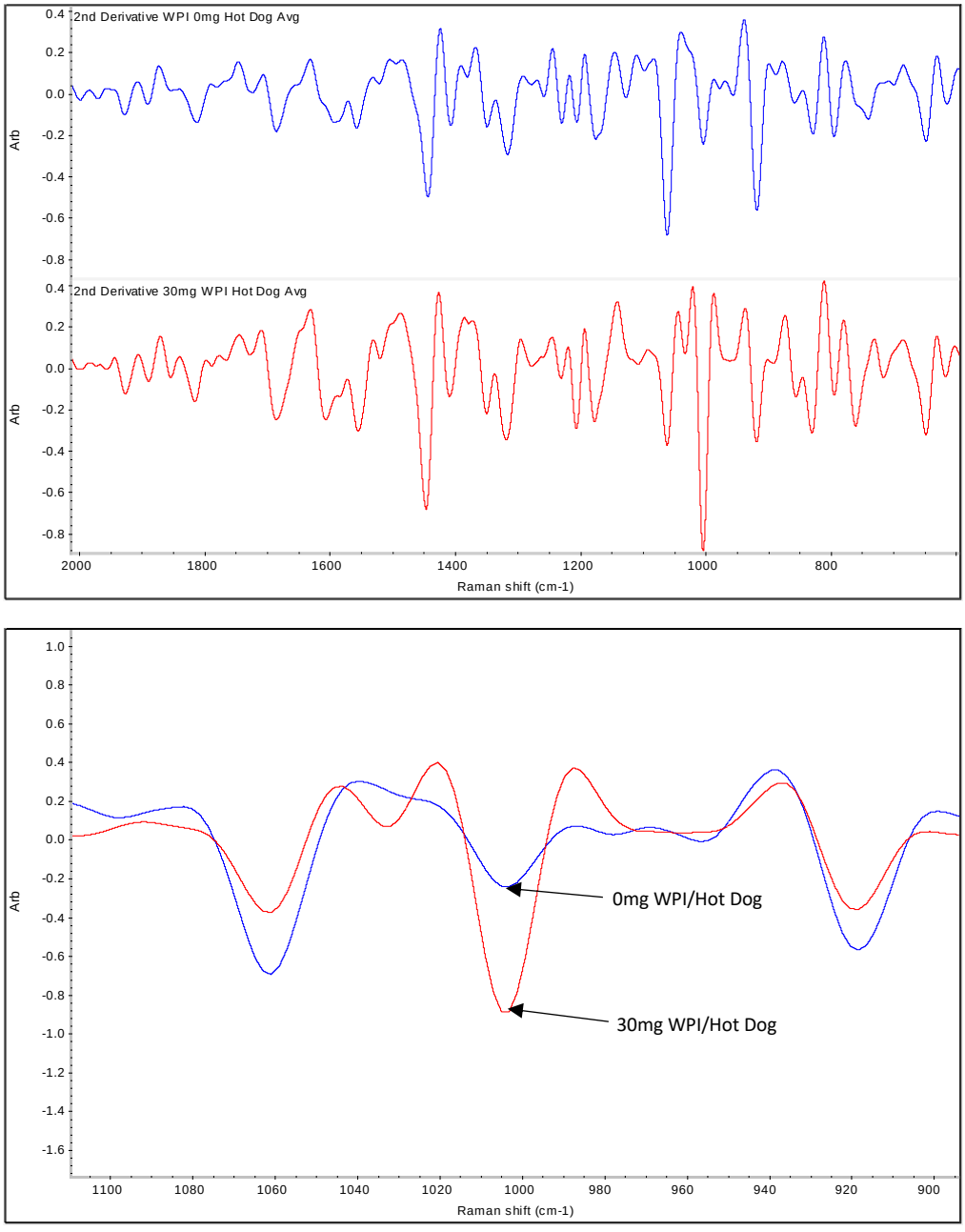

Figure 4.14: 2nd derivative spectra from the 30mg spiked hot dog IMS experiment. The top spectra shows the negative control and the spiked hot dog spectra side by side, while the bottom spectra shows them stacked over each other to better show the difference between the two.

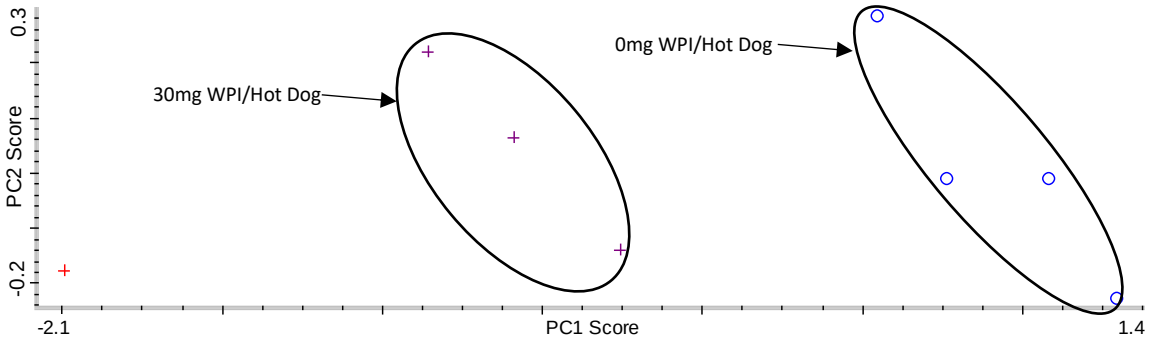

Figure 4.15: PCA graph of the 30mg WPI/hot dog and the negative control. The two separate groupings show that the two sets of spectra are distinguishable from each other.

Again, the 2nd derivative shows a larger peak at the 1004cm^{-1} mark than the negative control hot dog, though the peak for the 30mg hot dog was noticeably smaller than the peak from figure 4.11. This probably has to do with the extraction step and it not being 100% effective in pulling all of the whey into solution. There have been studies to show that whey proteins have interaction with fat molecules, Singh and Ye, which could account for some of the whey not being in solution (Singh and Ye, 2010). Another possibility would be since the hot dogs are cooked at 150°F, the whey proteins would unfold, expose their hydrophobic core, and interact with other hydrophobic molecules. Also, the whey proteins could also be forming disulfide bonds with other protein molecules, thus being prevented from being pulled into solution during the extraction step (Ye et al, 2004). Whatever the reason for the less than 100% extraction, the 30mg WPI hot dog was still able to be differentiated from the negative, as demonstrated by the PCA graph in figure 4.15. The negative and 30mg WPI hot dog are in clearly separated groupings, with the negative control being a little more tightly grouped

than the 30mg WPI hot dog. This, again, was most likely from the hot dogs not having the same extraction efficiency as one another. It could also be from the WPI not being distributed evenly, since only a small amount of whey was added to the original hot dog mix. This could explain the slightly uneven peaks and why one point for the 30mg WPI hot dog is so far from the others in figure 4.15, outside of the circle with the other, similar peaks.

Once we were able to prove that the method was able to detect whey in a real world product under realistic conditions, we decided to try and see if the method could go further and detect lower concentrations of whey in a hot dog. Again, the staff in the meat science laboratory created hot dogs with whey added to them, but this time at a concentration of 15mg of WPI per 50g of hot dog. The experiment was repeated, the same as the 30mg WPI/ hot dog, and the Raman spectra for the experiment can be seen below in figure 4.16.

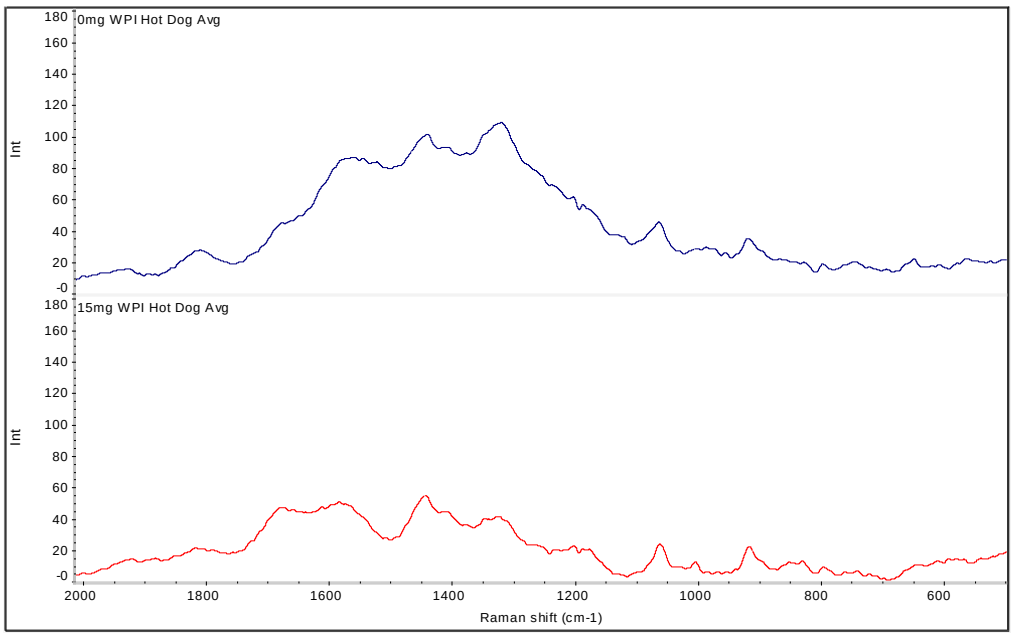

Figure 4.16: Average Raman spectra of the 15mg WPI hot dog and the negative control. While there appears to be a small peak on the 15mg WPI hot dog, it was shown to be not significant enough to differentiate between a 0mg WPI hot dog and the spiked hot dog group.

The spectra above appear to show a small peak at the 1004cm^{-1} mark for the 15mg WPI hot dog, but it is noticeably smaller than the 30mg WPI hot dog. A 2nd derivative of the average spectra was created to further see if the 15mg WPI hot dog could be differentiated from the negative control.

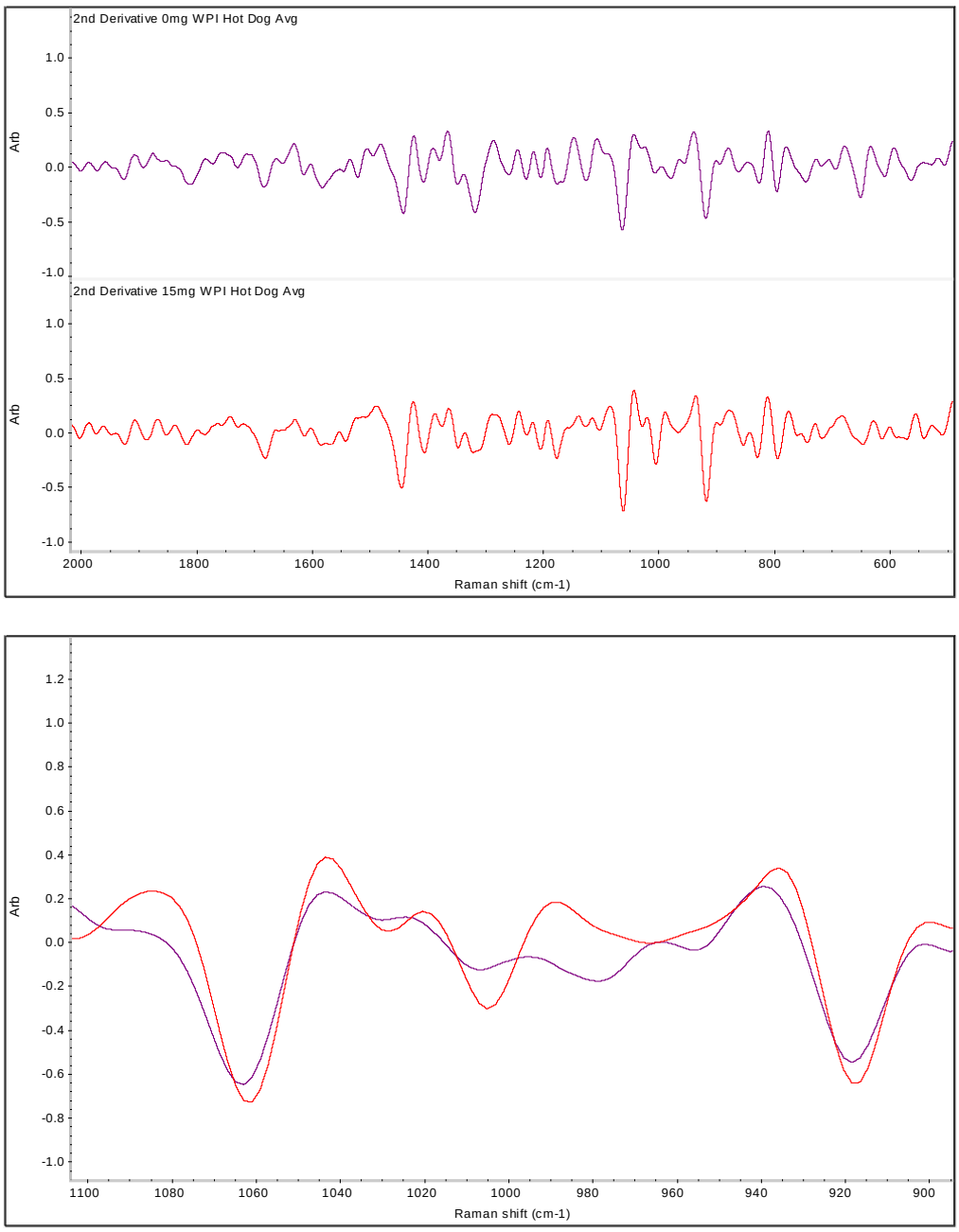

Figure 4.17: 2nd derivative of the 15mg WPI hot dog and the negative control. The 2nd derivative shows that while the bump on the raw spectra might be present, it doesn't end up being that distinguished from the negative control.

The 2nd derivative spectra shows that that the two hot dogs aren't that different, even though the 15mg WPI hot dog's raw spectra did contain a small peak at 1004cm^{-1}. As with the other hot dogs throughout the experiment, a PCA graph was created to give a nonobjective decision if the 15mg WPI hot dog could be separated from the negative control, which can be seen below in figure 4.17.

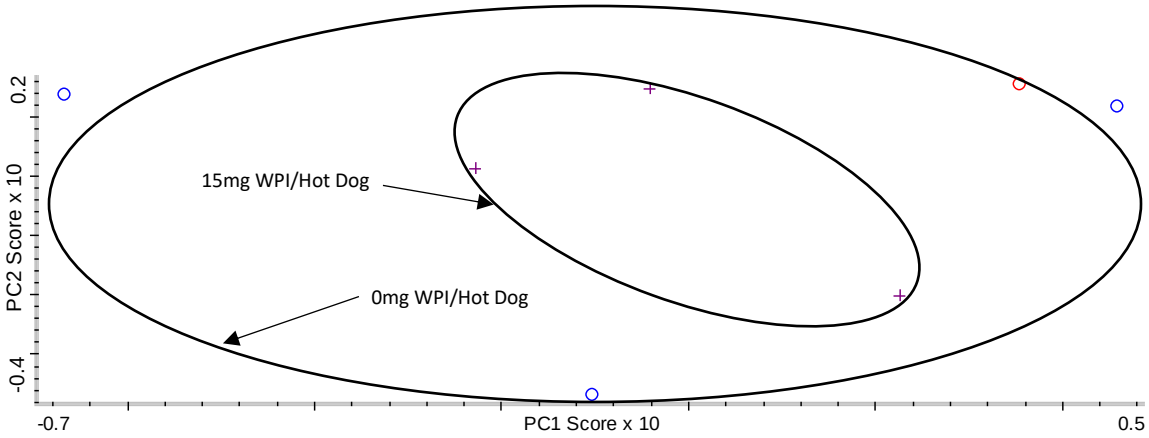

Figure 4.18: PCA graph of the 15mg WPI hot dogs and the negative controls. The 15mg WPI hot dogs are surrounded in the graph by the negative controls, meaning that the 15mg WPI hot dogs are not indistinguishable from the negative controls.

