QUANTIFICATION OF FLOW AIDS IN SHREDDED CHEESE BLENDS USING ENZYMATIC STARCH ANALYSIS AND FOURIER TRANSFORM NEAR-INFRARED SPECTROSCOPY

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Andrew Joseph Zumbusch

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Dedication

I dedicate this master's thesis to my wife Jennie, and our three children Teagan, Mallory, and Archer. To my wife for believing I could do this long before I did and always supporting me in this very, very long journey. To my children, to show them that if you care about something, and you never let what anyone else says get in the way of your work ethic, you can make amazing things happen. I could not have done this without their love and support, or being able to come home to them at the end of the day.

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1 Introduction

In the production of shredded cheese, it may be necessary to use production aids (flow aids) to maintain product quality and shelf life. The two most common ingredients in flow aids are starch and cellulose. These additives are odorless and tasteless and do not adversely affect the flavor or texture of the product. Starch and cellulose can be used to increase product quality by preventing agglomeration of the product during storage and transportation. They are also used as carriers for antimycotics and oxygen scavengers to increase shelf life and prevent mold growth. When cheese goes through the shredding process, the exposed surface area is drastically increased. Greater surface area increases the likelihood of mold growth. To combat this, and increase shelf life, antimycotics and oxygen scavengers are incorporated into the flow aid. The most common antimycotic used by the cheese industry is natamycin. Natamycin is a naturally occurring antifungal compound that is produced by the bacteria *Streptomyces natalensis* during fermentation (Ollé Resa et al., 2014). It inhibits the growth of mold and yeasts on the surface of the cheese while allowing the bacteria in the cheese to continue to function. Glucose oxidase is incorporated to scavenge any remaining oxygen in the package, thereby preventing the growth of mold as it requires oxygen to grow (Bankar et al., 2009).

With these additions comes the need for controls. The cheese industry, and the dairy industry as a whole, has tightly controlled standards related to their products. These standard are in place to maintain a high product quality while maintaining an even playing field for the industry. The USDA has a limitation that states at no point may the quantity of flow aid exceed 2% of the total weight of the product (for Cheddar cheese), and flow aids must be kept to the minimum amount to achieve the desired effect (USDA, 2001). Quantification methods for starch and cellulose have been a standard in the grain industry for many years, but none have been tailored to work on dairy products, and there is currently no standardized method for quantifying these flow aids in cheese. The methods in current use in the grain industry are time

consuming wet chemistry methods. These methods involve multiple steps by trained personnel, and can take as long as a week to perform.

There is an ever-increasing demand for product quality improvements. This has led to the gradual transition away from time consuming analytical techniques (GC, HPLC, MS) and nonspecific control procedures (temperature, pressure, and pH) to more specific and environmentally compatible analytical tools. This increasing need for fast, cheap, and high-throughput methods of analysis has led to the increased application of infrared spectroscopy. In order to insure shelf life stability, and that the standards of the product are met, there needs to be a rapid method for quantifying the flow aid in the final product. In an attempt to shorten this time frame, a Fourier Transform Near-Infrared Spectroscopy (NIR) method was developed. Since the 1960's, near-infrared spectroscopy has been successfully employed in the rapid analysis of moisture, protein, and fat content for a variety of agricultural and food products (López et al., 2013). NIR technologies have increased in popularity due to its speed and accuracy, the lack of necessity for chemical extractions removing any environmental concerns, and its simple sample preparation requirements. NIR methods can be very rapid, with the total time to test a sample being less that ten minutes. An NIR is also a piece of equipment many food companies already have in their analytical laboratories, making the cost of anlysis very low. NIR has emerged as an extremely powerful tool for quality control and process monitoring.

In addition to the development of an NIR method to quantify flow aids, a wet chemistry method based on modification of the Megazyme Total Starch Assay was developed. The Megazyme total starch assay is a general method for analyzing the total starch content in a cereal, plant, or food products. Until now, this procedure has not been tested and optimize to be used for shredded cheese. This method does involve wet chemistry, but with proper tailoring it can be a more rapid and accurate

method then the ones currently available. Optimization of this method provides a valuable tool for the industry.

2 Literature Review

2.1 Cheese Industry

The cheese industry has continued to be a strength of the dairy industry. Cheese makers are adding peppers, herbs, and fruits to their product, as well as using the nutrition label and on-package massages to communicate about the natural, non-GMO, high protein aspects of cheese to attract younger consumers (Finkel, 2014). This is in response to a shift in consumer demand to a more natural product that also offers bold flavors, mainly driven by the emerging millennial generation (Finkel, 2014). Highlighting the protein content has shown to be the biggest potential to increase for growth for the industry, with a consumer report produced by the market research firm Mintel reporting 55% of consumers seeing cheese as an inexpensive source of protein (Finkel, 2014). This portion of the literature review will focus on the economics, cheese making process, and standards of the cheese industry. Specific focus will be paid to Cheddar cheese and the cheese industry in the U.S.

2.1.1 Economics

Despite the fact that consumption of fluid milk has declined recently, the number of total dairy products consumed has continued to grow (USDAERS, 2016). This is due to the continued growth in the cheese market, now accounting for 40% of the milk fat and 15% of the nonfat milk solids produced by dairy farms (USDAERS, 2016). In 2016, the United States produced over 12 billion pound of cheese, a 2% increase from the previous year and a 4.9% increase from 2014 (USDAERS, 2016). In 2013, total cheese production of natural cheese in the U.S. exceeded 11 billion pounds for the first time (Statista, 2016). By 2015, that number rose to 11. 8 billion pounds (USDAERS, 2016). This growth correlates with an increase in the per capita consumption of cheese, growing from 31.4

pound per person/year in 2005, to 35.0 pounds per person/year in 2015, an 11.5% increase (USDAERS, 2016). The Food and Agricultural Policy Research Institute (FAPRI) projects this growth to continue, and rise to 37 pounds per year by the year 2025 (Statista, 2016). However, not all sectors of the cheese market have experienced the same levels of growth. Natural cheese sales have continually grown 0.7% per year between 2009 and 2013, while over the same time period, processed cheese sales experienced a 3.8% drop (Finkel, 2014). On a global scale, U.S. cheese production is second only to the European Union (EU) (Statista, 2016). Cheddar cheese specifically, is the second most popular cheese in the United States in terms of consumption and production, only being surpassed by mozzarella (Statista, 2016, USDA, 2016). In 2015, Wisconsin alone, the U.S. largest cheese producing state, produced over 2.8 billion pounds of cheese, 600 million pounds of which were Cheddar cheese coming from 138 plants. (Statista, 2016, Wisconsin Milk Marketing Board, 2016) Cheddar cheese production accounted for 3.2 billion in 2013, and 3.4 billion pound in 2015 (Statista, 2016, USDA, 2016). Cheddar cheese per capita consumption in 2013 increased 1% from the previous year to 9.66 pounds (Statista, 2016).

In 2014, the value of the U.S. product shipments of cheese totaled $44.7 billion (Statista, 2016). Natural cheese accounted for $35.6 billion (Statista, 2016). FAPRI projects that cheese production will grow from just over 11 billion pounds in 2016, to over 13.3 billion pounds by 2025 (Statista, 2016). U.S. domestic use of cheese is projected to increase from just under 12 billion pounds (5.4 million metric tons) in 2016, to 14.3 billion pounds (6.5 million metric tons) by 2025 (Statista, 2016). This correlates with an increase in price per metric ton from $5,500 to $6,700 U.S. dollars (Statista, 2016). IDFA data shows that shredded cheese accounted for 550 million pounds in sales in 2014 (Statista, 2016). When combined with shredded fine and grated cheese, the other types of cheese that incorporate flow aids, it accounted for 1 billion pounds of sales (Statista, 2016). This translates into $5.2 billion in sales for 2014 (Statista, 2016). Cheddar cheese accounted for the highest volume of total sales with over 760 million

pounds, totaling $3.9 billion U.S. (Statista, 2016). With the continually increasing demand for cheese and the high market value, it is important for the industry to have robust analytical methods to control their product. This will insure consumer confidence stays high, and continued growth for the industry.

2.1.2 Cheese Making Process

Every cheese has unique nuances of production, but in general cheese is categorized as ripened, unripened soft, semi-hard, hard, or extra hard product produced from dairy ingredients where the whey protein/casein ratio does not exceed that of the starting dairy products (CODEX 283, 1978). Approved dairy ingredients include milk, skimmed milk, partly skimmed milk, cream, and whey cream or buttermilk, and may be used in any combination the cheese-maker see fit (CODEX 283, 1978). All cheeses are produced through the partial, or total coagulation of dairy proteins from dairy ingredients by enzymes (chymosin or other enzyme sources) and/or acid (CODEX 283, 1978). The whey that separates as a result of the coagulation is drained off, leaving the curd that contains a higher protein content than the initial milk (CODEX 283, 1978). Cheddar cheese is a specific type of hard cheese that is ripened and has to meet the general standards of cheese, as well as the standards for Cheddar cheese (FDA, 2016). The term ripened cheese refers to a cheese that has gone through the ageing process. Ageing involves being held at certain temperatures and conditions to promote the necessary biochemical and physical changes produced by the cheese cultures that characterize the specific type of cheese (CODEX 263, 1978). The process of making “Cheddared” curd cheese (Cheddar) begins with warming the milk ingredients and adding both lactic acid producing bacteria culture, and one or more of the approved clotting enzymes (FDA, 2016). After the dairy proteins are allowed to coagulate, they will form a mat of curds on the surface (FDA, 2016). When the mat has achieved the right consistency, it is cut and stirred while heating continues (FDA, 2016). This encourages the separation of the curd and whey before draining (FDA, 2016). The whey is then drained from the bottom of the tank, and the curd is matted into a slab (FDA, 2016). The slab is cut into sections and

piled on top of one another to encourage the remaining whey to drain, and for acidity to develop (FDA, 2016). Slabs are then cut into pieces, salted, stirred, and allowed to sit for a final drain (FDA, 2016). If the cheese-maker wants to remove any excess surface whey, they can rinse the pieces before salting (FDA, 2016). After salting and draining, the pieces are pressed into forms, and stored in a ripening room (FDA, 2016). After ripening, the color of the Cheddar can range from near white to a light yellow or orange color depending on if, and what colorant is added to the original milk (CODEX 263, 1966). It has a smooth and waxy texture that is firm when pressed.

2.1.3 Standards

Many cheese have a standard of identity that must be met to carry their label. In order to meet these standards, the composition of the cheese must be within the allowable limits set forth in the Code of Federal Regulations by the United States Food and Drug Administration (FDA). In order to meet with the standard of identity for Cheddar, cheese must contain a minimum milkfat content of 50 percent by weight of the solids, and the moisture content has a maximum allowable limit of 39 percent by weight (FDA, 2016). If the Cheddar cheese is made from raw, unpasteurized dairy ingredients, it must be cured at a temperature above 35^{o}F for no less than 60 days (FDA, 2016). Acceptable dairy ingredients include milk, nonfat milk, and cream, obtained from ether cow or buffalo (FDA, 2016). Clotting enzymes, such as rennet, can come from animal, plant, or microbial sources (FDA, 2016). Other optional ingredients that are acceptable to use and maintain the standard of identity of Cheddar are coloring, calcium chloride, enzymes, antimycotics, and hydrogen peroxide (FDA, 2016). The amount of colorant used is based on the type colorant. Annatto is the most common colorant used in Cheddar cheese and is only limited by good manufacturing practices (FDA, 2016). Calcium chloride can be used as an aid for coagulation, but must not exceed 0.02 percent of the weight of the dairy ingredients (FDA, 2016). Antimycotics may only be applied to the surface of the cheese, but only in the case when it has been sliced or grated for consumer consumption (FDA, 2016). Hydrogen peroxide must not exceed 0.05 percent of the weight of the milk, and a

sufficient amount of catalase must follow to eliminate the hydrogen peroxide, but it must not exceed 20 parts per million of the weigh to the treated milk (FDA, 2016).

2.2 Processing Aids in Shredded Cheese

In the manufacturing of shredded cheese, processing aids (flow aids) are often used to retain shelf life and product quality. The four most common processing aids are starch, cellulose, natamycin, and glucose oxidase. Starch and cellulose are naturally derived from plant material and are commonly added to shredded cheeses as a flow aid. This prevents the cheese from sticking to production equipment, and prevents the shreds from matting together in the package during storage and transportation. These are important properties to maintain, as the USDA standards for shredded Cheddar cheese state that "Shredded Cheddar cheese shall be free flowing and shall not be matted" (USDA, 2001). The use of an anticaking agent is regulated and must be kept to the minimum amount to achieve the desired effect, and at no point can the content exceed 2% of the weight of the final product (for Cheddar) (USDA, 2001). Starch and cellulose also serve as carriers for antimycotics and oxygen scavengers, as one of the biggest and most important problems concerning shelf life and product safety is mold development (Ture et al., 2011). The most common antimycotic used in the cheese industry is natamycin, while glucose oxidase is the most common oxygen scavenger. In the following section we will discuss what each of these processing aid are, how they are used in the industry, and regulations in place to insure the high quality standard of the cheese industry.

2.2.1 Starch, Cellulose, Natamycin, and Glucose Oxidase

Starch

Starch granules are the principal energy storage unit of plants and is mainly found in tubers, roots and seeds (Pérez, 2009). The two major macromolecular components of starch are amylose and amylopectin, with amylopectin, one of the largest polymers known, being the majority (Pérez, 2009). In normal starches used in the food industry,

such as potato, corn, wheat, and rice, the ratio is between 70-80% amylopectin, to 20-30% amylose (Jane, 2009). Both amylose and amylopectin are made up of D-glucose units, but they differ in their branching. Amylose consists of mainly linear α(1→4)-linked glucose units and can have a degree of polymerization (DP) up to approximately 600 sugars (Pérez, 2009). Amylopectin has the same linear α(1→4)-linked glucose, but, in addition, has approximately 5% α(1→6) branch points, creating significant physical and biological differences between the two (Pérez, 2009). An example of these differences is in the way they react during film and gel formation. Amylose tends to retrograde and create strong films and tough gels, while amylopectin is more stable in an aqueous dispersion, and creating weaker films and softer gels (Jane, 2009).

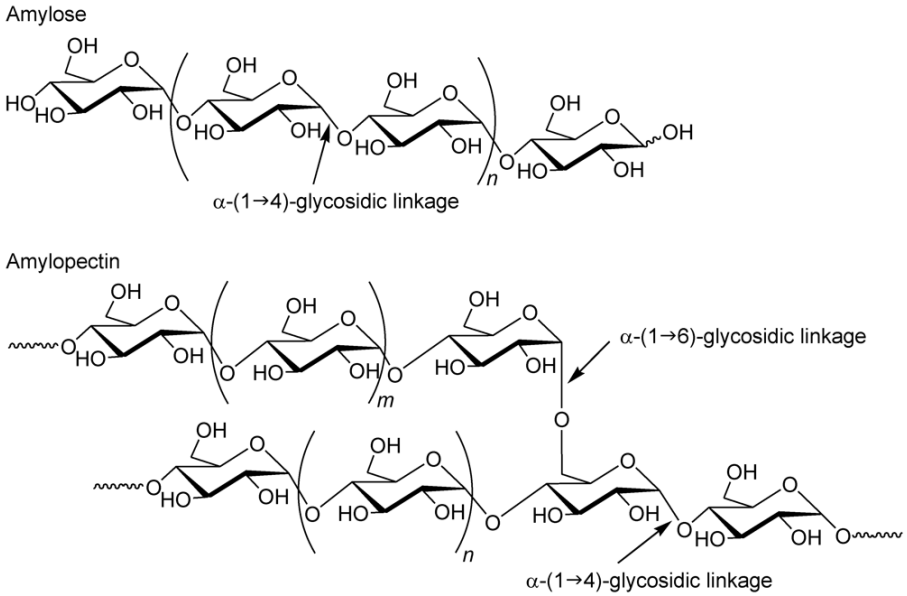

Figure 1: Structural configuration of amylose and amylopectin (Yang, 2016)

Starch can be easily isolated from plant matter in its granular form by sedimentation through centrifugation of an aqueous extraction and filtration (Jane, 2009). Through subsequent washing and processing starch can be physically, chemically, and enzymatically modified to meet industry needs (Jane, 2009). In 1995, 950,000 metric tons of starch were using in the food industry (Mason, 2009). For applications in food, starch has a wide variety of uses. They include thickening, gelling, emulsification, mouthfeel

enhancement, and extending or replacing more expensive ingredients (Mason, 2009). However for the purpose of this literature review, the most important aspects in its use in the food industry are as a flow aid.

Cellulose

Cellulose is present in the cell walls of plants, and is the key structuring element, adding rigidity and strength (Zugenmaier, 2008, Krawczyk et al., 2009). It is a naturally occurring polysaccharide consisting of glucose units, however the repeating unit is two consecutive glucose anhydride units known as cellobiose units (Krawczyk et al., 2009). The glucose units are linked through a β(1→4) glycosidic linkages and degree of polymerization can range from 50 to 3500 units (Krawczyk et al., 2009).

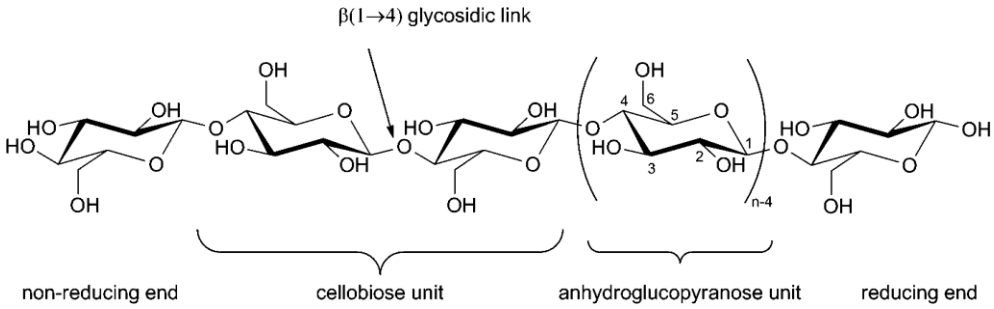

Figure 2: Structural configuration of Cellulose (Eyley et al. 2014)

The main source of cellulose is from wood pulp (Krawczyk et al., 2009). In order to be usable as a flow aid, it must go through several processing steps and be converted into microcrystalline cellulose. First the wood pulp goes through a pulping process to remove lignin, other polysaccharides, low molecular weight cellulosic material, and extractive, leaving high molecular weight cellulose fiber (Krawczyk et al., 2009). To remove the amorphous cellulose portions, hydrolysis with a strong mineral acid is used to leave cellulose crystals (Krawczyk et al., 2009). After neutralization, washing and filtering, the purified microcrystalline cellulose is diluted with water and spray-dried to produce microcrystalline cellulose (Krawczyk et al., 2009).

Microcrystalline cellulose has many advantages in the food industry. It has been in use for over 30 years and has earned GRAS status from the FDA (Krawczyk et al., 2009). It is a non-digestible, insoluble fiber that adds no caloric value to a food product (Krawczyk et al., 2009). Research has shown that addition of dietary fibers to a product can have probiotic effects and help with intestinal regularity and weight management (Nsor-Atindana et al., 2017). It has been used as a stabilizer in emulsion, suspensions, and foams (Nsor-Atindana et al., 2017). It is stable at high temperatures, as well as low pH products (Krawczyk et al., 2009). It has been used as a fat replacer in meat and baked products (Nsor-Atindana et al., 2017). It can also be used as the wall material for encapsulation and as an edible film on fresh produce (Nsor-Atindana et al., 2017). For the benefit of a flow aid agent, microcrystalline powder is odorless and tasteless so it does not affect the flavor of the cheese (Krawczyk et al., 2009). The powder itself is white, so in large concentrations in a cheese with color, like Cheddar, it can be visible on the shreds.

Natamycin

A major concern in the dairy industry is spoilage during refrigerated storage. This is a major issue due to the economic loss suffered from spoilage and health concerns associated. The most common reason is fungal spoilage (Ollé Resa et al., 2015). *Penicillium* species are the most prevalent type of spoilage fungi, with *Penicillium commune* being one of the most common (Cheong et al., 2014). *Saccharomyces cerevisiae* is one of the most common spoilage yeasts, however yeast spoilage is less common (Ollé Resa et al., 2015). Contamination from these species will reduce shelf life by causing undesirable flavors and will alter the visual appearance of the product (Ollé Resa et al., 2015). There is also the danger of mycotoxins release during the growth of mold (Cheong et al., 2014). It is possible to prevent the growth of pathogenic and spoilage microorganisms and extend the safety and shelf life of dairy product through the addition of antimicrobial agents (Franssen et al., 2004).