The PCA graph in figure 4.17 shows that the two hot dogs are in fact not distinguishable from each other. This is a great example of how a PCA graph gives a clear method to distinguish spectra from each other free from human objectivity. This can be even better seen when you add the 30mg WPI spectra to the PCA graph, as was done for figure 4.18 below.

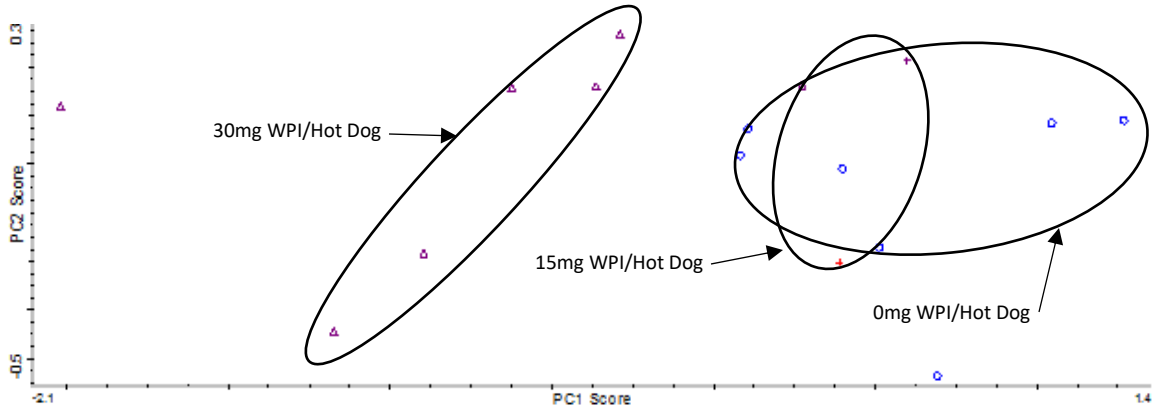

Figure 4.19: PCA graph of a 30mg WPI/hot dogs and 15mg WPI/hot dog with the negative control hot dogs.

One thing that was observed when testing with hot dogs was that the negative controls showed slightly more variability than the spiked hot dogs. The peak at 1004cm^{-1} on the 2nd derivatives for the negative controls ranged anywhere from 0 in intensity all the way to -0.4. This variability could possibly be from some nonspecific binding to the beads (Biorad Manual, 2015), which in turn leads to a slight peak in the spectra. Even with the use of washing agents, such as Tween-20 to the washing step, the variability was still present. With this variability in the negative controls, the detection limit for this method was determined to be 30mg of WPI per 50g of hot dog. With the variability seen by some of the negative hot dog spectra, the 15mg WPI hot dogs couldn't be significantly distinguished from the negative hot dogs, as seen in figure 4.18.

4.3.1 Detection of Whey Using Aptamer IMS Coupled with SERS

As with the antibody IMS, we started the aptamer IMS off by trying to detect whey in a pure solution. The difference here was that instead of using 10mM PBS, 10mM NaCl was used to dissolve the WPI. This was done because it was found out during optimization that the aptamer we used was better able to bind to the whey in 10mM NaCl versus 10mM PBS. We used circular dichroism, which detects the difference in left and right handed absorption of polarized light, to detect the aptamer forming what is called a G-quadruplex, which is the conformation of our aptamer that best allows it to bind with whey. Using circular dichroism, CD, we scanned our aptamer in different solution and found 10mM NaCl to be the optimum solution for our aptamer to be able to bind to whey. The results of the CD plot are shown below in figure 4.19.

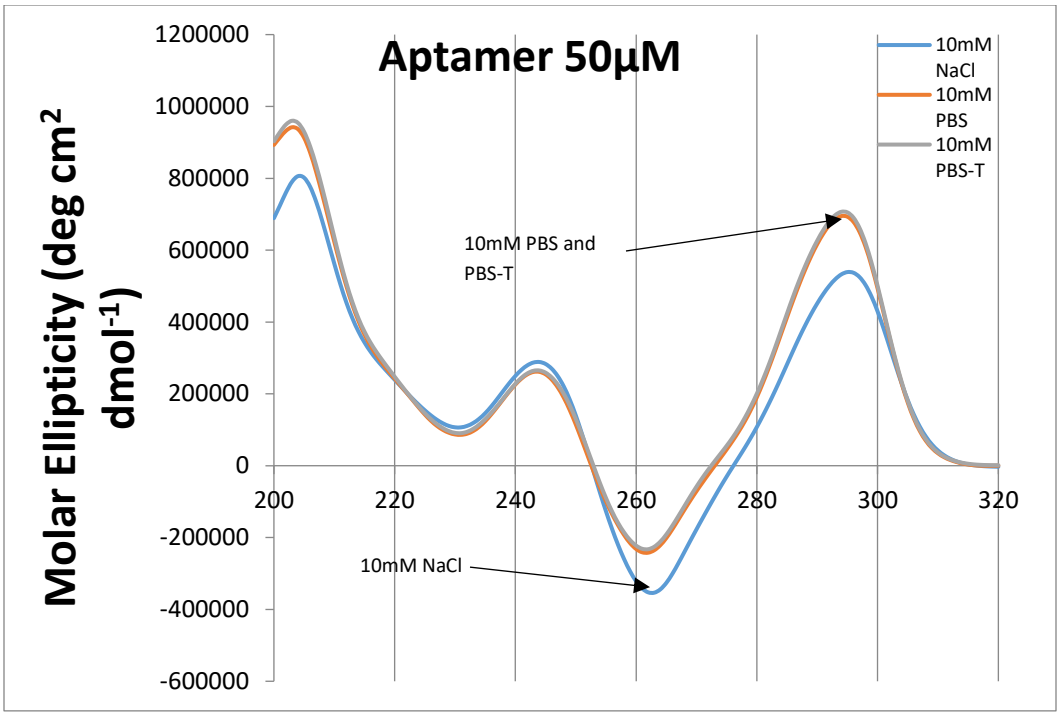

Figure 4.20: CD plot of the aptamer in different solutions. The three solutions that were chosen were 10mM PBS, and 10mM PBS-T. The 10mM NaCl had a larger peak at roughly 265nm and a smaller one at 295 meaning it was better suited to help form a G-quadruplex.

The CD graph showed us that the aptamer forms an antiparallel formation based off of the dip at 260-265nm and the peak at about 295-300nm (Li et al, 2005). The deeper dip and higher peak shown by the aptamer in 10mM NaCl indicated that it resulted in a better formation that was suitable to bind to its target molecule.

Once the aptamer solution was decided on, we started to test the aptamer IMS based method in 10mM NaCl with various WPI concentrations. The aptamer IMS method was tested at various concentrations of WPI, 1000, 500, 250, and

125µg/mL to see if the aptamer would maintain its binding capacity once it was attached to the magnetic beads. The results of the IMS run can be seen in figure 4.20 below.

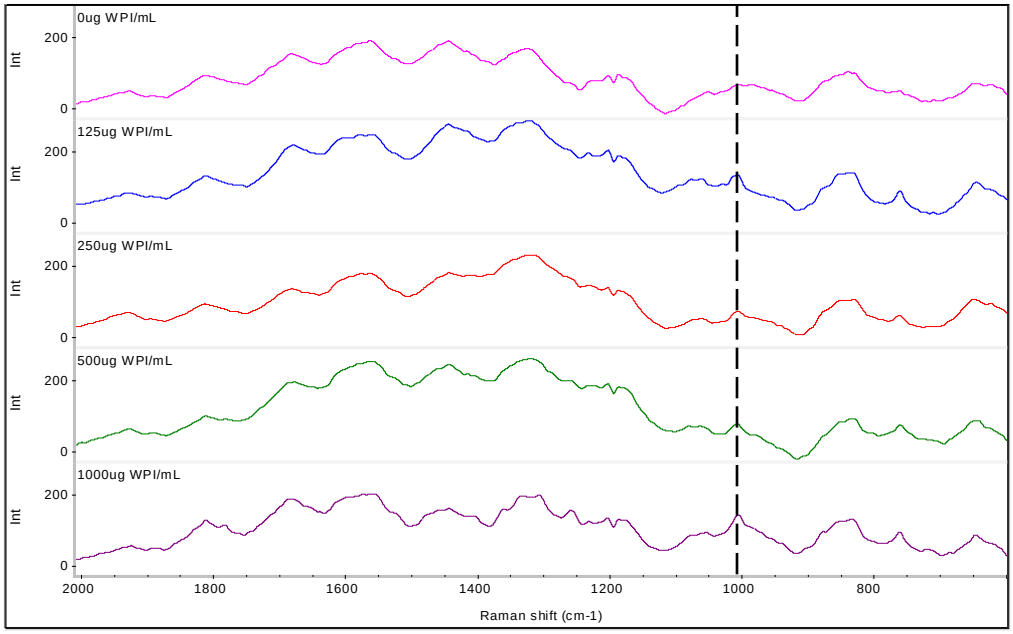

Figure 4.21: Average spectra from the aptamer IMS run in 10mM NaCl solution.

The spectra that were collected from the solution showed a strong peak at 1004cm^{-1} for the 1000 and 125µg WPI/mL and slight less intense peaks for the 500 and 250µg WPI/mL concentrations. This difference could be associated with the location of where the readings were taken with on the dried sample or some other small variability while conducting the experiment. The 2nd derivative spectra and PCA graph can be seen below in figure 4.21 and 4.22 further showing the difference between the positive samples and the negative control.

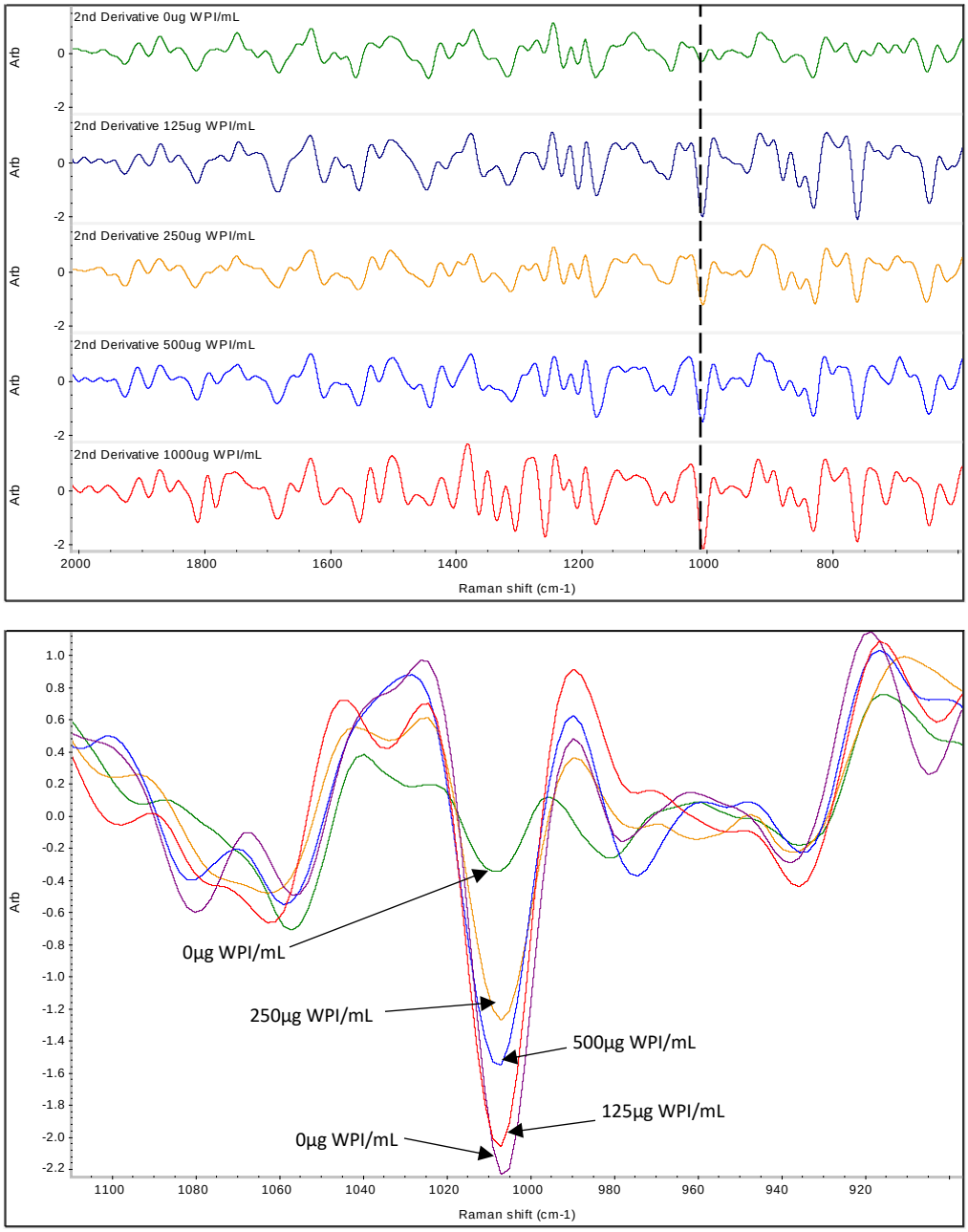

Figure 4.22: 2nd derivative spectra were created to further show the difference between the positive samples and the negative control. The 125µg showed a slightly more intense reading than expected, possibly a result of higher than average duplicates taken during the scanning process.

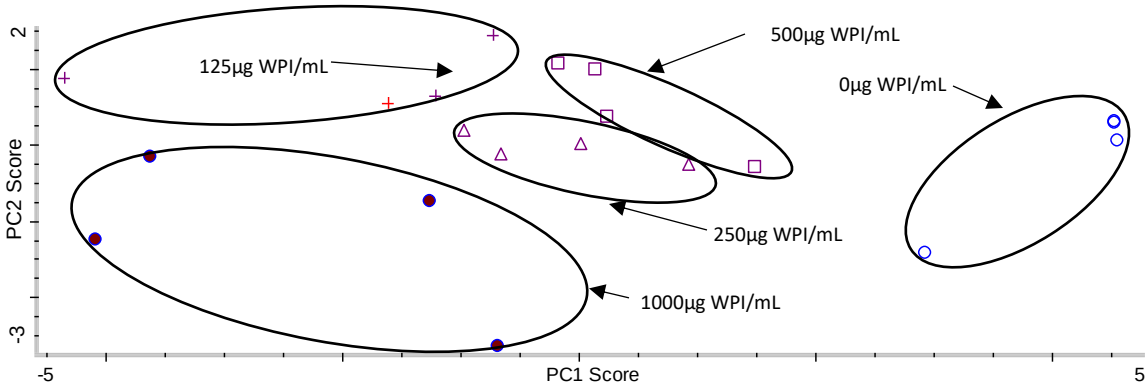

Figure 4.23: PCA graph of the 0-1000µg WPI/mL IMS run. Similar to what was shown in figure 4.21, the 125µg/mL sample was a little higher than expected, most likely because of a reading that was more intense and pulled the average higher than expected.

Again, the 125µg WPI/mL had a slightly more intense peak at 1004cm^{-1} than expected, this most likely being from one or two of the spectra being more intense than expected and thus skewing the average a little higher. This again shows why taking multiple readings per sample is advantageous as it will better capture the true average versus only doing two or three readings per sample. Once we were able to show that the aptamer IMS could function in a pure solution, we moved on to using the spiked hot dogs, just as was done for the antibody IMS.

4.3.2 Detection of Whey in Spiked Hot Dogs Using Aptamer IMS/SERS

Just as with the antibody IMS, we prepared the control hot dogs for the aptamer IMS using the extraction method outlined in section 3, except that we used 10mM NaCl instead of PBS as the extraction solution. After allowing the hot dogs to extract for at least 8 hours and then filtering out the larger particulates,

the aptamer based IMS was run using the same method that was used with the pure solution. Figure 4.23 below shows the spectra for the negative control hot dogs versus the 30mg WPI hot dogs.