Natamycin, also known as pimaricin, is a polyene antifungal produced by the aerobic fermentation of *Streptomyces natalensis* that prevents the growth of yeasts and molds (De Oliveira et al., 2007, Ollé Resa et al., 2014, Mattia et al., 2002). It is effective against almost all mold and yeast types even at low concentrations (Stark et al., 2003). A key to the mechanics of its functionality is in the difference between eukaryotic and prokaryotic organism. Eukaryotic organisms have sterols imbedded in the cell membranes, whereas prokaryotic organisms do not (Stark et al., 2003). The sterols are important for the organization of the membrane and are required for the cell to function properly (Ollé Resa et al., 2014). In molds and yeasts the major sterol in ergosterol (Stark et al., 2003). Natamycin binds to the ergosterol and disrupts the cell membrane causing the cell to lyse (Stark et al., 2003). The specificity of binding allows it to function against yeasts and molds, while not effecting bacterial cultures (Ollé Resa et al., 2014). Natamycin's activity against yeasts and molds, but not bacteria, makes it a useful tool in foods that require ripening (Mattia et al., 2002) In addition, like starch and cellulose, natamycin is a white powder with little to no taste or odor, so the treated product will not be negatively affected (Stark et al., 2003).

To collect natamycin, *Streptomyces natalensis* is allowed to go through fermentation for several days, and the natamycin is isolated through a broth extraction (Mattia et al., 2002). Natamycin is an approved food additive in over 40 countries, and has received GRAS status with the FDA for use in cheese, yogurt, and tea and fruit beverages (Ollé Resa et al., 2014). Natamycin is incorporated into starch and cellulose based flow aids and is applied to the surface of the cheese. Incorporation into flow aid does not affect the efficiency of natamycin (Ollé Resa et al., 2014). Incorporation into the flow aid also allows the natamycin to stay on the surface of cheese. Natamycin has shown very little diffusion when applied to the cheese surface, allowing it to act only on the surface organisms, and not interfere with the beneficial bacterial growth occurring within the product (De Oliveira et al., 2007).

There has been no evidence of fungi developing a resistance for natamycin, even after several years of use (De Oliveira et al., 2007). In one laboratory experiment, researchers collected yeast and mold strains from cheese warehouses and attempted to induce resistance to natamycin (Mattia et al., 2002). The isolated strains were transferred 25-30 times to media with increasing concentrations of natamycin (Mattia et al., 2002). None of the isolated strains became less sensitive to the effects of natamycin (Mattia et al., 2002). In another study, 26 fungi collected from eight warehouses where natamycin was used, and 2 warehouses where natamycin was not used, were tested and insensitivity to natamycin was not found (Mattia et al., 2002).

Glucose Oxidase

Glucose oxidase is an enzyme produced from the fermentation of the fungi *Aspergillus*, *Penicillium*, and *A. niger* (Bankar et al., 2009). All produce strains of glucose oxidase, with *A. niger* being the most commonly used in industry (Bankar et al., 2009). Glucose oxidase has proven to be a valuable tool in many industries. While it has been in industrial use since the 1950's, its applications in the last few years has grown in the chemical, pharmaceutical, clinical chemistry, biotechnologies, and the food and beverage industry (Bankar et al., 2009). As far as its use in the food industry, it has many applications, but one of the most important uses is as a preservative (Wong et al., 2008). The most important from the aspect of the application on shredded cheese, is its ability to consume oxygen. This is done by glucose oxidase catalyzing the reaction of one glucose molecule and two oxygen molecules to produce gluconic acid and hydrogen peroxide as end products, as shown in Figure 3 (Wong et al., 2008).

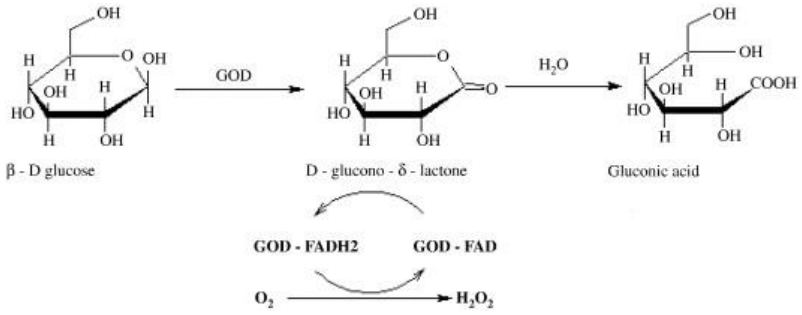

Figure 3: Oxidation of D-glucose by glucose oxidase (Banker et al., 2009)

This is the reason it is common to add D-glucose to processing aids containing glucose oxidase. The gluconic acid that is produced from this reaction is safe for human consumption and the World Heat Organization (WHO) has not set any limitation of its daily acceptable intake (Wong et al., 2008). The other product of this reaction is hydrogen peroxide (Bankar et al., 2009). Hydrogen peroxide exhibits anti-fungal and anti-bacterial properties, which can help further prevent spoilage of the product (Wong et al., 2008). An additional benefit to used glucose oxidase is it has received GRAS status from the FDA (Wong et al., 2008). With its GRAS status and its function as an oxygen scavenger, and the consumer demands to replace chemical food additives with more natural ones, glucose oxidase has become an ideal choice of oxygen scavenger for the cheese industry.

2.2.2 Industry Standards

To maintain the standards of identity of cheese, certain restrictions have been placed on the levels of additive allowed. When starch and cellulose are used as anticaking agents, they must be kept below certain levels. In the standards for Cheddar cheese, the USDA indicates the use of an anticaking agent must be kept at a minimum, and only as much as needed to achieve the desired effect is allowable, with an additional restriction that the amount must remain below 2.0 percent of the total weight of the product (USDA, 2001). However, the CODEX standards for Cheddar cheese states the amount of anticaking agent used is only limited by good manufacturing practices (CODEX 263,

1966). For natamycin, the concentration in the final product should be below 1mg/dm^2, and should not be detectable below a 2 mm depth from the surface of the cheese (CODEX 268, 1966, De Oliveira et al., 2007). There are currently no limitations for glucose oxidase addition in cheese by the FDA, USDA, or the CODEX standard.

2.2.3 Adulteration in the Industry

In recent years, the adulteration of grated and shredded cheese products with an overuse or undeclared use of starch and cellulose has made news headlines. This issue has been more pronounced in the hard Italian cheese market, specifically Parmesan. An article in Food Quality & Safety magazine stated that some Parmesans were found to be between 20 and 40 percent starch/cellulose (Schuman, 2015). Another article from Bloomberg News states that cheese labeled as 100% Parmesan contained as much as 8.8 percent cellulose (Mulvany, 2016). However, it needs to be noted that there are no stated methods of analysis on how these percentages were derived. It should also be noted that the FDA has only sent one warning letter for adulterated and mislabeled Parmesan to Castle Cheese in 2013 (FDA, 2013). Upon inspection of the Castle Cheese facility, it was found that cheese labeled as 100% Romano and 100% Parmesan were composed of various other types of cheeses and contained starch and/or cellulose to increase the weight of the product (FDA, 2013). In 2016, Michelle Myrter, president of Castle Cheese, plead guilty to fraud as was sentenced to three years' probation, as well as a $5,000 fine and 200 hours of community service to be completed at a county food bank (Mulvany and Skerritt, 2016). Data is not yet available on if news articles pertaining to adulteration have had any effect on sales or consumer confidence in the cheese industry.

2.3 Near-Infrared Spectroscopy (NIR)

Over the last 20 years, major technical advances have made spectroscopy equipment smaller, easier to use, and more user friendly (Chalmers et al., 2011). The development of the Fourier transform in the 1700s, and the addition of the interferometer that improved

the mathematical transformation was one of the most historically important events in the development of spectroscopy (Marchi et al., 2014). It was not until the 1980 that Fourier-transform infrared spectrophotometers were integrated into personal computers and became widely known as a versatile and cost effective method of analysis (Marchi et al., 2014). Near-infrared spectroscopy (NIR) is a technology that has continued to gain more traction over the last decade in the food industry. Its popularity has continued to grow due to its unusually fast speed, its non-destructive nature, and the fact it can require little to no sample preparation for analysis (Burns and Ciurczak, 2008). It is a remarkably versatile tool that can analyze almost any sample containing C-H, N-H, or O-H, and will yield acceptable results as long as the concentration of the analyte is above 0.1% of the total composition (Burns and Ciurczak, 2008). While NIR was originally used primarily for grain and grain products, applications for NIR analysis rapidly expanded in the 1980's and applications have now been developed and are in use in agricultural products, plastics, pharmaceuticals, petrochemicals, textiles, the beverage industry, and most importantly for the aspects of this literature review, dairy product (Williams, 2008). In order to better understand its application to the food industry, it is important to understand the principles of how NIR functions.

For the purposes of this literature review, information in the NIR section is approached from two perspectives. General information on the function and mechanics of NIR will be from a broad perspective. Detailed information will be based on the specific instrument and software used during calibration, the Buchi NIRFlex N-500 FT-NIR spectrometer with the associated NIRCal 5.2 Chemometric software.

2.3.1 Instrumentation

Before going into the theory of spectroscopy, it is important to have a basic understanding of the mechanics of the NIR equipment. The basics for all NIR instrumentation is the same. The instrument projects a beam of near-infrared light into the sample from a source of radiation where the beam and sample interact (Lewis et al.,

2007). After interaction with the sample, the reflected radiation is directed to a sensitive detector and a spectra is produced (Lewis et al., 2007). Each equipment manufacturer has their own design to optimize their instrument. In the case of the Büich N-500 NIRFlex, the internal design has been set up as shown below in Figure 4.