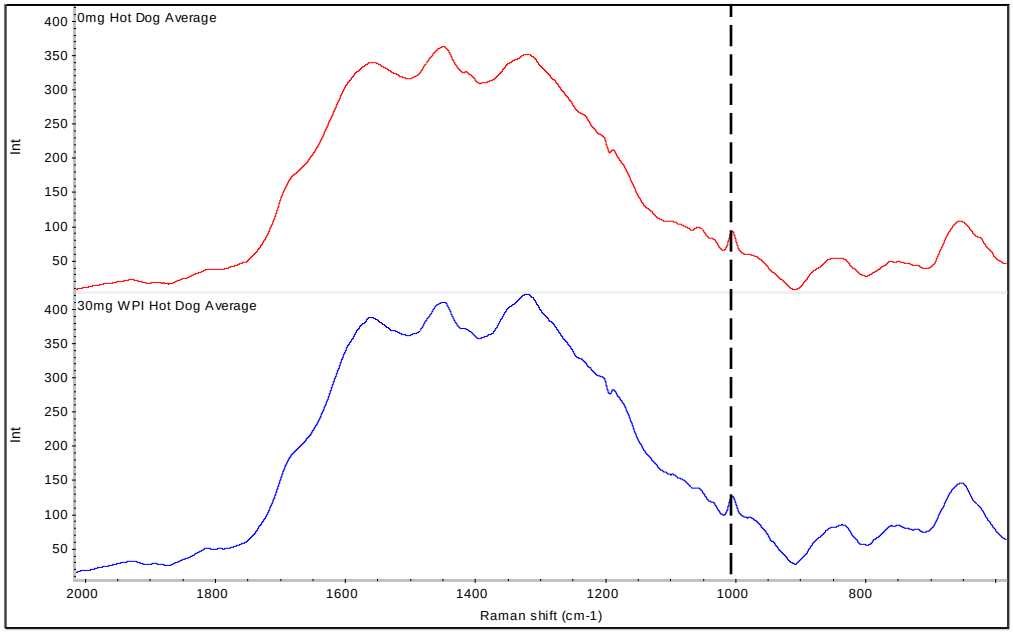

Figure 4.24: Spectra from the aptamer IMS run using the spike and negative control hot dogs. Both spectra show a peak at 1004cm^{-1}, seemingly indicating that they are both "positive" results.

Both spectra came back with peaks at 1004cm^{-1} and appear almost identical to each other. Since the presence of a sharp peak at 1004cm^{-1} has been the indicator for a "positive" during this experiment, having the negative control exhibit a peak at that point indicated something wasn't working. A 2nd derivative plot was created to see if a baseline correction could yield a clearer picture, seen in figure 4.24.

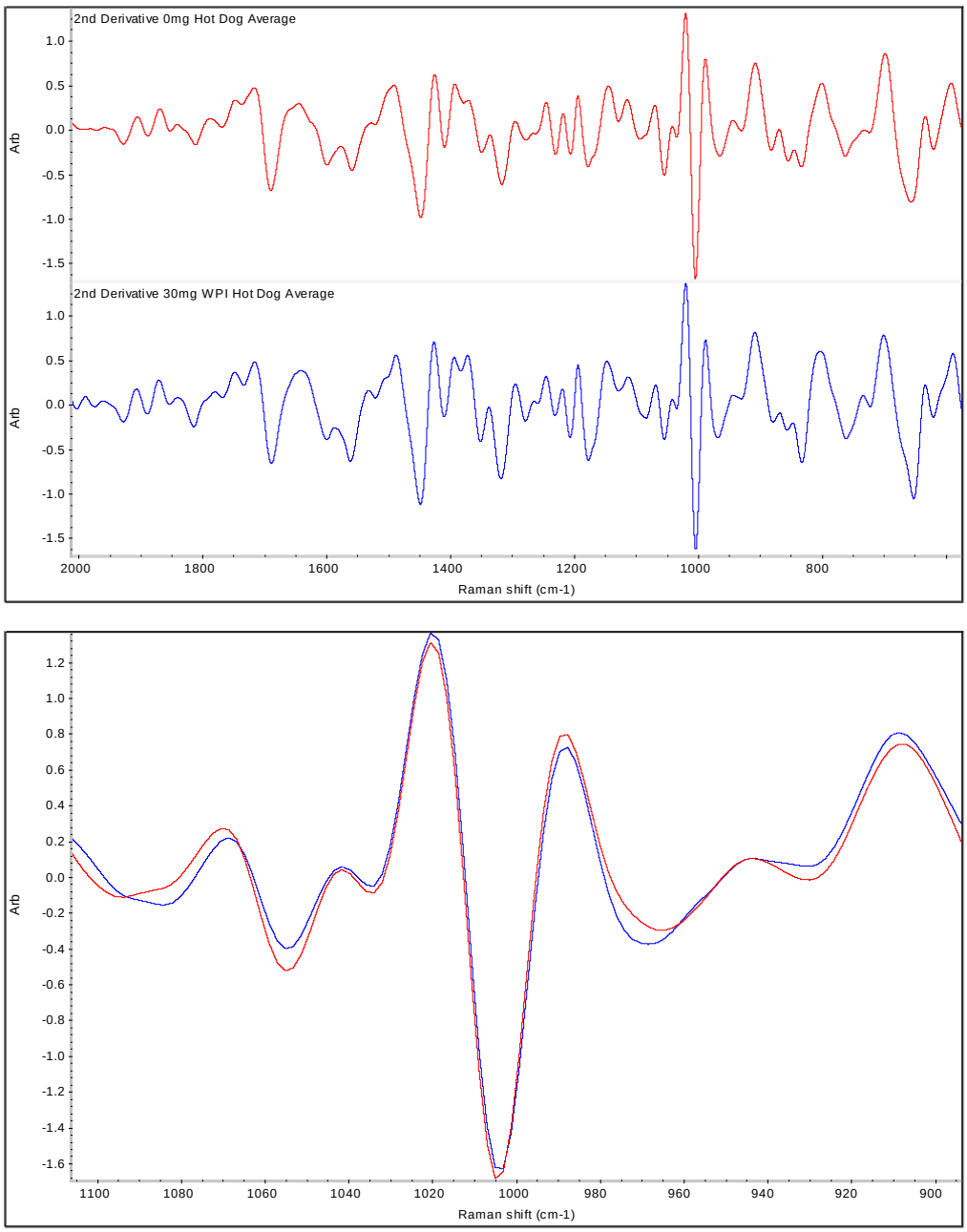

Figure 4.25: 2nd derivative spectra again show how the two are identical to each other.

The 2nd derivative spectra give even a better picture of how similar the two spectra are. Figure 4.24 shows the two spectra overlaid and how they almost

appear as a single spectrum, with both being almost unrecognizable from each other.

As with the other IMS runs, a PCA graph was created to see if maybe an outlier was skewing the average enough that the spectra look similar to each other. The graph in figure 4.25 below shows that that was not the case and that something was giving off a signal in both hot dogs, masking their spectra and giving off a false positive result for the negative control.

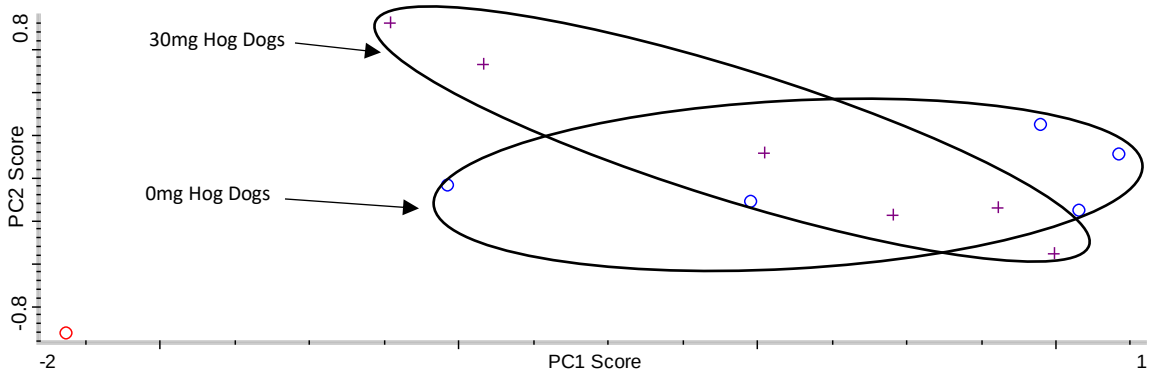

Figure 4.26: PCA graph of the 30mg hot dogs and the negative control hot dogs. The intertwining points of the two spectra show that the two can't be differentiated from each other.

Looking at the PCA graph and the 2nd derivative spectra for the run, it is clear there is some type of interference going on. Since the IMS-SERS worked with the antibodies, the negative control hot dogs were deduced to not contain any whey or whey product in them, since their spectra didn't give off such a high signal as the negative control in the aptamer IMS run. This left only a few possibilities; that there possibly was some type of small molecule sticking to the aptamer/bead complex or that some protein was sticking to it and giving off a

signal at 1004cm^{-1}. There have been studies that mentioned similar issues with magnetic beads and some of the issues that non-specific binding can have on the final results (Chalmers et al, 2009). They mentioned that the magnetization from the beads possibly causes an increase in the non-specific binding of cells, most likely through their surface proteins. While that paper focuses on the non-specific binding of cells to magnetic beads, the theory behind their argument, that the magnetic field created by the beads could affect non-specific binding, could be what is responsible for the issue with the aptamer IMS results.

To see what kind of non-specific binding was occurring, the aptamer IMS run with hot dogs was run again, except this time, the eluent wasn't used for SERS analysis, but instead was used for gel electrophoresis. The theory was that if the non-specific binding was protein related, we would see bands appear on the gel indicating that protein was interacting with the bead complex. If not, that would show that the source of the non-specific binding was smaller than a protein and most likely some Raman active small molecule. Aptamer IMS was repeated under the same conditions as before and the eluent collected. Next, gel electrophoresis was performed under standard conditions. The resulting gel was then photographed and the resulting images can be seen in figure 4.26 below.

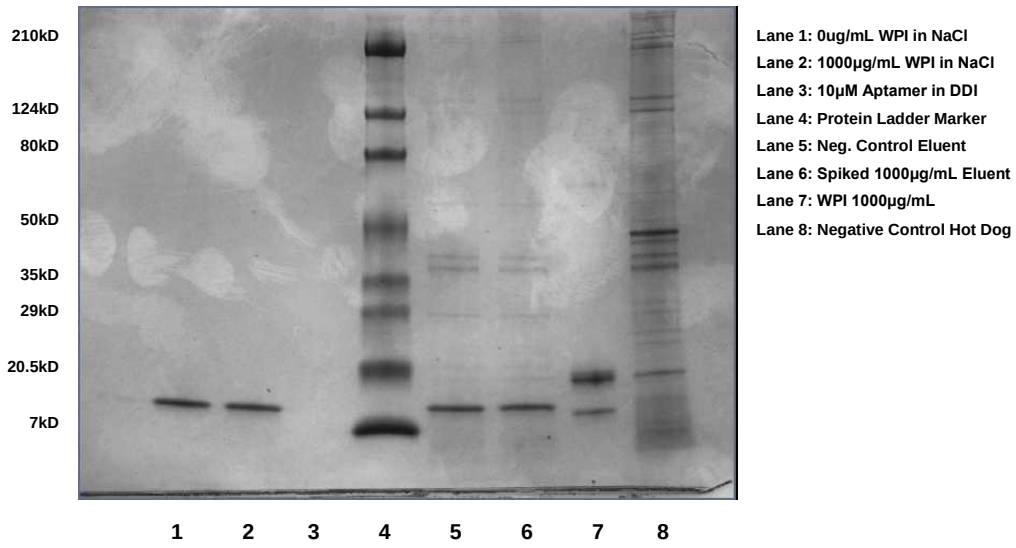

Figure 4.27: Image of gel with various eluents ran on it. Lane 1 was the negative control for the aptamer IMS ran in 10mM NaCl, Lane 2 was the positive control for the aptamer IMS ran in 10mM NaCl, Lane 3 was the aptamer in DDI water, Lane 4 was the protein ladder marker, Lane 5 was the negative control during the hot dog aptamer IMS, Lane 6 was the positive control for the hot dog aptamer IMS, Lane 7 WPI in DDI, and Lane 8 was the negative control hot dog slurry in 10mM NaCl.

The image of the gel shows quite a few protein bands on the hot dog aptamer IMS eluents, indicating that the non-specific binding occurring is most likely protein based. When lanes 5 and 6 are compared to lane 8, the negative control hot dog slurry, there are some obvious similarities that point to protein from the hot dog are somehow managing to stick with the beads despite the washing steps used during the IMS procedure. Most notable are the bands at 40-45kD, which appear in all three hot dog lanes. Along with those bands, a slightly fainter band at 27-28kD appears in all three hot dog lanes as well. It is worth noting that while there appears to be a band matching the WPI band at about 17kD in lane 8, the matching band in lane 8 is most likely bovine myoglobin,

which has a similar molecular weight of about 17-18kD (Uniprot ID P02192, 2016), since the main ingredient for the hot dogs is beef from a cow. Also, the small band found on all the lanes with aptamer IMS eluent was deduced to be a small subunit of streptavidin that is most likely cleaved off at higher temperatures, like those used during the elution step of the aptamer IMS protocol (Howarth et al, 2008). It was determined that this subunit was not responsible for the signal interference since the subunit is present in the aptamer IMS run in NaCl, but didn't cause the same interference found in the hot dog aptamer IMS.

Once we figured out the source of the signal interference, we tried a blocking method to see if that would prevent the interfering protein from attaching to the bead/aptamer complex. For our blocking agent, we went with BSA, as it is commonly used in other detection methods to prevent non-specific binding. The BSA blocking step was added to the protocol just before incubation with the hot dog samples, with the beads being blocked for 1 hour under constant rotation. The spectra below in figure 4.27 is the result from the BSA blocked aptamer IMS run.

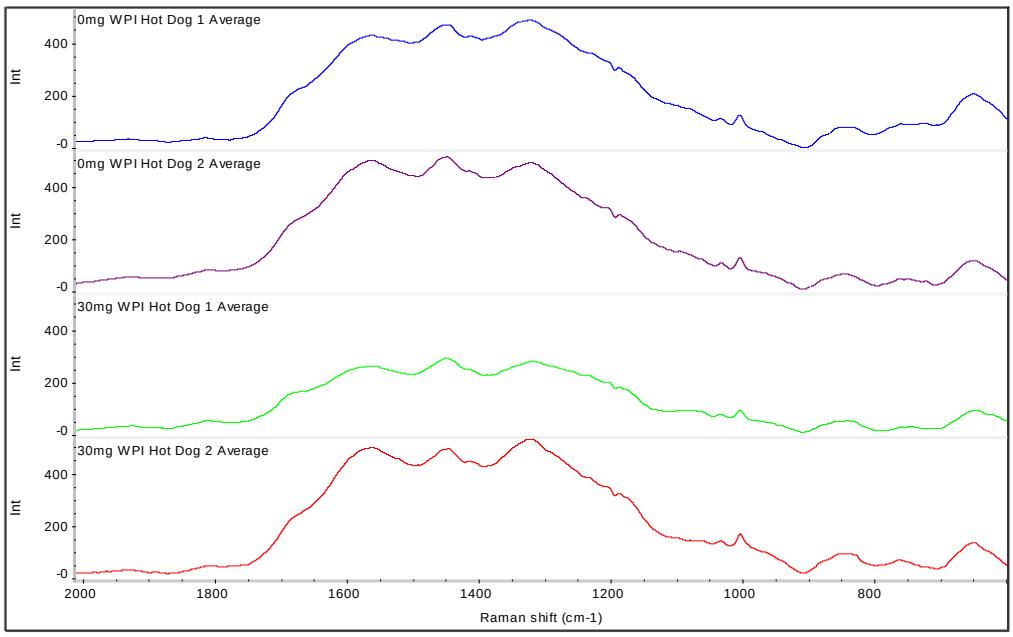

Figure 4.28: Raman spectra of the spiked and negative control hot dog run. A blocking step was used prior to the incubation with the hot dog samples to try and see if the interference could be eliminated.

Looking at figure 4.27, you can see that the BSA blocking didn't eliminate the peak that was interfering at 1004cm^{-1} on the negative hot dogs. It's possible that since the interference with the signal in the first place was protein in origin, then using a protein to block that signal wasn't going to work. Either the protein binds to the beads stronger than the BSA or that the BSA binds in a similar fashion as whatever protein is interfering in the first place and now the BSA is causing the interference. Either way, there is still interference and it appears that the BSA didn't help. A 2nd derivative spectra was created to get a baseline look at how the two groups looked compared to each other.

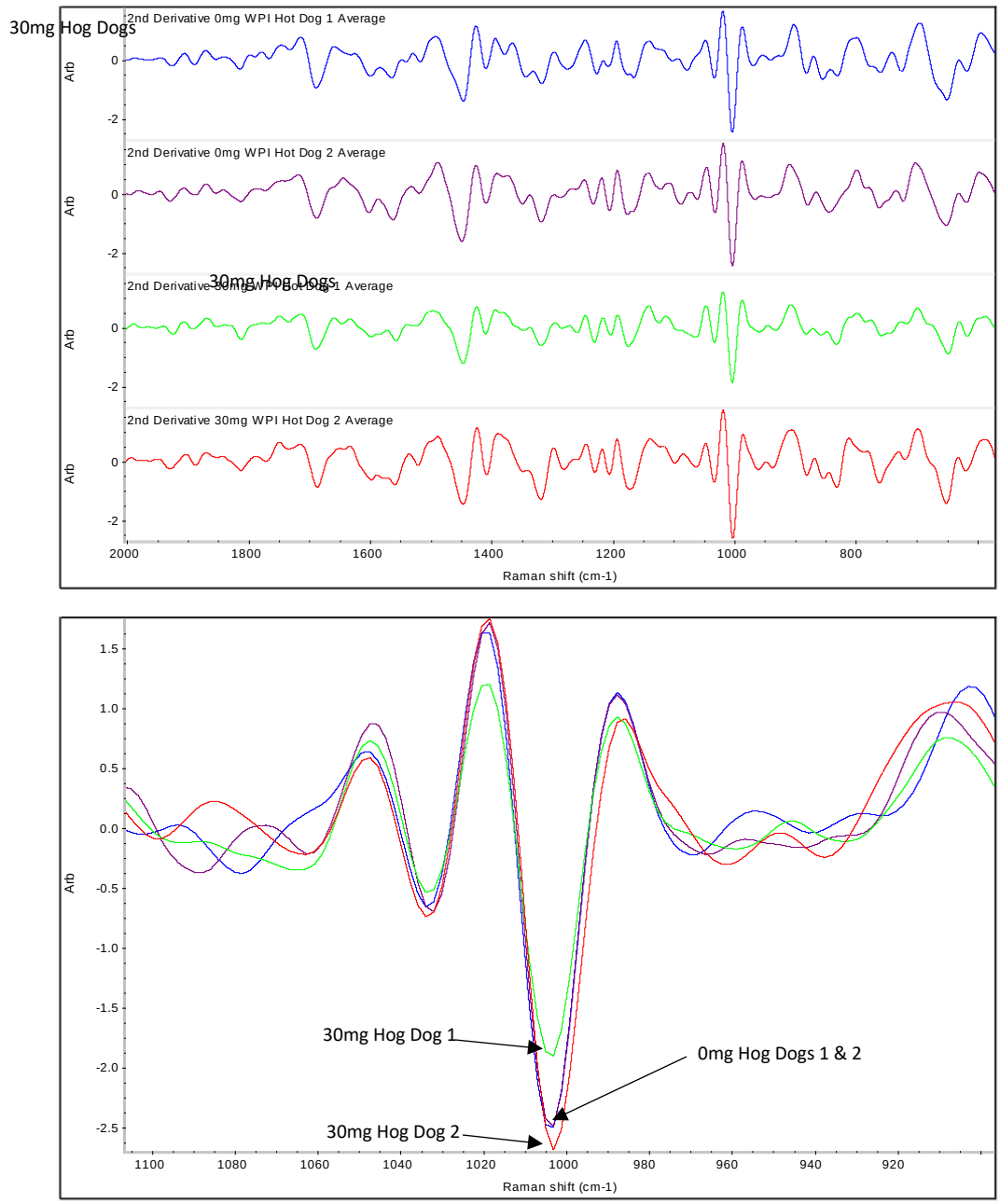

Figure 4.29: 2nd derivative spectra from the BSA-blocked aptamer IMS run.

The 2nd derivative spectra created showed that one of the spiked hot dogs actually had less intense of a signal at 1004cm^{-1} than both the negative control hot dogs. This shows that the signal interference is sporadic and prevents us

from implementing up with a baseline correction that could be applied to the spectra. If there was some consistency in the interference or background, we could either subtract it from the spectra or adjust the baseline for the negative control. Without some way of removing this interference or at the very least finding a way to mitigate it so it gives a consistent interference so a baseline can be created, this method cannot be used to detect whey in a hot dog matrix at this time.

5 Conclusions

5.1 Comparison of Antibody and Aptamer IMS/SERS

The two methods used during this study were very similar in nature; the primary difference being the capture agent that was used to separate the whey protein from the sample hot dog matrix. The antibody based system relied on antibodies, which attach to an allergenic protein by targeting their specific epitope or epitopes. This mechanism allows an antibody to work in a variety of different conditions and still function properly versus an aptamer. The aptamer, on the other hand, is more durable in nature being able to survive harsher conditions, such as high heat, acidic conditions, denaturation of the aptamer itself, and still revert back to its working configuration (Meyers et al, 2004). With regards to availability of the two agents, antibodies are more commercially available than aptamers are, with large companies supplying a broad range of antibodies to the scientific community (Sigma Aldrich and Abcam, 2016). Aptamers can be purchased from scratch, but a lot of the services available require researchers to foot the bill of the entire creation process of the aptamer from scratch, which can end up costing anywhere from thousands to tens of thousands of dollars depending on the selected antigen (Base Pair Biotechnologies and Aptagen, 2016). While antibodies are more readily available, if one knows the process needed to create aptamers and has a lab that with the needed equipment, new aptamers can be created in a much shorter time than it would be needed to create a brand new antibody (Mosing et al, 2009). Using a method such as the

SELEX process, the turnaround time for a new aptamer can be as short as two weeks, whereas some monoclonal antibodies can take up to 4-6 months depending on which host animal is chosen and the purity required. Another difference between the two is the cost for the individual capture agents. Per milliliter, the antibody for this experiment cost about $65, while the aptamer cost about $32.50 (Sigma Aldrich and Integrated DNA Technologies, 2015). Again, this most likely goes back to the amount of work needed to create each agent, with the aptamer requiring far less labor to create. While it may cost less, the aptamer could have taken up to 2-3 weeks to get to us, depending on if the company was successful in creating the needed sequence in the correct concentration. Finally, the other difference between the two would be the cost of their respective magnetic beads needed during the IMS procedure. This time, the antibody's magnetic beads ended up being cheaper, being about $50/mL, where the aptamer's magnetic beads were about $225/mL (BioRad and Thermo Fisher, 2015). This most likely has to do with the fact that antibody IMS has been around longer, and as such, has had longer to find ways to cut down on production cost versus the aptamer's magnetic beads.

The two methods used in this experiment both were successful in being able to separate and detect whey in solution. Both methods, in solution, were able to create spectra with an identifying signal at 1004cm^{-1} that signaled that whey was present, as seen below in figure 5.1. Both the antibody and the aptamer IMS were able to signal a positive result, showing that method, at the

very least, was capable of working in a basic solution. It also appears that the difference in incubation solutions, 10mM PBS-T and 10mM NaCl with Tween, didn't. Also, both methods were able to be run from start to finish in almost the same time, about an hour and forty-five minutes. That time included all prep work, pipetting, aspirating washed solution, incubation, drying, and reading using the Raman spectrometer.

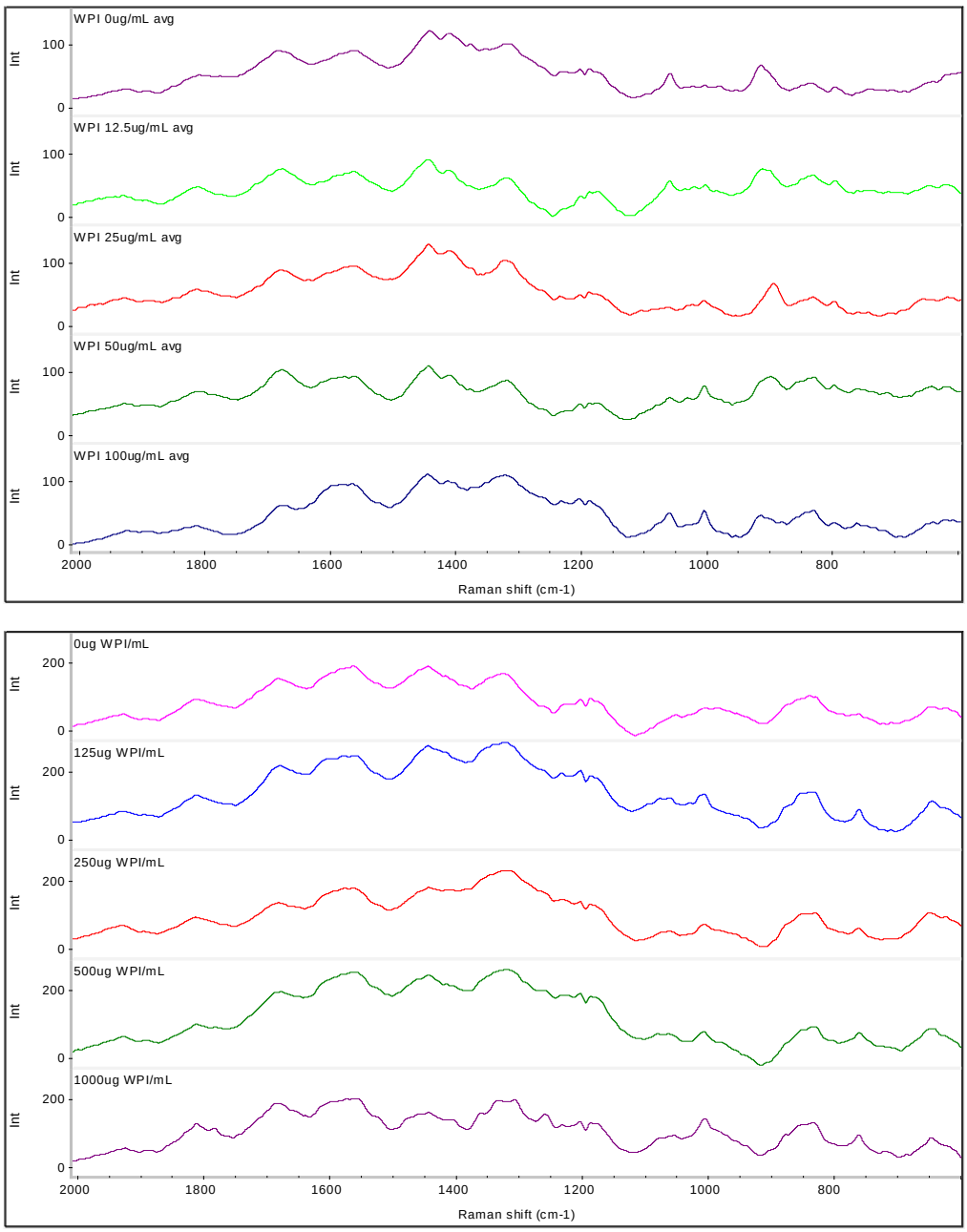

Figure 5.1: Spectra of the antibody IMS (top) and the aptamer IMS (bottom). Both were able to detect whey in a pure solution, showing that their respective methods could be used to detect whey.

But once a real life sample was used, the antibody based IMS method proved to be the effective method in detecting whey, whereas the aptamer

method was unable to differentiate the negative control hot dogs from the spiked hot dogs. The antibody method was able to detect whey in a hot dog matrix at a concentration of 30mg of WPI per 50g of hot dog or about 600µg of WPI/g of hot dog. The aptamer on the other hand, was unable to differentiate between the spiked hot dog and the negative control. It was deduced that the reason behind the failure to differentiate was an interfering signal from a protein found in the hot dog matrix. Gel electrophoresis was used on the eluent from the IMS procedure to determine this, finding similar bands in the hot dog matrix and the negative control eluent. We also ran gel electrophoresis on the antibody IMS to make sure this interference wasn't occurring. The images of the gels ran for both methods can be seen below in figure 5.2.

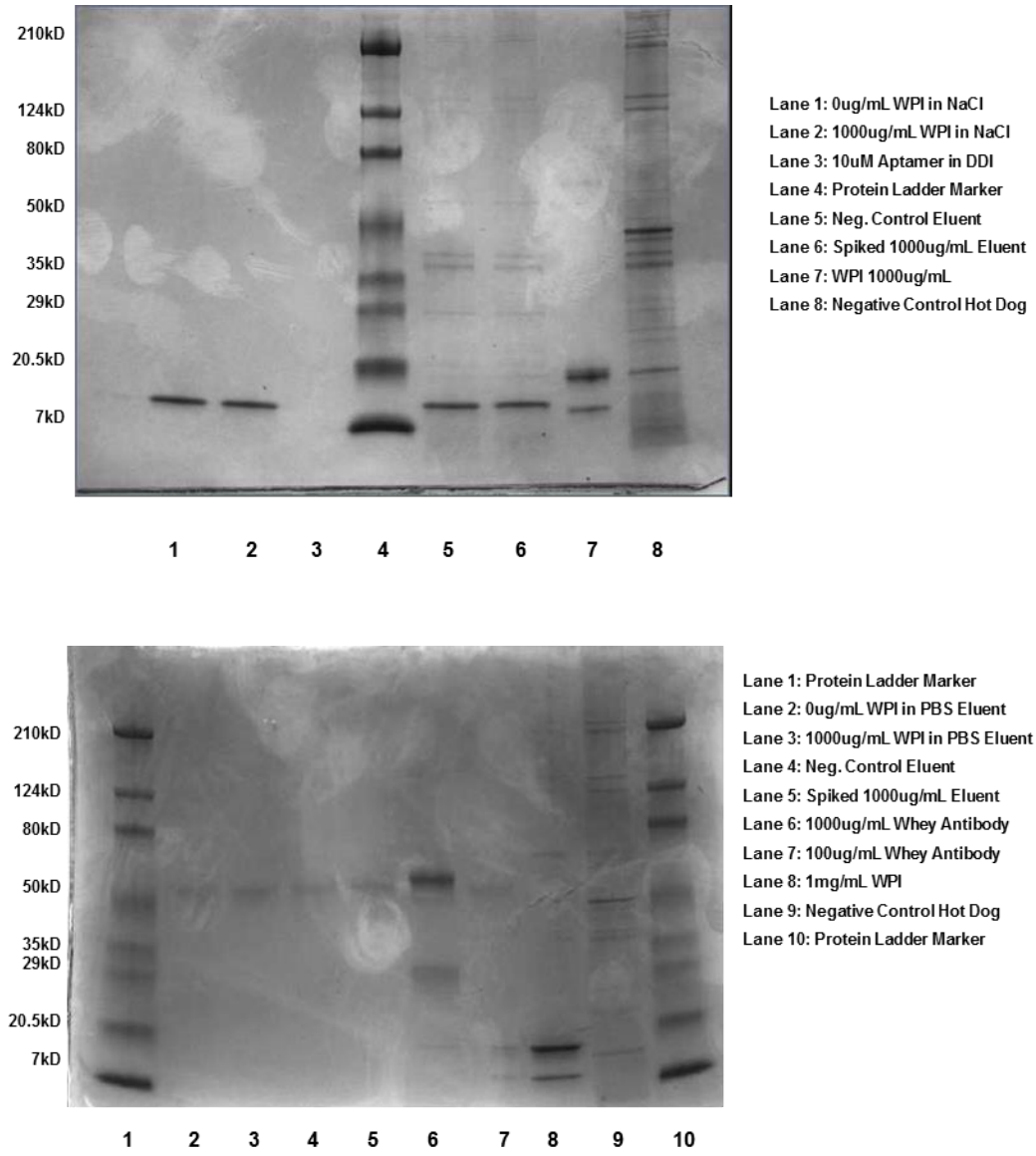

Figure 5.2: Images of gels from the aptamer (top) and antibody (bottom) IMS procedures. The aptamer eluent gel shows bands on the negative control hot dog that match up with the hot dog solution. The negative control hot dog eluent for the antibody IMS does have some bands on it, but not as many.