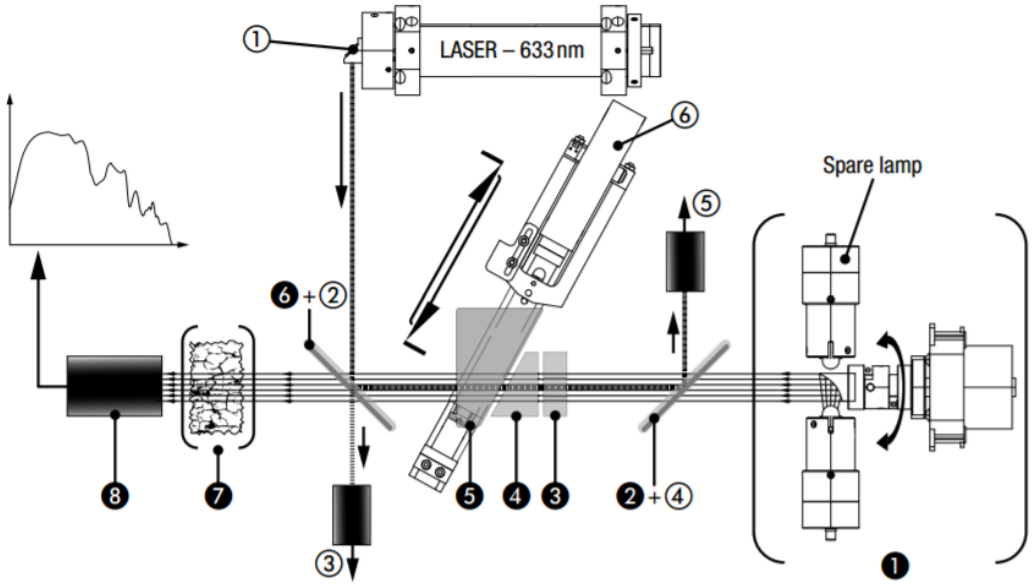

Figure 4: Internal components of the Büchi NIRFlex N-500 Fourier Transform Near-Infrared Spectrometer (Büchi, 2007)

The instrument works as follows, as described in the Buich NIRFlex N-500 Technical data sheet. The light source ❶ generates an undefined polarized light. The light passes through the polarizer ❷, were it becomes a well-defined polarization output. The light is then broken down into two orthogonally polarized beams with a small, static phase shift by passing through the double refracting block ❸. The light then passes through two refracting wedges ❹ and ❺. Wedge ❹ is a stationary, while ❺ continuously shifted back and forth by a linear-drive shaft. These wedges change the thickness in the light path, creating ongoing phase shift between the light beams. Another polarizer ❻ converts the beams into a single light output with intensity variations. The infrared beam then interacts with the sample ❼ where it is refracted and picked up by the detector ❽. The portion of the diagram with the white numbered circles shows the internal reference laser system. It

ensures proper functioning of the internal components, but is not critical to understanding the workings of the instrument. The light beam can interact with the sample in a variety of ways to match the optical properties of the sample. In this instance the beam interacts with the sample by diffuse reflectance, as shown in Figure 5a. Diffuse reflectance is used when scanning non-translucent solids where light penetration is limited. The light beam will interact with the sample, be refracted and diffusely reflected back to the sensor.

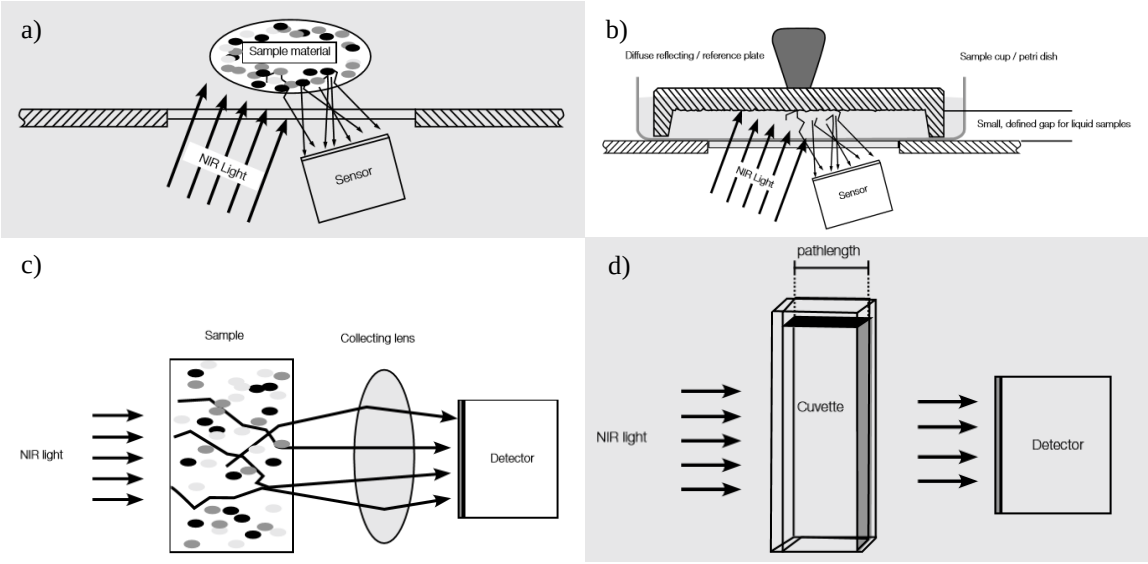

Figure 5: Measurement setups for NIR for samples with different optical properties. a) Diffuse reflectance; b) Transflectance; c) Diffuse transmission; d) Transmission (Büchi, 2007)

Other measurement modes include transflectance, Figure 5b, diffuse transmission, Figure 5c, and transmission, Figure 5d. Transflectance and transmission are better suited for liquid samples. Diffuse transmission is used for light conducting materials such as tablets and crystal powders. Each measurement mode comes with its own auxiliary equipment attachments.

2.3.2 Spectroscopy methods

There are several different techniques used in spectroscopy, but the most important ones are Raman, Mid-Infrared (MIR), and Near-Infrared (NIR). Commercial Raman, NIR

and MIR equipment differ widely in their application, configuration, and performance, with each showing strengths in different application (Chalmers et al., 2011). All three were developed off the same basic principal of analyzing the molecular vibrations of a chemical compound, but vary in several aspects of how they function, as shown in Figure 6 (Burns and Ciurczak, 2008). Raman spectroscopy uses a scattering technique, while MIR and NIR are based on the absorption of radiation (Siesler, 2008). Each system offers their own advantages and disadvantages. Infrared analysis can be hampered by the presence of water or hydrated chemical species (Chalmers et al., 2011). The limited ability to read low wavenumbers, below 400 cm^{-1} can limit the characterization of drug polymorphs and heavy metal inorganics (Chalmers et al., 2011). Raman spectroscopy is dependent on molecular polarizability, unlike infrared that realize on dipole moments (Chalmers et at., 2011). This means polar groups like OH and C=O are more effectively analyzed through infrared spectroscopy than Raman (Chalmers et al., 2011). However homopolar bonds such as C=C and N=N are more effectively analyzed using Raman spectroscopy (Chalmers et al., 2011). All three of these techniques have gained acceptance in a variety of industries, and with the continued development of more powerful software, there applications continue to grow. This portion of the literature will focus on the comparison of these three vibrational spectroscopy methods.

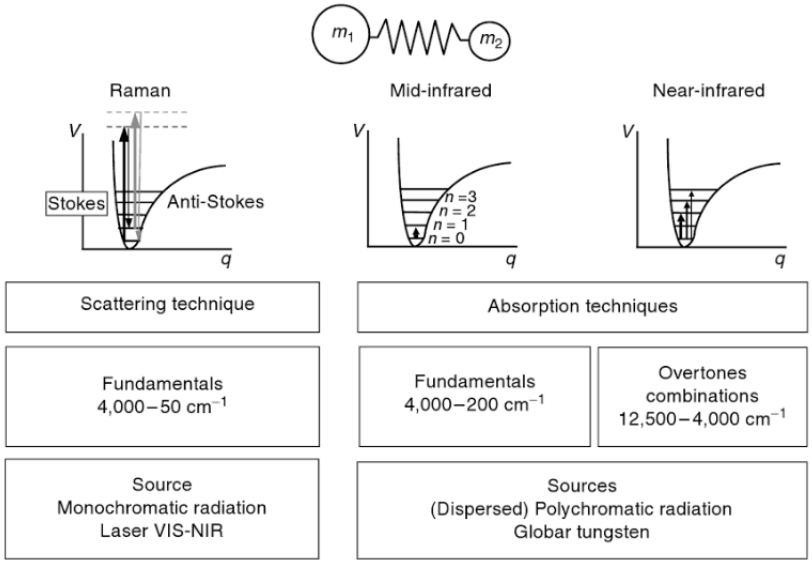

Figure 6: Raman, Mid-infrared, and Near-infrared comparison (Siesler, 2008)

Raman

The basic mechanics of a Raman spectrometer are similar to that of other spectrophotometers include a light source, monochromator, sample holder, and detector. Light sources that create the excitation can vary depending on application, but typical sources are krypton ion, helium/neon, argon ion, and diode lasers (Rostron, 2016). Raman spectroscopy measures the frequency shift of inelastic scattered light when the photon from the incident light hits the molecules in the sample, shifting the energy of the outgoing photon, creating a scattered photon with a different wavelength (Rostron, 2016, Long, 1977). This change in the molecular polarizability is a result of the displacement of atoms in the compound from their equilibrium position, and is proportional to the magnitude of the change in polarization (Rostron, 2016). There are two types of Raman scattering, stokes and anti-stokes (Larkin, 2011). Stokes Raman scattering occurs when the out coming scattered light from the sample, whether from reflectance or transmittance, has a lower frequency than the original photon (Rostron, 2016). When the out coming light has higher frequency than the original photon, it is anti-Stokes Raman scattering (Rostron, 2016). The type of scattering can be explained through the transfer of

energy between the incident radiation, and the scattering system (Long, 1977). When an electron in the system is transferred from a lower energy state, to a higher energy state, the necessary energy must be acquired for the incident radiation (Long, 1977). This causes the returning photon to be at a lower frequency, and is defined as Stokes Raman scattering. Conversely, if the system is already in a higher energy state, there is available energy for the incident radiation to incorporate and a higher energy photon returns to the instrument (Long, 1977). In this case, it produces anti-Stokes Raman scattering. These frequency changes create a shift in the wavelength of the scatted light in response to the chemical composition of the molecules in the sample and can be detected by the instrument (Rostron, 2016). The spectrum obtained can be read as a fingerprint of different compounds, and can be used to qualitatively analyze a mixture of compounds, or identify an unknown sample (Rostron, 2016).