The gel from the antibody IMS showed what we had already known from the Raman spectra, that there weren't any interfering proteins in the antibody

based IMS procedure. The negative control hot dog did show some bands sticking to it, but there were some bands that were missing in the antibody IMS gel that were present in the aptamer IMS gel. The most noticeable bands that were missing were the bands around the 40-45kD mark. These were speculated to be some of the bands of protein that could be causing the interference with the aptamer IMS method. The fact that they were missing in the antibody IMS method adds some credence to the theory that these proteins were the culprit of the interference for the aptamer based IMS procedure.

Overall the antibody based IMS method was the more successful method for detecting whey. With the antibody IMS being able to not only detect a smaller amount of whey than the aptamer IMS in pure solution, but being able to also detect whey in a hot dog product, the antibody IMS method is the better choice. Besides the performance difference between the two methods, there wasn't many other differences. The overall time needed to complete detection for both was about the same and the cost was about the same, if you take into account that while the cost of the capture agent for the antibody IMS was more expensive, the magnetic beads needed to run the method were cheaper for the antibody IMS. A table comparing the two methods can be seen below in table 5.1.

Table 5.1: Side by side comparison of the antibody based IMS method and the aptamer based IMS method.

	Aptamer	**Antibody**
LOD in Pure Solution	125ug/mL	50ug/mL
LOD in a Food Matrix	X	30mg WPI/50g Hot Dog
Total Run Time	~1 hour 45 min	~1 hour 45 min
Cost for Capture Agent	$32.50/mL	$65/mL
Cost for Magnetic Beads	$225/mL	$50/mL

5.2 Possible Use in the Food Industry

Now that we've shown this method to be able to work on a real life food product, we can now look to how this method could be used in the food industry. While there are other commercially available methods to detect allergens such as whey, they either take too much time to run a sample or use up expensive reagents with every test. SERS presents a system that not only is rapid, but after the initial startup cost of purchasing the spectrometer, can be run relatively cheaply compared to other methods. This method could be used in the food industry as a screening tool to test batches of product for allergens. The first step would be to create a library of positive result spectra that future samples will be compared with. This library of standards would then be uploaded to a program such as TQ Analyst, where the groupings of positive samples would create zones

that can be used as a reference to compare samples to. An example of this is seen in figure 5.3 below.

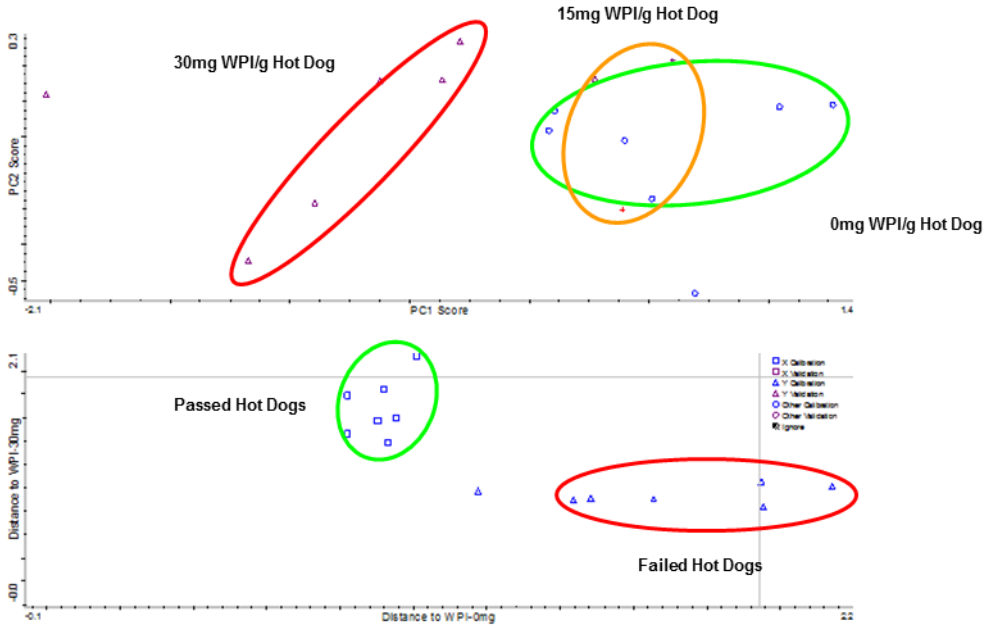

Figure 5.3: Example of “zones” created in TQ Analyst using known standards. The red circles show “failed” samples, ones with whey, and the green circles show samples that would “pass”. The top graph shows a multi-variable PCA, which could be used to show different amounts of whey, while the bottom is a simpler pass/fail graph where anything outside the green circle would be considered contaminated.

Once a standards library is uploaded into TQ analyst, all future test samples can be quickly compared to it and the software will compare the unknown spectra to the standards library, telling us if the sample is either contaminated or is clean. A more in-depth PCA graph can be created that not only could show if a sample is contaminated or not, but will also show how

contaminated it is. The only drawback to this would be that the company would have to invest in creating the standards library for each additional level, but this would allow a company to tell just how contaminated a sample was. This could also be used to help with products that have an allotted tolerance for an allergen. The different zones would allow to see the level of the allergen in the product and if a batch of product was close to the tolerance or if it was well under the tolerance level.

6 Future Research

While the method proved to be able to detect whey in a hot dog, there is still room for improvement. The extraction method for this method was a simple one, with the blended hot dog sitting in a solution for a few hours and then the heavier particulates strained and separated. This could be looked into more and is one area that could allow for a lower limit of detection by being able to extract more of the whey into the solution. Also, if there was a way to combine the bead/antibody complex overnight and then remove the beads, this would save tremendous amount of time and allow for a next day turn around to get the results. And while it was much worse in the aptamer IMS, the reduction of background noise and interference would allow for the limit of detection to be reduced even further. As for the limit of detection, I would like to see an in depth study performed to evaluate the effects of processing on the allergenicity of whey in a hot dog. If the effects of the processing are known, the process can be changed and then the limit of detection wouldn't need to be as low for it to be successful. And finally, while the allergenicity of milk proteins has been fairly well documented in literature, notably by the risk analysis studies done by Dr. Steven Taylor and his lab at the University of Nebraska (The Food Allergy Research and Research Program), the allergenicity of whey and milk proteins isn't well known and comparing how the proteins behave and induce allergic reactions would better allow the industry to make smarter safety guidelines. This could be done

by performing an in depth risk analysis for whey in hot dogs similar to what Dr. Taylor and his lab has done for other allergens in other food matrices.

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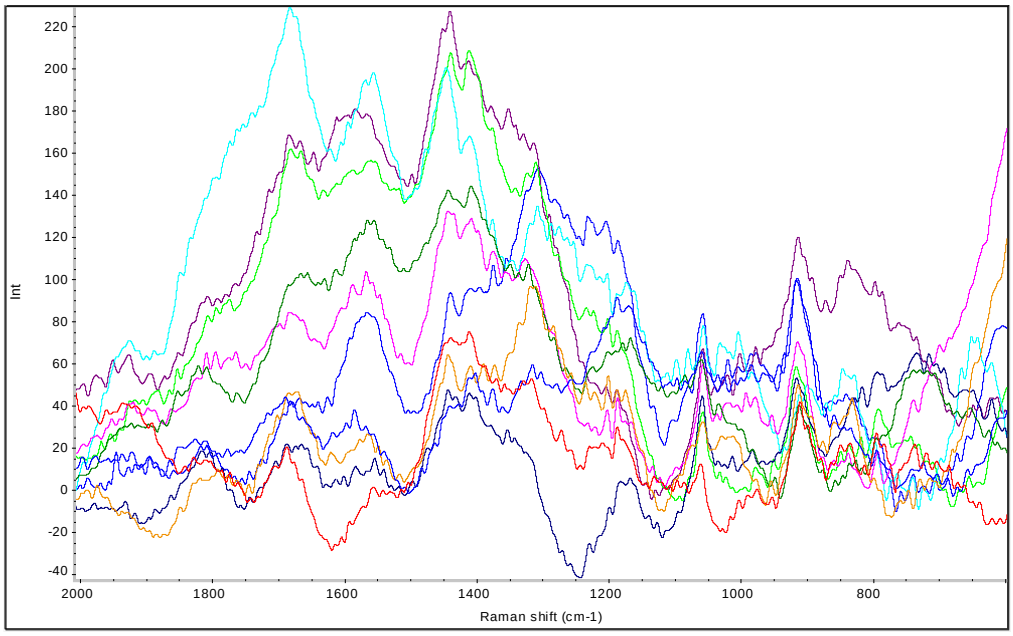
8 Appendices

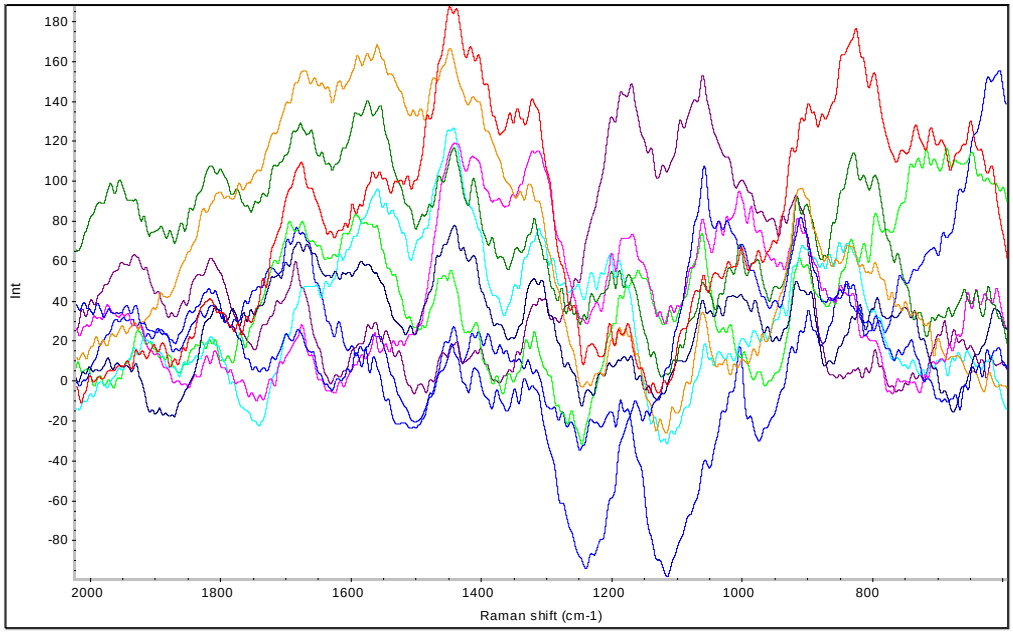
Appendix A- Antibody IMS/SERS Spectra

The following spectra are individual replicates that made up the averages shown throughout the paper.

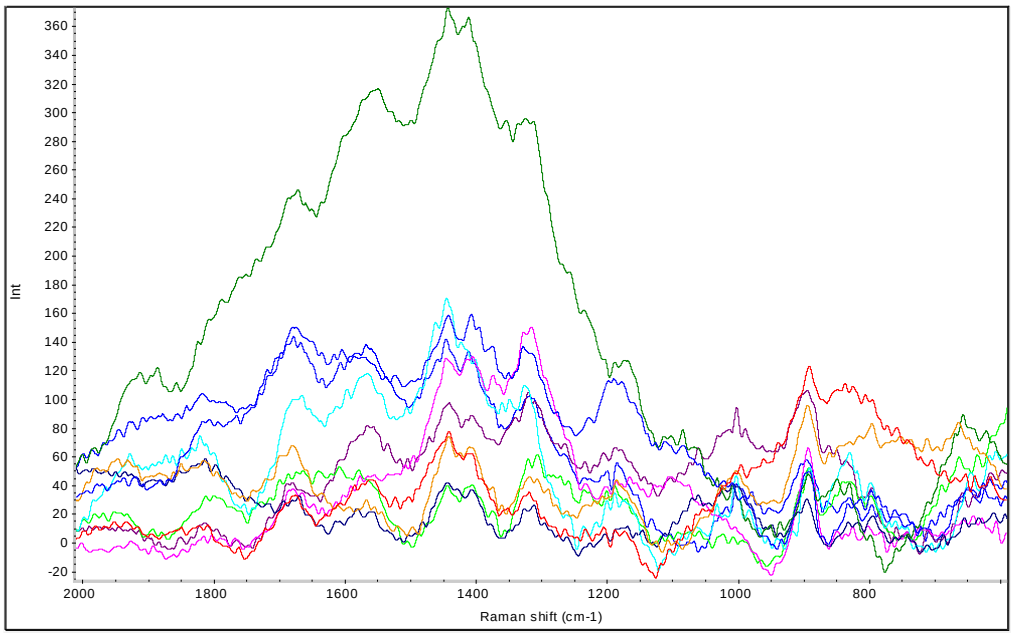
0mg WPI/mL in 10mM PBS



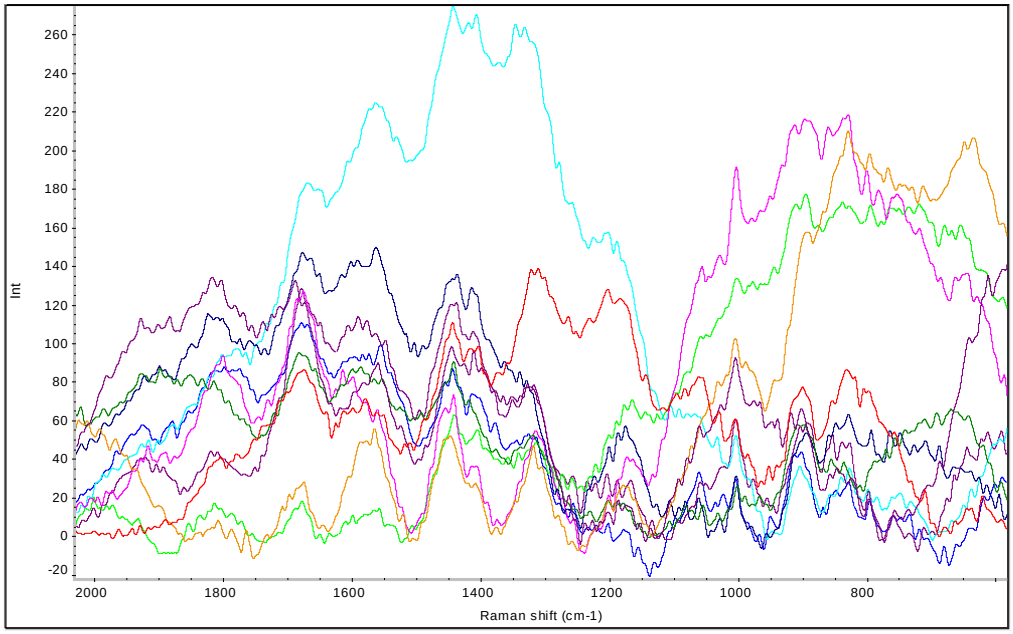
12.5mg WPI/mL in 10mM PBS



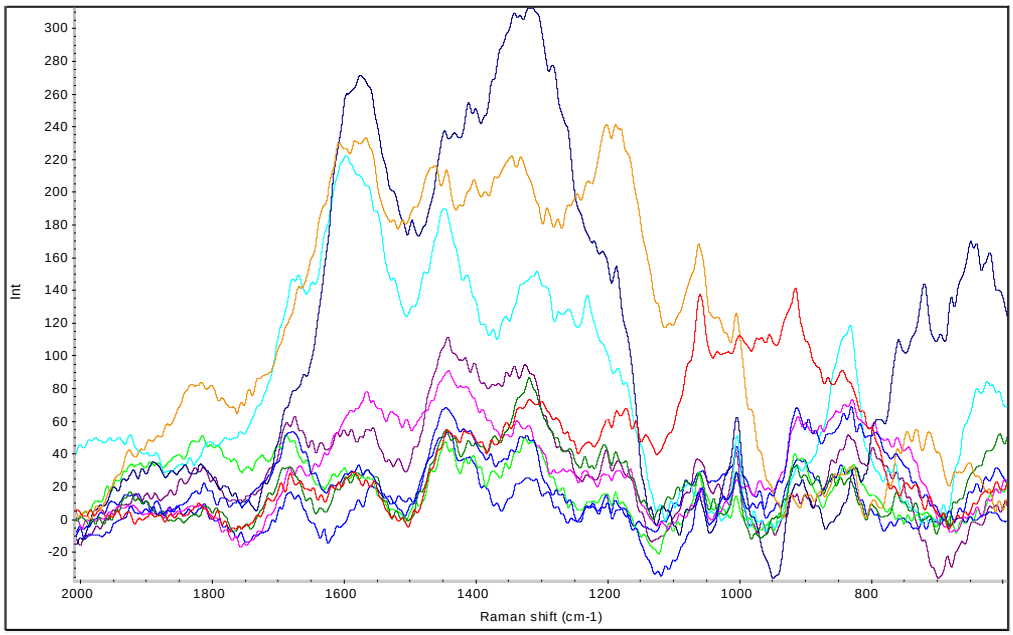
25mg WPI/mL in 10mM PBS



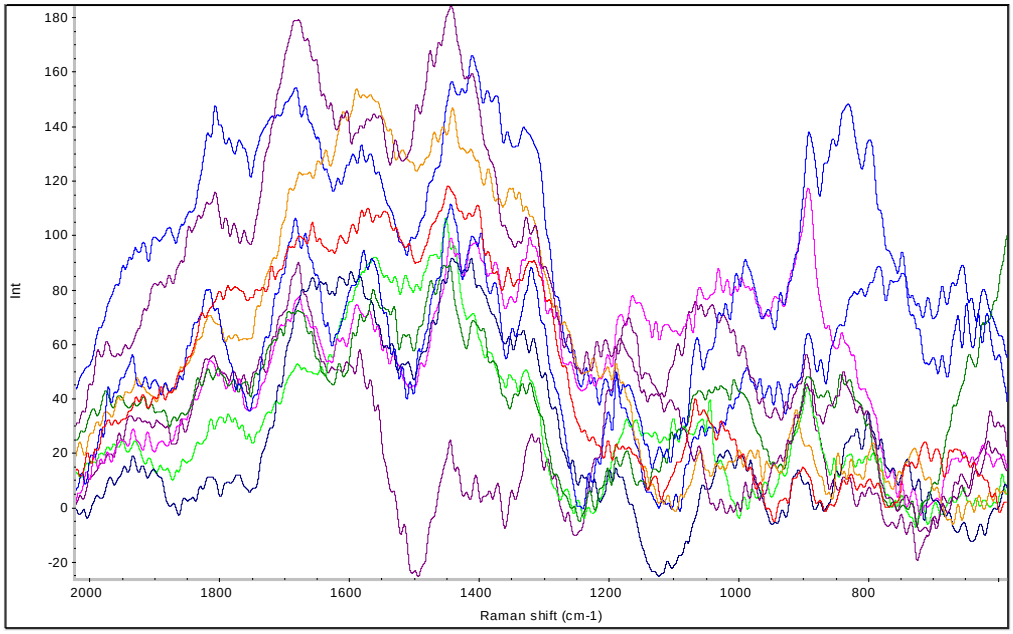
50mg WPI/mL in 10mM PBS



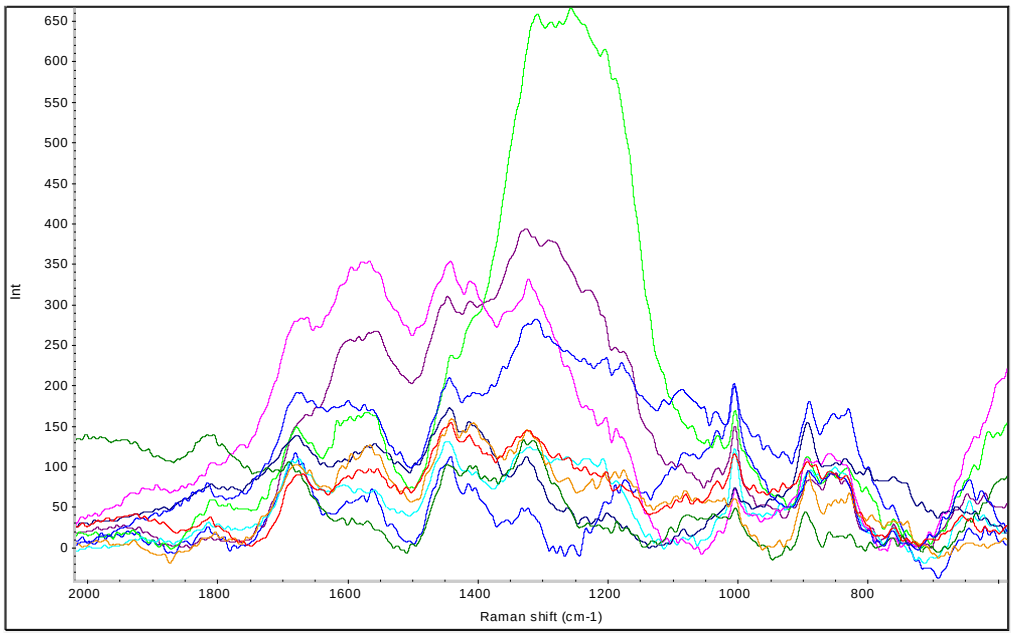
100mg WPI/mL in 10mM PBS



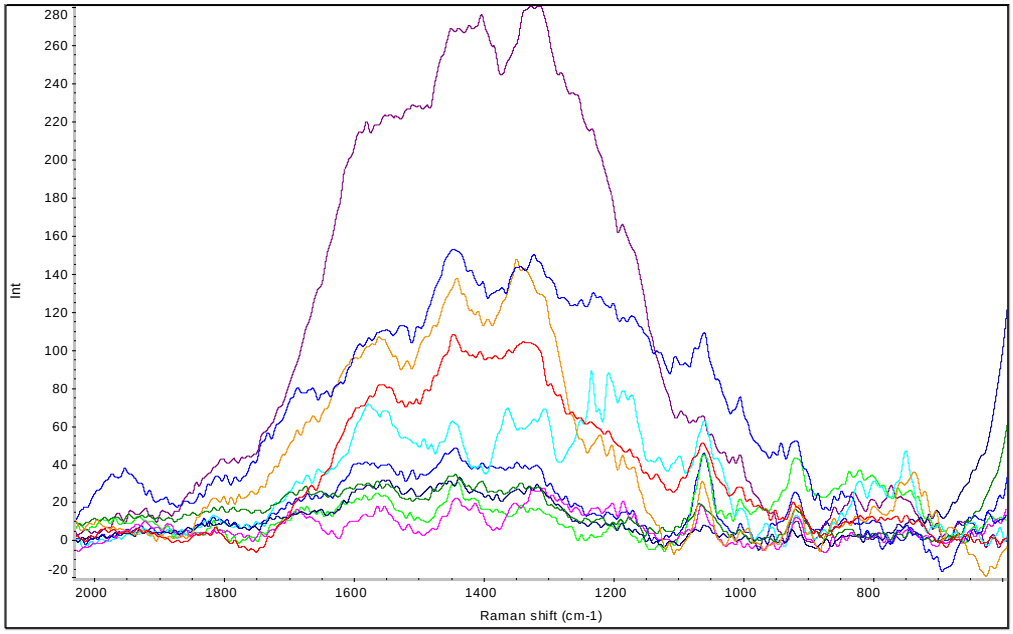
0mg WPI/mL with Spiked Hot Dog Matrix in 10mM PBS



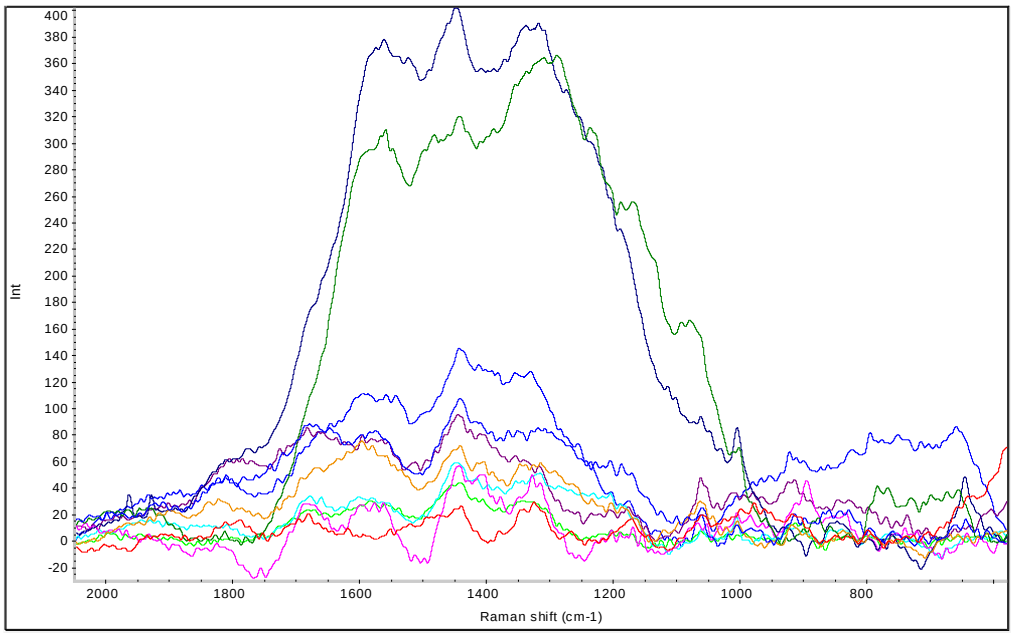
1mg WPI/mL with Spiked Hot Dog Matrix in 10mM PBS