Mid and Near-Infrared

Mid and near-infrared spectroscopy have the advantage of having a lower limit of detection than Raman (Chalmers et al., 2011). Both work off the same principal of transmitting electromagnetic radiation into a sample, and measuring the amount of energy absorbed (López et al., 2013, Marchi et al., 2014). When a sample is crossed by the electromagnetic radiation, the bonds in the sample that are active in the infrared range create vibrational, or rotational movements marked as an absorption in the corresponding spectra (Chalmers et al., 2011, Marchi et al., 2014). The interactions between the sample and energy follows Beer-Lambert's Law, where the "absorbance at any wavelength is proportional to the number or concentration of absorbing molecules present in the path of the radiation" (Lopez et al., 2013). These infrared active vibration are shown as absorptions bands in the mid and near-infrared wavelength region of 2.5-25.0 μm and 780-2500 nm respectively (Chalmers et al., 2011). Most of the molecular vibration will take place at a specific characteristic frequency between 10^{12} to 10^{14} Hz, where they interact with light at the same frequency (Büchi, 2016). The wavelength, λ, is related to the frequency, ν, and the speed of light, c, in a vacuum by:

$$\lambda = \frac{c}{\nu}$$

However, most spectra recorded and displayed currently are represented in abscissa linear wavenumber format where the unit is a reciprocal centimeter, cm^{-1}, ranging from 400 to 4000 cm^{-1} for mid and 12800 to 4000 cm^{-1} for near-infrared (Chalmers et al., 2011). The wavenumber, $\tilde{\nu}$, represents the number of waves in a unit length (Chalmers et al., 2011).

$$\lambda = \frac{c}{\nu} = \frac{1}{\tilde{\nu}}$$

Electromagnetic radiation comprises different regions according to the following wavelengths: x-ray 0.5-10 nm, UV 10-350 nm, visible 350-800 nm, near-infrared 800-2500 nm, mid-infrared 2500-25000 nm, microwave 100 μm-1 cm, and radio frequency 1 cm-1 m, as shown in Figure 7 (Marchi et al., 2014). Through the use of the Fourier transform mathematical treatment, and the known supplied energy and amount absorbed by the sample, the chemical composition of the sample can be determined (Marchi et al., 2014).

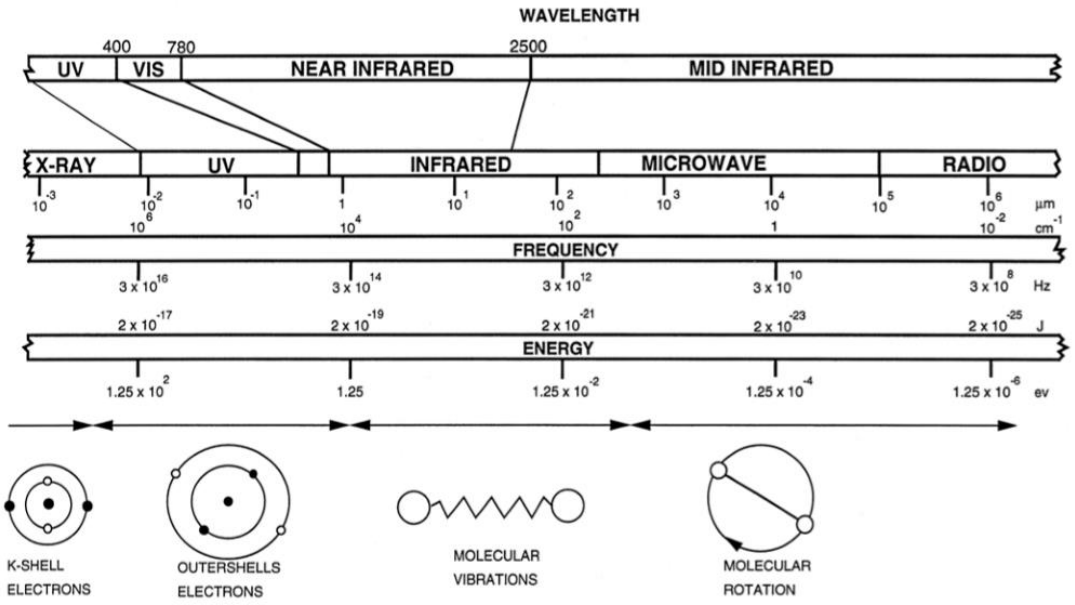

Figure 7: Summary of the electromagnetic spectrum (De Marchi et al., 2014)

Near Infrared

NIR measures some of the fundamental vibrations through measurements of the broad overtone and combination bands, making it an excellent technique for accurate, and rapid quantification (Larkin, 2011). As stated above, NIR measures in the region of the electromagnetic spectrum between 800 and 2500 nm, or between 4000 and 12800 cm^{-1} (López et al., 2013, Marchi et al., 2014). This region is characterized by overtones and combinations bands that are indicative of CH, NH, and OH bonds in the analyte, making it mainly suitable for organic compounds (López et al., 2013, Büchi, 2007). An advantage to working in this range is the relatively low degree of absorbance of these overtones and combinations, giving a high degree of reflectance in solids (Büchi, 2007). The concentration of the substance being detected can be as low as 0.1% of the total composition of the sample, and still yield accurate results (Burns and Ciurczak, 2008, Büchi, 2007).

NIR is considered one of the most advanced nondestructive quality assessment techniques (López et al., 2013). However, one of the biggest hurdles to overcome in the

establishment of an NIR calibration is that the initial phase of development of the calibration depends on the reference method (López et al., 2013). In order to develop a calibration, a mathematical relationship needs to be established between the spectral and reference data (López et al., 2013). In order for this to be true, time must be spent on the preliminary work, where the instrument is "taught" what is important when analyzing the samples (Burns and Ciurczak, 2008). A minimum of 40 calibration samples are required for an adequate calibration (Frankhuizen, 2008). With the establishment of a quality calibration, the time spent developing is well worth it.

2.3.3 Chemometrics

"Chemometrics is the science of relation measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods." [International Chemometrics Society]

Spectra obtained from NIR analysis are characterized by multiple broad oscillating peaks, making visual evaluation virtually impossible (Büchi, 2007). When comparing differences in spectra of similar make up, only small shifts and changes are detectable in the wide absorption bands. Analysis of these small changes must take place through the mathematical model of the chemometrics software. Most samples have a heterogeneous chemical nature, requiring specific data analysis of the absorption bands relating the electromagnetic information collected in the form of spectra, with the information on chemical composition obtained from reference methods (Lopez et al., 2013). Chemometric software uses a series of mathematical and statistical procedures to analyze and interpret the large amount of data obtained from the spectra and finds statistical correlations between the data, and the known property values of the samples used in the calibration (Büchi, 2007). If the correlations found by the software are found to by systematic, it will be able to predict the desired values of an unknown sample through the same evaluation of the unknown samples spectra (Büchi, 2007). To obtain the maximum benefit, several hundred intensity values in the spectra region are incorporated into the calibration, and principle component analysis (PCA) is applied (Büchi, 2007).

Calibration methods

Calibration methods can be broken down into qualitative and quantitative methods. Qualitative calibration allows for the identification of differences in substances, or the same materials of different quality through PCA (Büchi, 2007). The two primary methods for qualitative calibrations are cluster, and soft independent modeling of class analogies (SIMCA) (Mark, 2008). The cluster calibration is a useful solution when a sample needs to be assigned to a specific category (e.g. check raw materials to confirm their identity and quality). This can be accomplished through scanning samples that cover the full range of allowable variation of the quality product. To cover the full range, several samples from at least five to fifteen independent batches produced over a range of at least six months must be incorporated (Büchi, 2007). All samples being incorporated into the calibration need to be tested with reference analysis methods to ensure accuracy (Büchi, 2007). The calibration will recognize well separated tolerance regions (clusters) where each one represents only one of the possible properties (Büchi, 2007). These clusters are created through the selected secondary principle components (PC) that show a clear separation of the substances and their scores (see principle component section) (Büchi, 2007).The major differences in the two methods is that the cluster method is used for a group of similar substances, while in SIMCA, a calculation is performed for each substance individually (Büchi, 2007). SIMCA is a relatively new method of analysis that incorporates a combination of method that have been utilized by calibration software for a while (Mark, 2008). SIMCA uses a principal component analysis for each substance or property, however each calibration can contain only one substance (Büchi, 2007). This method is primarily used for the identification of a substance.

Quantitative calibrations generally use three different calculation procedures either individually or in combination: multiple linear regression (MLR), principal component regression (PCR), and partial least squares regression (PLS) (Büchi, 2007). These three techniques establish a mathematical relationship between the information gathered in the spectra, and the quantity of the component measured (Lopez et al., 2013). MLR is an

extension of linear regression and is based off a few selected wavelengths (Büchi, 2007). Principal components are not use in this calibration, and the properties are calculated with intensity values and correlation coefficients in the selected wavelengths (Büchi, 2007). With these limitations, this is not a recommended method, and is only suggested for filter instruments (Büchi, 2007). To make use of the full wavelength, PCR and PLS are the preferred methods. PCR is a principle component analysis followed by a MLR. This is a two-step method with first scoring the principle components, and then MLR using the scores and property values from the principle component analysis (Büchi, 2007). With the principle components and MLR taking place in separate steps, any number of parameters can be simultaneously included in the calibration (Büchi, 2007). However, this also means that the principal components of the desired property are not necessarily the ones making the biggest spectral variation (Büchi, 2007). Work has continued to improve the prediction accuracy of PCR calibration by incorporating more of the variations present in the sample set (Brown et al., 1994). PLS, on the other hand, calculates the principle components with spectral information and property values being taken into account simultaneously (Büchi, 2007). With the quantitative reference values being taken to account from the beginning of the calibration, PLS is considered a more up to date method than PCR. This also allow PLS to use the PC with the highest correlation to the changing variable as the first PC, shifting the first focus from the most dominate dimension, to the most relevant dimension (Büchi, 2007). PLS has become the preferred calibration for quantitative analysis for its robustness and its ability to not be effected by outliers (Brown et al., 1994). All of these methods can be tested through the use of independent validation samples, or by using cross validation with calibration samples. Cross validation is not as robust as independent validation and should only be used when the sample set is smaller than 50 (Büchi, 2007). In order to obtain the best results for a calibration, the wavelength selection, pretreatments of data, and optimal number of principle components need to be adequately chosen (Büchi, 2007).

Wavelengths

An advantage to running a calibration based on principle components is there is no need to run a wavelength search to select the proper wavelengths (Mark, 2008). Wavelength selection can be difficult due to random noise effecting the spectra, as well as adjacent wavelengths being equivalent in their importance (Mark, 2008). Through the use of PC, this issue can be alleviated by using the whole range of wavelengths, despite the number of PC selected (Mark, 2008). The range of wavelength or wavenumber depends of the instrument type and its resolution. In this instance, the recommended range of the NIRFlex N-500 for solids and liquids is 4,000-10,000 cm^{-1} (Büchi, 2007). Ideally, the range of wavelengths should be as wide as possible, as the selected wavelengths for calibration defines the spectrum range used by the mathematical algorithm of the software (Büchi, 2007). Although it is important to keep the wavelength range as wide as possible, it is also important to remove any section of the wavelengths that is only adding noise to the calibration. Removal of these sections can lead to improvements in the calibration (Büchi, 2007).

Pretreatments

Pretreatments are used to control for variations of chemical and physical properties of the samples, and changes at the spectrometer that can affect the spectrum (Büchi, 2007). Some of these property differences can be due to differences in particle size or other variations that are not of interest. This can be a particular problem when analyzing food products due to their complex matrix and natural variations. Data pretreatments can help eliminate false differences, from variations that occur during sample preparation and presentation (Büchi, 2007). While the pretreatments do not affect the original spectra, it can help overcome these issues by improving the quality of the spectra through mathematical transformations (Büchi, 2007). In the NIRCal chemometric software, there are 34 different pretreatment options available in 6 categories: normalization, offset, smoothing, derivatives, transformation, and filters (Büchi, 2007). Each of these

pretreatments can be combined with one another, and in different orders, resulting in an almost limitless number of combination. Each pretreatment group has a particular aim and use. Normalization is use to reduce baseline variations that can be caused by particle size effects and pressure differences (Büchi, 2007). Offset is used to make baseline corrections that are caused by light scattering (Büchi, 2007). This is seen as deviation in the reflectance (Y-axis) due to sample variability. Smoothing is used to reduce the noise levels in the spectra, but this will cause a loss in resolution, and can result in a loss of spectral information (Büchi, 2007). Derivatives reduce the baseline effects and increase small absorption peaks (Büchi, 2007). It is often combined with smoothing to minimize any loss in signal-to-noise that occurs (Büchi, 2007). Transformation is a pretreatment that is more often applied to liquids than solids and can be applied to modify the adsorption peaks (Büchi, 2007). A linear filter is a useful pretreatment when spectra have been recorded with different resolutions, such as spectra recorded from different instruments (Büchi, 2007). Pretreatments can be a powerful tool in developing a quality calibration, however, they need to be used cautiously, as over use will cause the data to become distorted, making it unusable.

Principle Components

The definition of a principal component is hard to nail down. The issue comes from separating principle components and principle component analysis. Generally, principle components can be thought of as the components of the data that, after applying mathematical techniques, account for the largest amount variation in the data (Mark, 2008). The American Society for Testing and Materials has not adopted any definition for principle components (Mark, 2008). With this in mind, principle components must be looked at as the software of choice defines them. In the case of the Büchi NIRCal chemometric software, PC are classified into two groups, primary and secondary PC (Büchi, 2007). Each classification serves a different function for the calibration. Primary PC are used for reconstructing the spectra after pretreatments and mathematical measurements have been applied (Büchi, 2007). When the optimal number of primary PC

have been selected, almost all noise will be disregarded during the analysis of the spectra (Büchi, 2007). If too many primary PC are selected, noise will be interpreted as critical data and the calibration will be overfit (Büchi, 2007). As a consequence, the calibration will only identify the calibration spectra correctly, while validation samples will be miss interpreted due to the incorporation of noise (Büchi, 2007). On the other hand, if too few primary PC are selected, the calibration not being selective enough and the calibration will be underfit (Büchi, 2007). Valuable information will be left out of the calibration and it will fail to fully identify the sample (Büchi, 2007). Selection of the optimal number of primary PCs in the Buchi NIRCal software should be based on:

- The X-PRESS function, which shows the PCs improve the reconstruction of the spectra. The smallest number that still show changes in the X-PRESS values should be selected
- The loading, which shows the individual PCs. PCs that are considered noisy should be removed.
- The Residuum spectra, which shows the amplitude of the spectra which should all be about the same.

The secondary PC are the components taken into account for the final calculations of the established variable and have slightly different purposes depending on whether the calibration is quantitative or qualitative. (Büchi, 2007). In a qualitative calibrations, secondary PC are responsible for the separation of the different substances based on their properties and are used to calculate the allowable tolerance areas for those properties (Büchi, 2007). This establishes how close two like samples have to be to each other in order for them to be classified as the same. For a quantitative calibration, the secondary PC will be used for the property value calculations for the variable (Büchi, 2007). They are also responsible for the parameter calculations, and there selection will have the biggest impact on the overall quality of the calibration (Büchi, 2007). The secondary PC selected need to show a clear separation of the substances, or accurate isolation of the variable being quantified where the scores are repeatable (Büchi, 2007). Selection of the number of secondary PCs should be chosen where:

- The plot regression coefficients the PCs, which have similar constant value, are food for the calibration, big deviation indicates over fitting.
- The SEP centralized cross validation is small.
- The V-set bias is around zero.
- The Q-value is high.
- The absolute value of the PCR B-matrix is high.
- The V- and C-set regression coefficients are as close to one as possible.
- The consistency is around 100.
- The V- and C-set press are as small as possible.
- The V- and C-set SEP and SEE (SEC) are as small as possible and are similar (constancy).

With these selection guidelines in mind, it is important to remember that there is an art to choosing principle components for the calibration. Various selections of PC should be chosen with these guidelines and the calibration recalculated to compare results. Each calibration and material will have their own ideal selection of the parameters discussed.

2.3.4 Factors Effecting NIR Scanning

Two types of error are considered when looking at factors affecting NIR analysis. There are errors associated with the creation of the calibration, and error associated with analysis of samples by reference methods. During the calibration process, the largest sources of error can be minimized by reducing the variations (Workman, 2008). A significant amount of issues can also occur from laboratory error. This error can be reduced drastically through in-house audits for procedures, equipment, and personnel, with extra emphasis on sample presentation, drying procedures, and moisture loss associated with any grinding procedures (Workman, 2008). A full list of calibration error sources, and recommended actions for correction can be found in Appendix 1.

The main factors affecting NIR testing have to do with samples, both their selection and preparation, as well as the reference methods used for analysis (Williams, 2008). There are roughly 30 different factors attributed to sampling that affect the accuracy and precision of an NIR calibration (Williams, 2008). A full list of these factors can be found in Appendix 2. As far as the sample is concerned, there are four main sources of error.

These are the source of the sample, the sampling method, and the sample itself, which includes preparation and presentation (Williams, 2008). One common issue that can occur in sampling plans is collecting a representative sample. In order to get an accurate analysis of the sample lot, the sample must be collected in a way that represent the whole population (Williams, 2008). One of the first problems that can cause issues is population sampling error. If a sample set does not accurately represent the total population, the maximum variation in the calibration will not account for the natural variations present in the sample (Workman, 2008). This problem can be multiplied with sampling itself, including sampling location and collection of sample (Williams, 2008). To address these issues, an extremely comprehensive dataset must be collected and entered (Workman, 2008). Sample handling, which includes packaging and transportation, is becoming less of an issue with better packaging material and transportation methods (Williams, 2008). However, if care is not taken during this step it can cause structural changes and increases the chance of the sample being contaminated.

After any issues with the sampling procedures are addressed, sample preparation should be considered. Sample preparation refers to any manipulation of the samples prior to scanning on the instrument (Williams, 2008). Things that need to be taken into account during sample preparation include the nature of the sample, physical size, texture, composition, amount, removal of any foreign material (more important in grains), grinding or any other method used to reduce size, and blending (Williams, 2008). A preparation step as simple as grinding can have a large effect on mean particle size, particle size distribution, and particle shape depending on the type of grinder and how long the sample is ground. The temperature can also increase during grinding, potentially resulting in moisture loss (Williams, 2008). This makes not only the type of grinder important, but the specific grinding procedure critical to get consistent results and good calibrations. Sample storage is normally not a large source of error as most samples are analyzed immediately after preparation or in-line during production. If sample storage is

required, it is important that the sample be presented to the instrument as close to its original composition as possible (Williams, 2008).

The final issue that must be considered is the presentation of the sample to the instrument. The first thing that needs to be considered is the sample presentation cell where the sample is presented to the instrument for scanning. Most NIR instrument manufacturers have multiple sample cells in varying shapes and sizes (Williams, 2008). The key parameters to consider with a sample cell are its ease of filling without stratification, ease of cleaning, and its ability to hold a sample layer thick enough to conduct diffuse reflectance from the sample only (Williams, 2008). After sample cell selection, loading the sample cell in a consistent way that produces accurate results is required. The bottom layer of sample in the sample cell is the surface the instrument actually scans (Williams, 2008). Loading variations can be compensated for by collecting as many measurements as possible (Workman, 2008). This can reduce the associated error in prediction by up to 70% (Workman, 2008). If stratification occurs during loading, both the precision and accuracy of the scans will be negatively affected (Williams, 2008). With a minimum of 40 calibration samples required for an adequate calibration and the sensitivity of NIR at detecting variations in a sample, all these variables must be carefully controlled for (Frankhuizen, 2008). However, with the establishment of a quality calibration, and proper training many of these variations can be avoided.

2.3.5 Current Uses of NIR in Dairy Industry

The use of NIR instrumentation has been growing in the dairy industry. The composition of dairy products are strictly controlled through economic and legal constraints, making rapid analysis an important and highly desirable tool (Frankhuizen, 2008). Butter is a perfect example of this. To meet the standard of identity and conform to the legal regulations, moisture content and solids nonfat content must meet certain criteria (Frankhuizen, 2008). With these standards in mind, the application of NIR analysis has taken three major application pathways; analysis of incoming milk

shipments, analysis of spray-dried products, and analysis of finished products such as cheese and butter to ensure manufacturing specifications and legal regulations are being met (Frankhuizen, 2008).