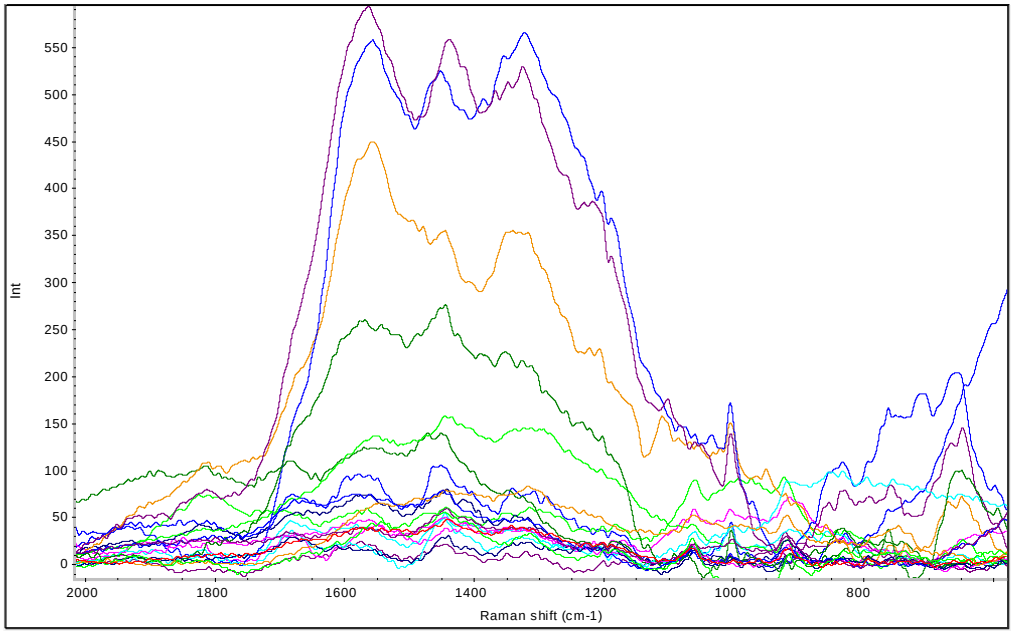
0mg Hot Dog #1



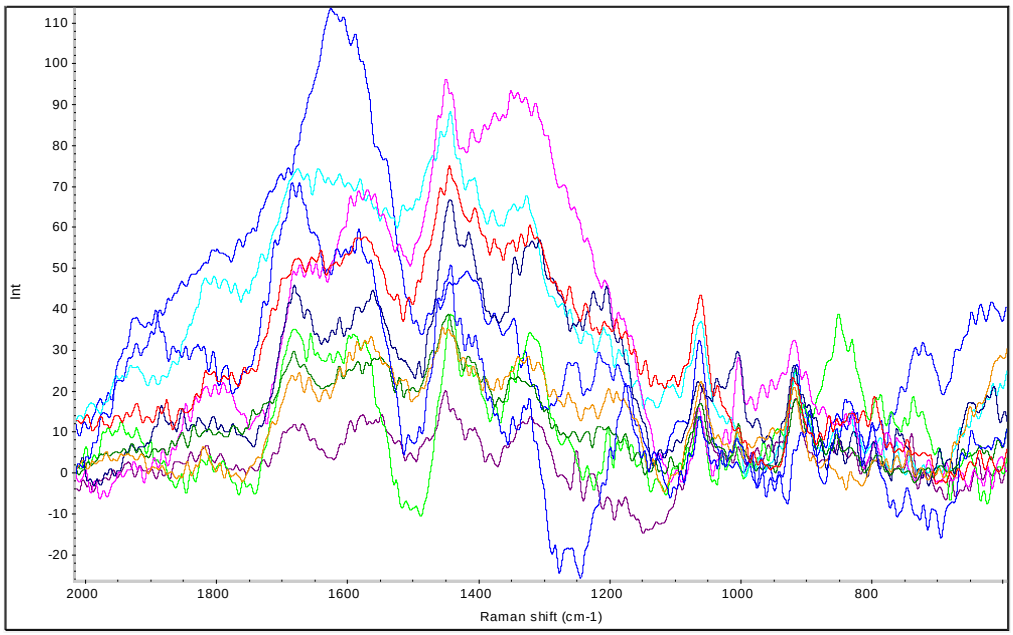
0mg Hot Dog #2



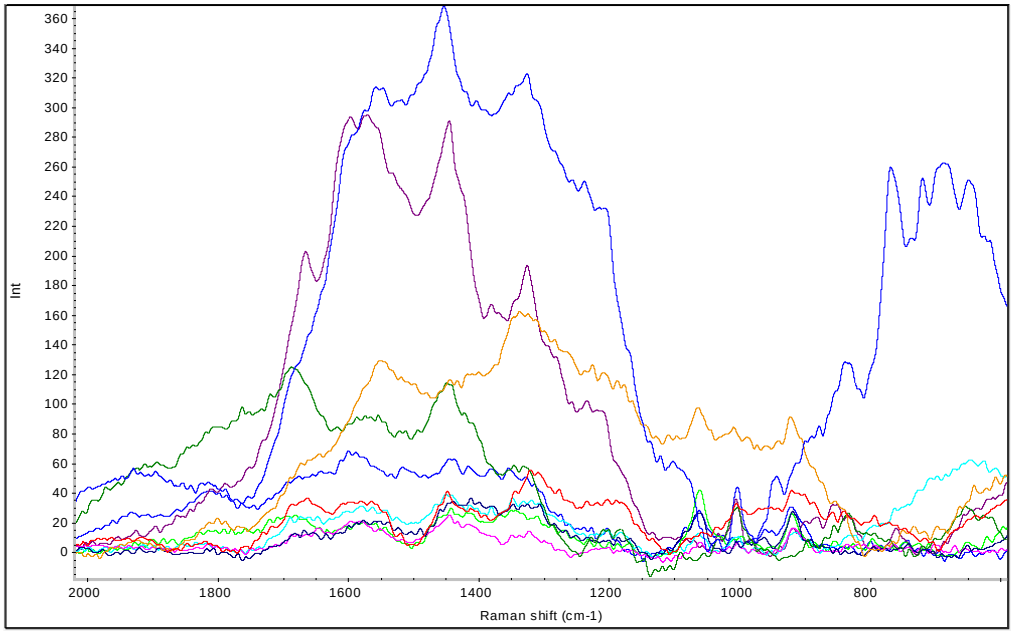
15mg Hot Dog #1



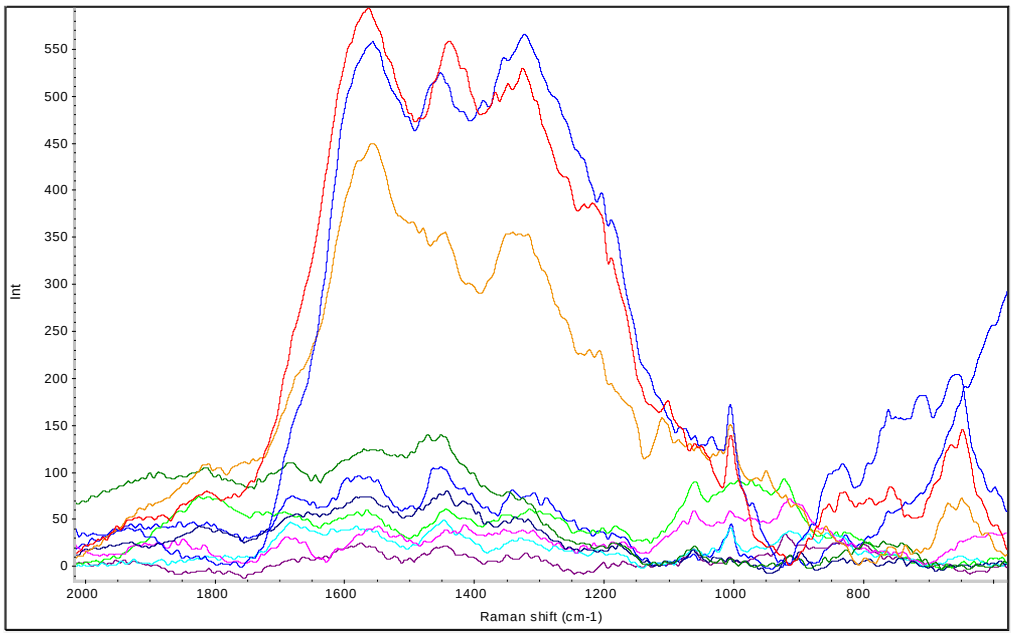
15mg Hot Dog #2



30mg Hot Dog #1



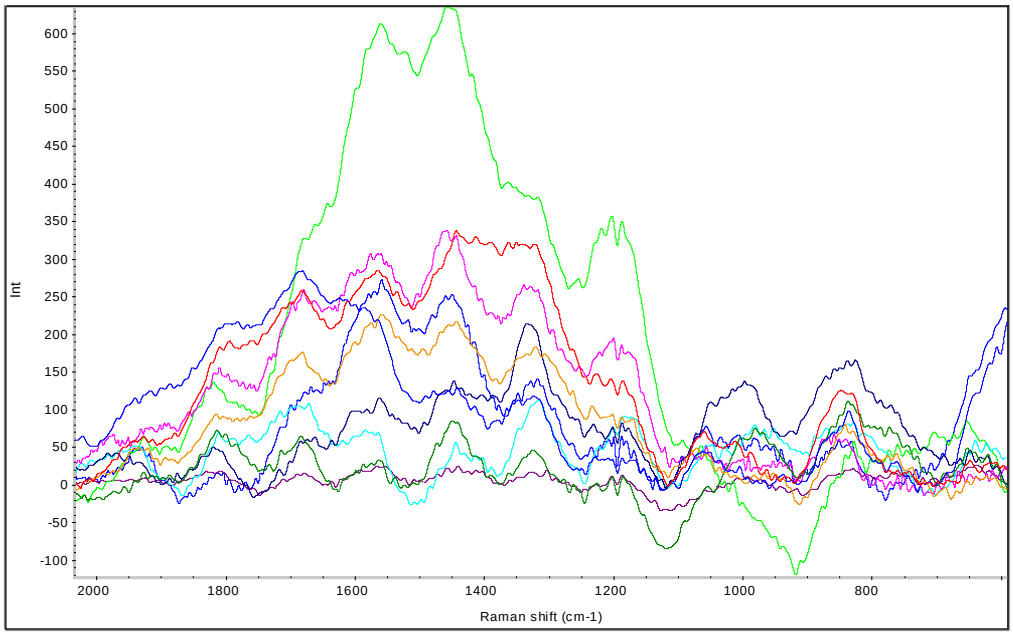
30mg Hot Dog #2



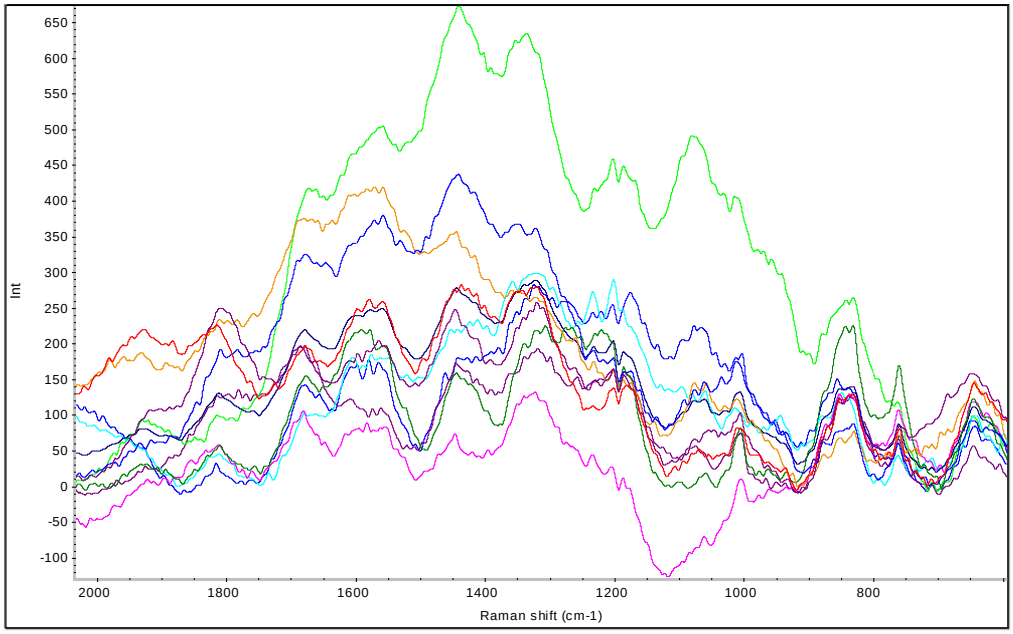
Appendix B- Aptamer IMS/SERS Spectra

The following spectra are individual replicates that made up the averages shown throughout the paper.