Fluid milk has had the fastest adoption of NIR analysis. It is currently used for fat, protein, lactose, and total solids content (Frankhuizen, 2008). Even though fluid milk was first to adopt the technology, milk powders have been found to be the most well suited products for NIR analysis (Frankhuizen, 2008). Milk powders are suited for NIR analysis because of their uniform particle size and shape, and the consistency of formulation (Frankhuizen, 2008). With milk powders being so well suited for NIR, calibrations have been developed for protein, moisture, fat, lactose, lactate, and ash (Frankhuizen, 2008). Ash is not commonly analyzed through NIR due to its inorganic nature not giving any characteristic reflection signals (Frankhuizen, 2008). However, with the instruments ability to accurately analyze the other major constituents, ash can be determined as the residual to a lower degree of accuracy (Frankhuizen, 2008). Some work has also been done to identify adulterants in milk powders. Researchers out of the University of Campinas in Brazil explored the possibility of using NIR to detect starch, whey, and sucrose addition (Borin et al., 2006). Using a least-squares support vector machine (LS-SVN) they were able to qualify adulterants, but results were not accurate enough to establish quantification (Borin et al., 2006). Researches did note that through further development, NIR with LS-SVN is a promising technique for the rapid analysis of isolated adulterants (Borin et al., 2006). Casein, caseinates, and butter are examples of products that do not have a lot of variation, where NIR can be used for screening. These products carry tight specifications, making collecting a range of compositional differences problematic. For these calibrations, samples that fall into specifications are analyzed. This allows NIR analysis to rapidly screen if products are meeting specifications. For casein and caseinates, moisture, protein, fat, and ash are calibrated, for butter, moisture and solids nonfat content are calibrated (Frankhuizen, 2008). Cheese has proven to be one of the most difficult dairy products to analyze with NIR due to the

difference in process, physical and chemical composition, and high levels of moisture and fat (Frankhuizen, 2008). This makes standardizing the sampling and sample preparation critical to achieve an accurate and representative measurement (Frankhuizen, 2008). Robust calibrations have been established for the major constituents for moisture, fat, and protein, while the minor constituents of salt, pH, water soluble nitrogen, trichloroacetic acid, soluble nitrogen, and water soluble primary amines are possible to quantify, but with lower accuracy (Frankhuizen, 2008). A combination of minor constituents were used to develop a calibration to determine the age of gouda and edam, but the issue of low accuracy persisted (Frankhuizen, 2008).

Accuracy of NIR equipment for the analysis of major constituents of dairy, such as fat, protein, and moisture has proven to be similar to the accuracy of the reference methods (Frankhuizen, 2008). NIR has also been analyzed for minor constituents such as salt, pH, and water soluble primary amines and demonstrated that it is possible, but the achieved accuracy of these calibrations is relatively low (Frankhuizen, 2008).

2.4 Starch and Total Dietary Fiber Analysis

Total Dietary Fiber

Total dietary fiber analysis of foods is a well-established method. The Association of Analytical Communities (AOAC) took final action on official method 985.29, Total Dietary Fiber in Foods, in 1986, and has since been a staple analytical method (AOAC, 2005). This method was also adopted as a CODEX Alimentarius defining method for enzymatic-gravimetric digest of total dietary fiber in special foods (AOAC, 2005). The principal of this method is extraction of the dietary fiber, and analysis through change in weight. A food sample is dried and defatted if fat content is greater than ten percent, and gelatinized with thermostable α-amylase, then enzymatically digested with amyloglucosidase and protease enzymes (AOAC, 2005). This degrades the proteins and starch from the sample before extraction. Components in the digested sample are then precipitated with ethyl alcohol, and filtered. After filtration, the filtrate is washed with

78% and 95% ethyl alcohol and acetone before drying (AOAC, 2005). The residue containing the fiber is weighed, and protein and ash are corrected for to yield the quantity of total dietary fiber in the sample (AOAC, 2005). These methods have been thoroughly studied and validated since the 1980's and have continually proven their worth (McCleary et al., 2012). It needs to be noted, however, that there is no official method for analyzing dietary fiber in cheese, or dairy products in general. This requires any dietary fiber method used to be tailored to function on a cheese matrix before its application.

Starch

Starch analysis assays can be broadly broken down into two categories, acid hydrolysis and enzymatic. Acid hydrolysis methods are limited in their scope and can only be applied to pure starch samples (Megazyme, 2015). These methods rely on the hydrolysis of starch using acids while boiling the sample in a water bath at atmospheric pressure (Moreels, 1987). With these conditions, the hydrolysis of starch can be incomplete. The degree of hydrolysis will vary based on the heat transfer characteristics of the water bath and flask system, the accuracy of timing of the hydrolysis before cooling, and influence of any clarification reagents and there amounts (Moreels, 1987). It is also known that under acidic condition D-glucose can produce 5-hydroxymethylfurfural, as well as D-fructose requiring quantification and a correction factor to determine starch content (Faithfull, 1989). Due to the lack of specificity and the dependence on dangerous, corrosive reagents, acid hydrolysis methods are no longer favored (Karkalas, 1985). One method that is still in use for spreadable cheese, and is an official method of the Ministry of Agriculture, Livestock and Food Supply in Brazil, is a modified version of the Lane-Eynon method (Sá Oliveira et al., 2015). In this method, the product goes through a clarification, filtration, acid digestion, a second clarification and filtration before titrating with the Fehling reagent to determine the reducing sugar amounts (Sá Oliveira et al., 2015). However this method has several disadvantages. The results are based on a precise reaction time, temperatures, and reagent concentrations, and it does not distinguish between reducing sugars. So the results can be inaccurate in the

presence of other reducing agents (McClements, 2003). This method also generates hazardous waste and has been found to under-estimate starch content due to loss during the first filtration step (Sá Oliveira et al., 2015).

Enzymatic methods employ the use of enzymes such as α-amylase and amyloglucosidase for the hydrolysis of starch (Moreels, 1987). One of the main benefits to using an enzymatic method is the specificity of the enzymes used. Enzymes are very specific in their action and for starch, hydrolyze only the starch and leave other materials unaffected (Moreels, 1987). The enzyme α-amylase will hydrolyze amylose and amylopectin starch chains into soluble branched and unbranched maltodextrins (Megazyme, 2015). Amyloglucosidase will hydrolyze maltodextrins into individual D-glucose units that can be further manipulated to quantify the total starch content (Megazyme, 2015).

While the analysis of starch in dairy products is limited, some methods have been investigated in processed cheese and powdered milk. Focus has been put on more rapid methods of analysis such and Raman and Near-Infrared spectroscopy. Researchers at the Federal University of Juiz de Fora in Brazil have created two Raman spectroscopy methods capable of identifying the presence of starch in spreadable cheese (Sá Oliveira et al., 2015). Researches first established a discrimination analysis calibration with a partial least squares discriminant analysis (PLS-DA) (Sá Oliveira et al., 2015). They were able to qualify starch adulteration with 100% accuracy and specificity (Sá Oliveira et al., 2015). For quantitative analysis, a PLS-DA calibration was established with a detection limit of 0.34% (w/w) with a limit of quantification of 1.14% (w/w) (Sá Oliveira et al., 2015). There were instances where starch free samples were recording a negative result, however with the prescreening of the discrimination analysis this was not seen as an issue, as non-adulterated samples would not be scanned for quantification (Sá Oliveira et al., 2015). Some work has also been done to identify adulterants in milk powders. Researchers out of the University of Campinas in Brazil explored the possibility of using

NIR to detect starch, whey, and sucrose addition (Borin et al., 2006). Using a least-squares support vector machine (LS-SVN) they were able to qualify adulterants, but results were not accurate enough to establish quantification (Borin et al., 2006) Researches did note that through further development, NIR with LS-SVN is a promising techniques for the rapid analysis of isolated adulterants (Borin et al., 2006). These methods show that spectroscopy can be a valuable tool for starch identification in the dairy industry.

2.5 Conclusion

With this information in mind, this research will focus on two analytical methods for analyzing flow aid concentration in shredded cheese. One method will employ an enzymatic starch assay to analysis the starch content in the cheese. The starch content will then be related to total flow aid concentration through the known starch content of the flow aid. The flow aid can also be analyzed using the same starch analysis method to confirm the starch composition. The other method will develop a preparation method and calibration for analyzing shredded Cheddar cheese on a fourier transform near-infrared spectrometer. This will offer a more rapid method, but will also require more upfront cost. Both of these methods will serve as valuable tools in the dairy industry for both manufacturers and regulators. It will allow manufactures to insure their flow aid addition is meeting their specifications to ensure accurate shelf life. Regulators will be able to use these methods to ensure the standards of identity are being adhered to, and prevent the overuse of flow aid.

3 Analysis of Flow Aid in Shredded Cheese through an Enzymatic Total Starch Assay

3.1 Introduction

Starch is a common ingredient in flow aids used in shredded cheese. It functions as both an anticaking agent, and a delivery system for antimycotics and oxygen scavengers. Flow aid addition is done by gravimetric calculation where a known weight of flow aid and cheese is tumbled together. This does not ensure even distribution of the flow aid or confirm what percentage adheres to the shreds. To maintain quality control and meet the standard set by the dairy industry, the amount of flow aid in the final product must be accurately quantified. To do this, an enzymatic starch analysis method was developed that is capable of accurately calculating starch in a cheese matrix. Most starch methods have been designed to analyze grain products, and do not take into consideration the moisture and fat content of a product like cheese.

Enzymatic starch assays employ α-amylase and amyloglucosidase enzymes to break down chains of starch into measurable components (Moreels, 1987). For this assay, α-amylase is introduced first to hydrolyze starch chains into branched and unbranched maltodextrins (Moreels, 1987). Amyloglucosidase is then added to hydrolyze the maltodextrins into D-glucose (Megazyme, 2015). To convert the D-glucose into a measurable form, hexokinase (HK) and adenosine-5'-triphosphate (ATP) convert D-glucose to glucose-6-phosphate (G-6-P) through phosphorylation (Megazyme, 2015). Glucose-6-phosphate is then oxidized by nicotinamide-adenine dinucleotide phosphate ($NADP^+$), in the presence of glucose-6-phosphate dehydrogenase (G6P-DH), to gluconate-6-phosphate (Megazyme, 2015). This also forms reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (Megazyme, 2015).

$$\text{Starch} + H_2O \xrightarrow{(\alpha\text{-amylase, pH 5.0, } 100^oC)} \text{maltodextrins}$$

$$\text{maltodextrins} + H_2O \xrightarrow{(AMG)} \text{D-glucose}$$

$$\text{D-glucose} + ATP \xrightarrow{(HK)} \text{G-6-P} + ADP$$

$$\text{G-6-P} + NADP^+ \xrightarrow{(G6P\text{-}DH)} \text{gluconate-6-phosphate} + NADPH + H^+$$

The amount of NADPH formed is stoichiometrically related to the amount of D-glucose. NADPH is measured with a spectrophotometer by absorbance at 340 nm. This method has not been tested for its effectiveness in a cheese product. Cheese does not naturally contain starch. For accurate quantification, the D-glucose in the sample must first be extracted before digestion of the starch. D-glucose can be present in cheese due to the hydrolysis of lactose by cultures, and due to its addition to flow aids to increase the functionality of glucose oxidase as an oxygen scavenger in the package (Wong et al., 2008). This research was undertaken to determine the modifications necessary to accurately quantify starch from flow aids in a cheese matrix.