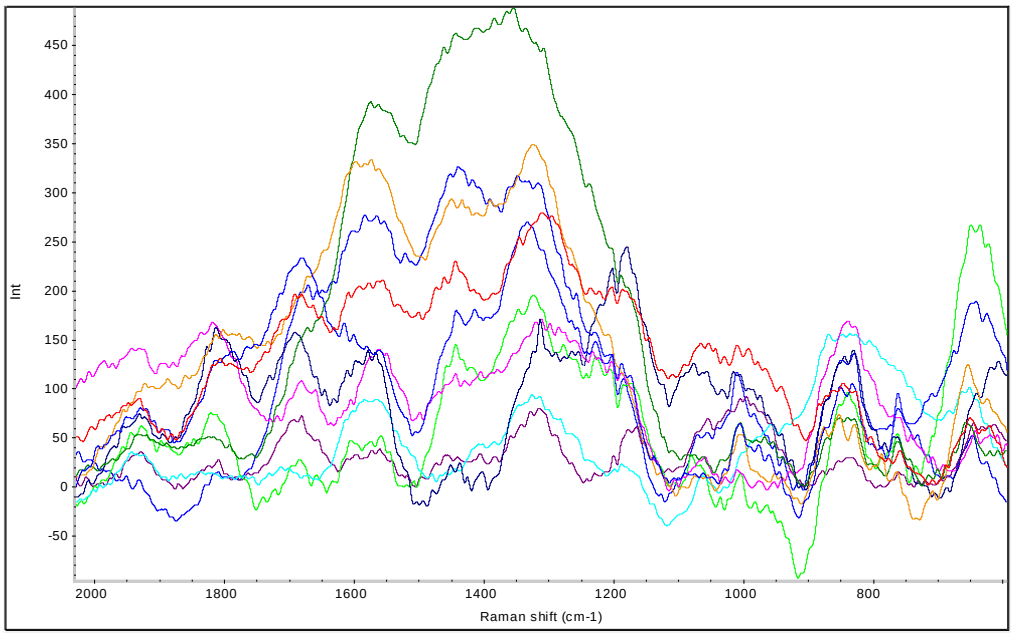
0mg WPI/mL in 10mM NaCl



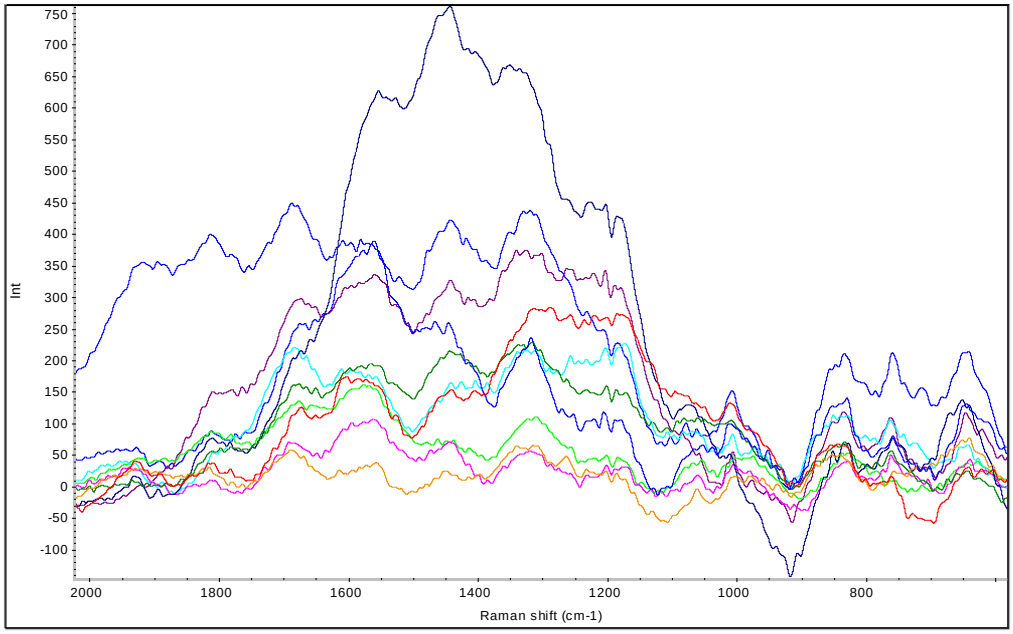
125mg WPI/mL in 10mM NaCl



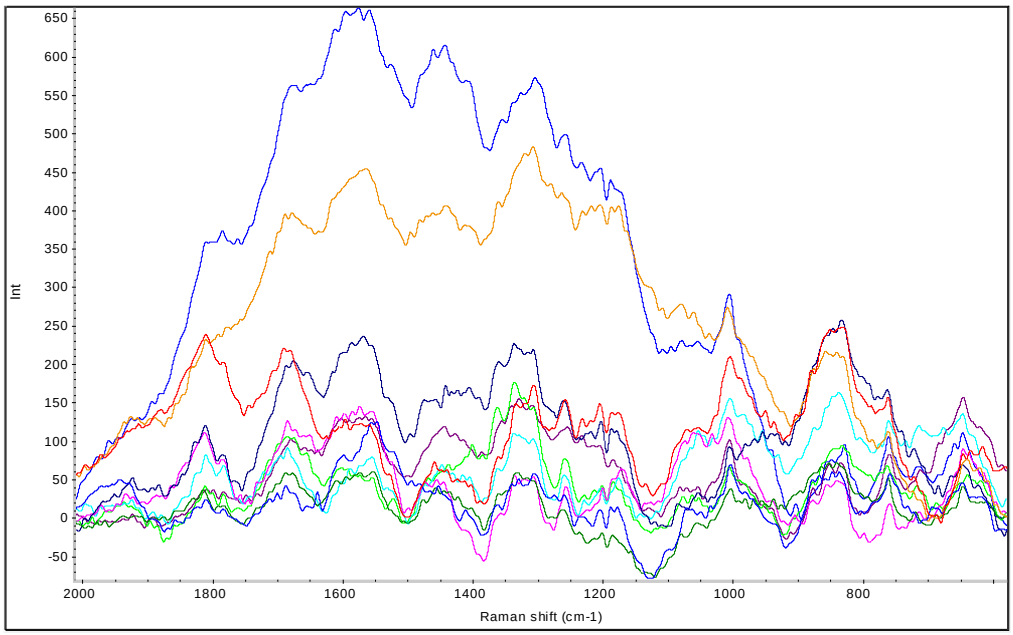
250mg WPI/mL in 10mM NaCl



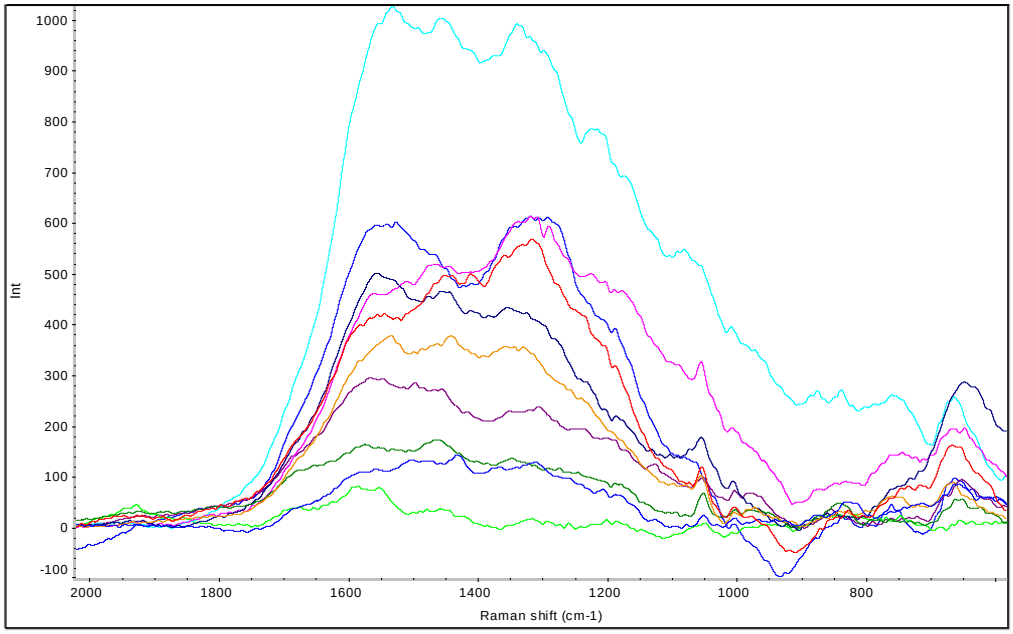
500mg WPI/mL in 10mM NaCl



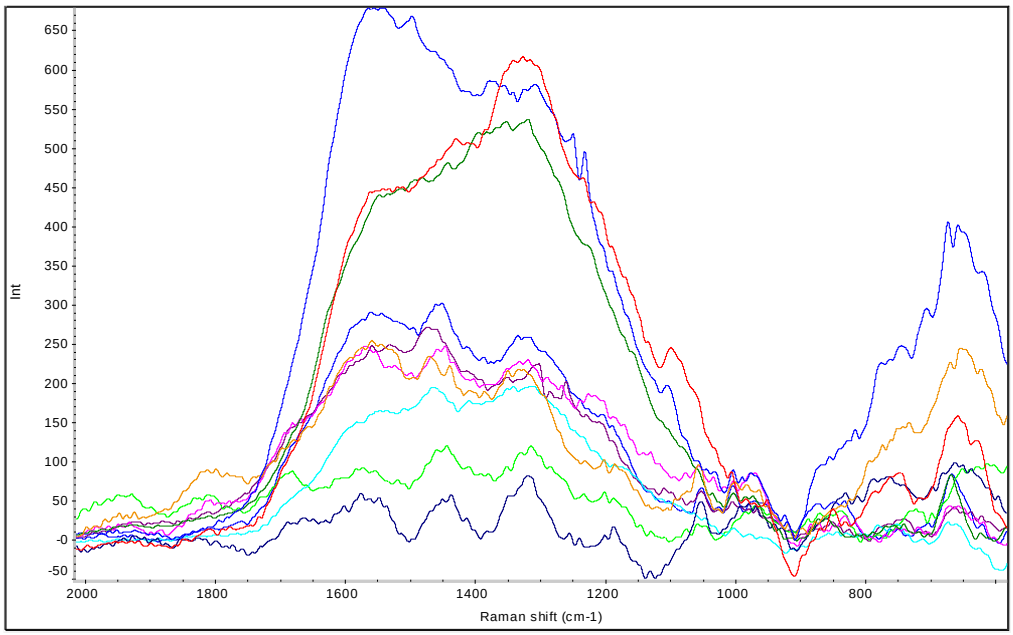
1000mg WPI/mL in 10mM NaCl



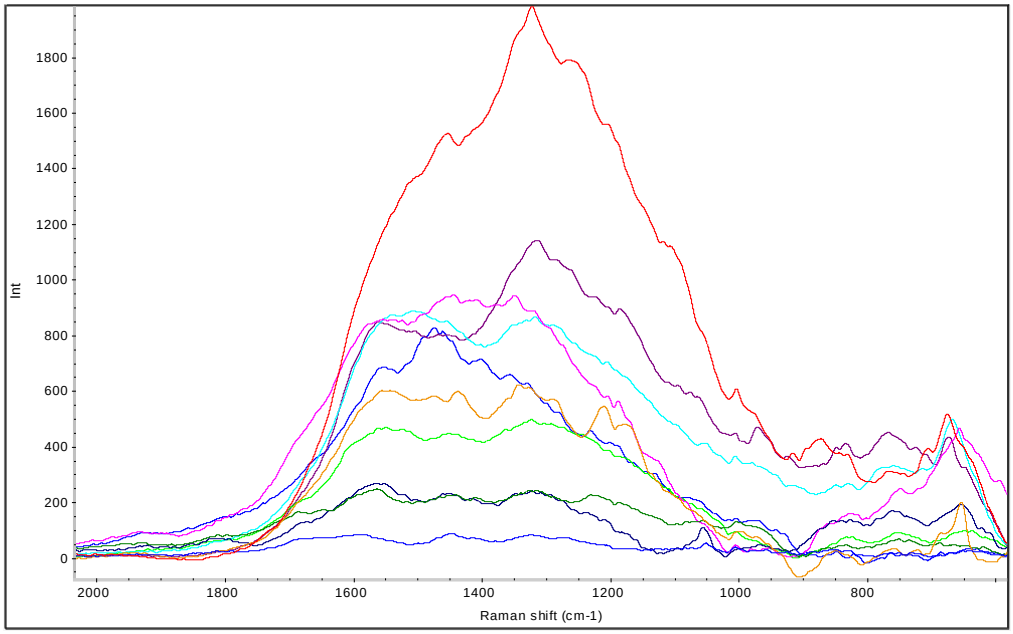
0mg WPI/mL with Spiked Hot Dog Matrix in 10mM NaCl #1



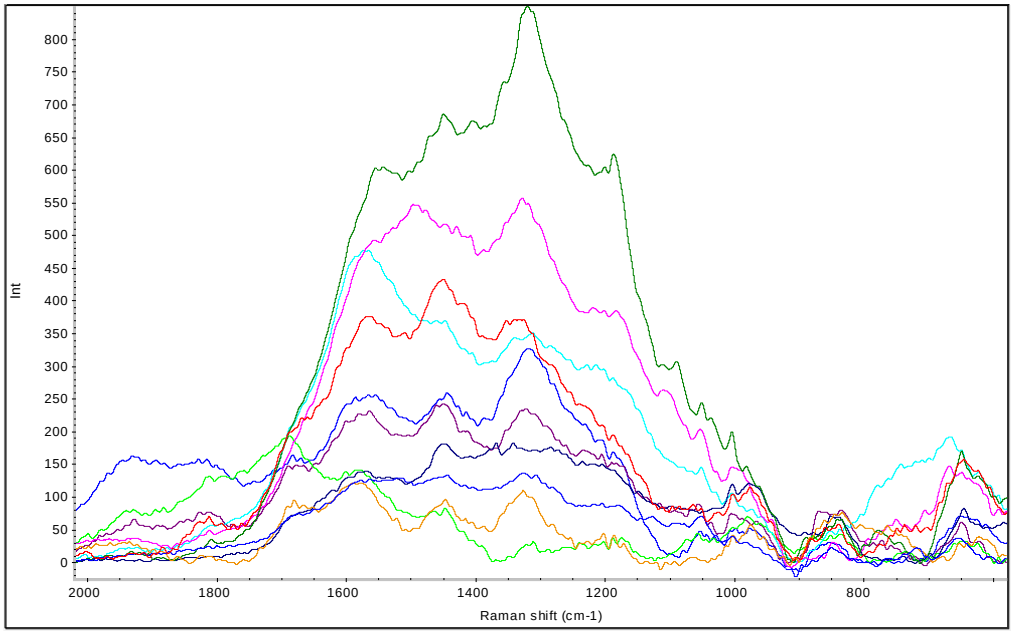
0mg WPI/mL with Spiked Hot Dog Matrix in 10mM NaCl #2



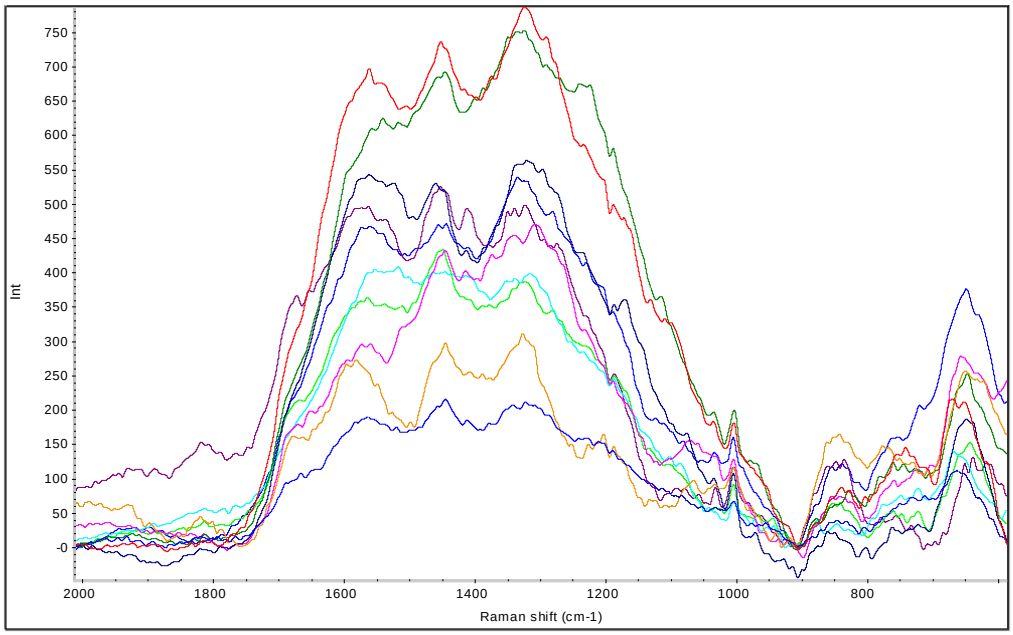
1000mg WPI/mL with Spiked Hot Dog Matrix in 10mM NaCl #1



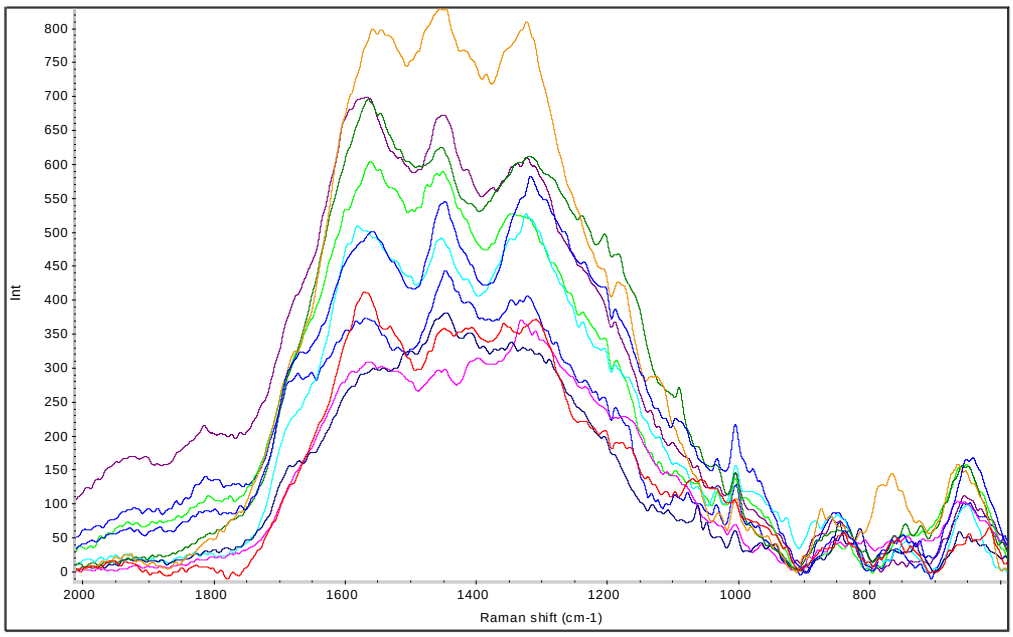
1000mg WPI/mL with Spiked Hot Dog Matrix in 10mM NaCl #2



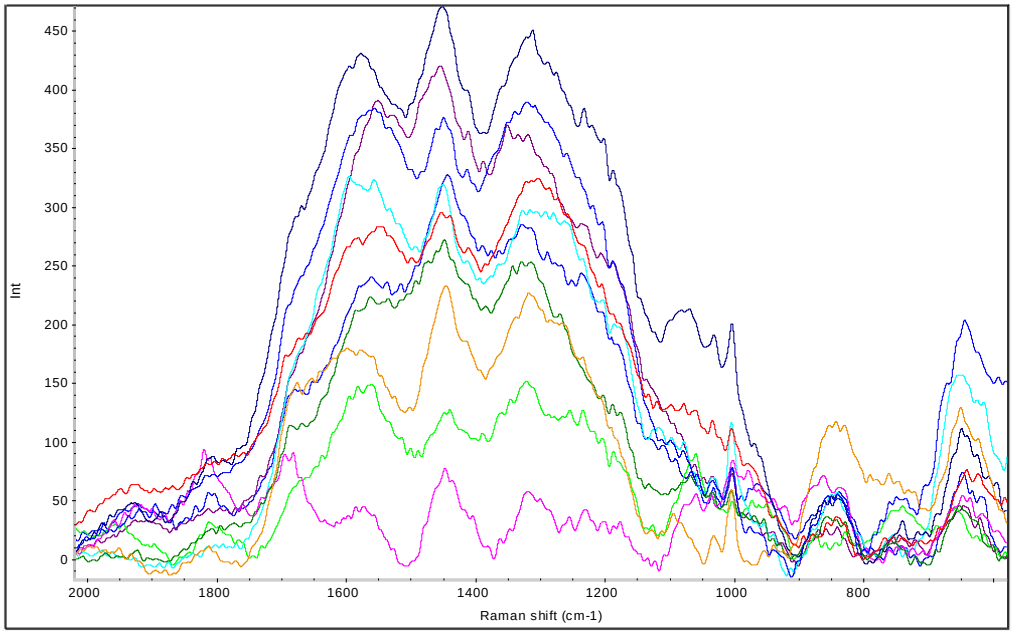
0mg WPI/mL with Spiked Hot Dog Matrix in 10mM & 5% BSA NaCl #1



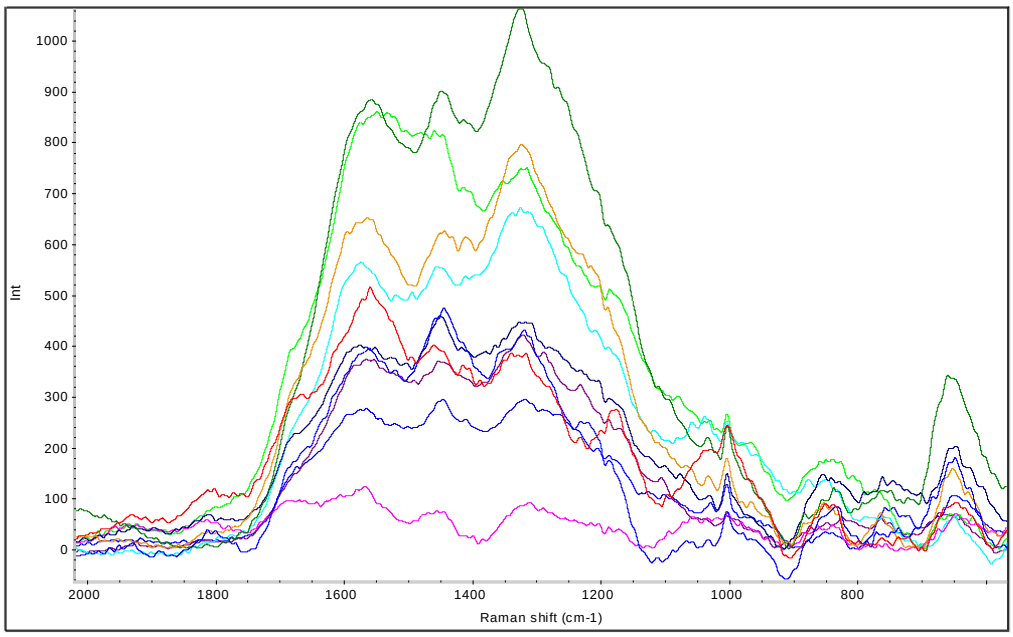
0mg WPI/mL with Spiked Hot Dog Matrix in 10mM & 5% BSA NaCl #2



1000mg WPI/mL with Spiked Hot Dog Matrix in 10mM & 5% BSA NaCl #1



1000mg WPI/mL with Spiked Hot Dog Matrix in 10mM & 5% BSA NaCl #2


Int
1000
900
800
700
600
500
400
300
200
100
0
2000
1800
1600
1400
1200
1000
800
Raman shift (cm-1)

Appendix C- Raman Specifications

Nicolet Almega XR Raman Spectrometer

Wavelength: 785nm

Aperture: 25μm Slit

Resolution: High

Estimated Spot Size: 0.5μm

Collect Exposure Time: 5 seconds

# of Exposures: 4 per replicate

# of Replicates per sample: 10