3.2 Materials and Methods

3.2.1 Cheese Samples and Preparation

Cheese samples were sourced from two locations. Cheddar cheese samples produced at the University of Minnesota by Dr. Tonya Schoenfuss were collected in 2.27 kg blocks. The blocks were trimmed, portioned into 0.45 kg blocks, and shredded with a Hamilton Beach Stack & Snap 10 cup processor with shredding disk. To increase shredability, sample were shredded directly after being in a 4ºC cooler. The second source was supplied from a food processor (Diane Bussell at Kroger). Kroger supplied 2.27 kg of pre-shredded Cheddar cheese free of flow aid, shipped directly the University of Minnesota for preparation and analysis. All samples were vacuum sealed and stored at 4ºC when not in use.

Blends of two hundred grams were created ranging from 2-8% flow aid. The flow aid used to create the blends was Allied Blending & Ingredients: Free Flow 2032, sourced

from Diane Bussell at Kroger. The amount to flow aid and shredded cheese was weighed into separate weigh boats. The shredded cheese and flow aid was transferred to a benchtop drum tumbler and the two were tumbled together for 60 sec at medium speed. This mimicked the method used in the Kroger manufacturing facility. Sample was then homogenized in a Hamilton Beach Stack & Snap 10 cup food processor (Hamilton Beach, Picton, Ontario) with a chopping S-blade insert for 60 sec. During the initial testing stage smaller portions of 20 g total were created. In this instance the shreds and flow aid were mixed by hand and homogenization was carried out by hand kneading.

3.2.2 Starch Analysis

The starch analysis method was based on the Megazyme Total Starch HK Assay. Flow aid addition to shredded cheese is typically between 1-4%, and range in their starch content. The Free Flow 2032 used in this research contained 62.2% starch. To achieve a proper starch concentration in the final analysis aliquot, initial sample size and analysis aliquot size needed to be increase above the 100 mg and 0.05 ml portions recommended. Samples were extracted using 10 ml aliquots of 80% aqueous ethanol 2 to 4 times, and the results were compared to determine how many washing steps were required for accurate results. Fat content of the samples also created issues during the assay. After centrifugation in the ethanol extraction step, the pellet became difficult to re-suspend with the vortex mixer. In addition, interference occurred in the analysis cuvette as the sample was pipetted for final analysis.

The final method developed was evaluated with different concentrations of starch added to Cheddar cheese. The method was as follows:

Two hundred grams of sample was homogenized in a Hamilton Beach Stack & Snack 10 cup food processor with S blade insert for 60 sec. 0.5 g of sample was added to a test tube containing approximately 15 glass beads. Five ml of 80% aqueous ethanol was added and tubes were incubated in 80-85ºC water bath for five minutes. After incubation, 5 ml of 80% aqueous ethanol was added and the tubes were vortexed before being

centrifuged at 3,000 RPM (approximately 2,000 g) for 10 minutes. Supernatant was carefully drained off and discarded. 10 ml of 80% aqueous ethanol was added and the tubes were placed back in the water bath for 30 sec to soften the pellet. Pellet was re-suspended with a vortex mixer and centrifuged as above. Supernatant was carefully drained off and discarded. 3 ml of thermostable a-amylase mixture was added and tubes were placed in a boiling water bath for six minutes. Test tubes were vortexed at 2, 4, and 6 minutes to keep homogeneity and prevent any of the sample from escaping as the alcohol evaporated. Test tubes were transferred to a 50ºC water bath for three minutes to allow the solution to equilibrate. 0.1ml amyloglucosidase was added and the tubes were incubated at 50ºC for 30 minutes. The entire contents of the tube were transferred to a 100 ml volumetric flask, the volume was adjusted with distilled water and mixed thoroughly. 30 ml aliquot of this sample was transferred to a centrifuge tube and centrifuged as above. 5 ml was then gravimetrically filtered through grade 1 filter paper into 10ml test tube.0.2 ml of filtered sample was then analyzed thorough the spectrophotometric at 340 nm with the method as shown below. Detailed procedures can be found in Appendix 3.

Wavelength: 340 nm
Cuvette: 1 cm light path, 3 ml (glass of plastic)
Temperature: ~25ºC
Final Volume: 2.27 ml
Sample Solution: 4-80 g of D-Glucose per cuvette

Pipette into Cuvettes	Blank	Cheese Sample	Flow Aid Sample
Distilled Water (~25ºC)	2.05 ml	1.85 ml	1.90 ml
Sample	-	0.20 ml	0.15 ml
Solution 3 (buffer)	0.10 ml	0.10 ml	0.10 ml
Solution 4 ($NADP^+$/ATP)	0.10 ml	0.10 ml	0.10 ml
Read the absorbance of the solution after ~ 3 minutes (A_1). Start reaction with addition of:			
Suspension 5 (HK/G-6-PDH)	0.02 ml	0.02 ml	0.02 ml
Read the absorbance of the solution at the end of the reaction (A_2). May take up to 30 minutes. Read absorbance in 5 minute intervals for the first 20 minutes and 2 minute intervals after that until absorbance remains the same.			

The full procedure for this method is available in Appendix 3.

To calculate the total starch, the absorbance difference between the blank and the sample was determined by subtracting the blank value from the sample value ($\Delta A_{D\text{-}glucose}$). The concentration of starch was calculated from the following formula.

$$c = \frac{V*MX}{\varepsilon*d*v} * \frac{162}{180} * \Delta A_{D-glucose} \quad (3.1)$$

Where:
V = Final volume (ml)
MX = Molecular weight of D-glucose (g/mol)
ε = Extinction coefficient of NADPH at 340 nm = 6300 ($l*mol^{-1}*cm^{-1}$)
d = Light path (cm)
v = Sample volume (ml)
162/180 = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch)

For cheese samples, the following equation was used:

$$c = \frac{2.27ml*180.26g/mol}{6300Lxmol^{-1}xcm^{-1}*1cm*0.20ml} * \frac{162}{180} * \Delta A_{D-glucose} \quad (3.2)$$

Therefore (for cheese samples):

$$c = 0.5842g/L * \Delta A_{D-glucose} \quad (3.3)$$

If the sample has been diluted, as it will be with analysis of flow aids, the result are multiplied by the dilution factor. To convert the content to g/100g use the following calculation.

$$\frac{c_{Starch}(g/L\ sample\ solution)}{Weight_{Sample}(g/L\ sample\ solution)} * 100 \quad (3.4)$$

3.3 Results and Discussion

Enzymatic starch assays are generally designed to be perform on grain products. In order to function properly on a high fat, high moisture, low starch product like shredded cheese, certain modifications needed to be made. The first issue than needed to be address was sample size required to obtain an accurate reading. Cereal grains range from 55-70% starch (Koehler, 2013). Standard sample size for the Megazyme Total Starch HK assay kit calls of 100 mg (Megazyme, 2016). However this value is based on the starch content of grains. Flow aid addition is shredded cheese generally ranges from 2 to 4% and will vary in their starch content (Bussell, 2016). The flow aid used during this analysis tested at 62.2% starch. In order to achieve a sufficient level of starch for analysis, an initial sample size of 500 mg was used. Sample sizes larger than this created further problems re-suspending the pellet during digestion. In addition, the analysis aliquot needed to be increased to register the proper concentration during spectrophotomic analysis. The analysis aliquot was increased from 0.05 ml (as called for in the original method) to 0.20 ml. An analysis size of 0.20 ml provided absorbance units above the 0.100, a concentration above the 0.08 g/L D-glucose, threshold required for

accuracy (Megazyme, 2016). With these sample sizes, accuracy was achieved for concentrations as low as 2% total flow aid.

Prior to the enzymatic digestion, any D-glucose present in the sample needed to be removed. Final starch content determination is based on the amount of D-glucose obtained from the digested starch. In order for accurate quantification, the D-glucose in the sample must first be extracted before digestion. D-glucose is added to flow aids to increase the functionality of glucose oxidase as an oxygen scavenger in the package (Wong et al., 2008). There is also a possibility of D-glucose being present in the cheese sample as a result of the cultures breaking down the lactose (comprised of galactose and glucose) during the cheese making process (Ryan, 2004). D-glucose is soluble in 80% (v/v) aqueous ethanol, however it needed to be determined how many extractions were necessary to completely remove it from the cheese sample. The standard method calls for 5 ml of ethanol to be added before being incubated in an 80-85ºC water bath for 5 minutes (Megazyme, 2016). An additional 5 ml is added before being centrifuged and the supernatant is drained off (Megazyme, 2016). An additional 10 ml is added, vortexed, centrifuged, and drained off (Megazyme, 2016). To determine if the standard procedure was sufficient to remove all D-glucose present from the flow aid in the cheese samples, 2 to 4 extractions were conducted and compared.

Table 1: Results of multiple extractions to remove D-glucose

Sample[1]	*Extractions*	*True Value*[2] *(%)*	*Method Value (%) (Ave ± SD)*
Cheddar – 3% Flow aid	2	3.00	2.96 ± 0.21
Cheddar – 3% Flow aid	3	3.00	2.93 ± 0.13
Cheddar – 3% Flow aid	4	3.00	2.96 ± 0.21

1: Samples blended by hand, 2: true value calculated gravimetrically

Results from multiple extractions (Table 1) showed that the standard method (2 extractions) was sufficient to remove any D-glucose present in the sample prior to digestion. However, an issue of pellet dispersion was encountered during this step. Initially, 15-20 glass beads were added to each test tube to help break up and aid in

dispersion of the sample after centrifugation. The results from the glass bead addition were mixed, with some pellets dispersing easily after the addition, while others remained in a pellet and required manual breaking of the pellet with a spatula. To alleviate this issue, the test tubes were placed back in the 80-85ºC water bath for 30 sec after the 10 ml of aqueous ethanol was added. This was enough to soften the pellet, and enable full re-suspension during vortexing.

The presence of fat in the sample also caused interference during spectrophotometric analysis after the final centrifugation step. When pipetting the final analysis sample volume from the centrifuge tube, fat at the surface was pulled into the pipette and transferred to the analysis cuvette. Carefully pipetting the sample from below the fat layer was not enough to completely eliminate the fat interference. To alleviate this issue, 5 ml of the centrifuged sample was gravimetrically filtered through Whatman, grade 1, 85 mm filter paper (GE Healthcare, Buckinghamshire, UK). This allowed the fat to be removed from the sample without affecting the D-glucose for analysis (Megazyme, 2016).

After establishment of the finalized method, samples ranging from 2-8% flow aid were analyzed.

Table 2: Starch Analysis of Hand Blended Cheddar Samples

Sample[1]	*Starch Content (g/100g) (Ave ± SD)*	*Total Flow Aid*[2] *(%) (Ave ± SD)*	*% Erel*[3]
Cheddar – 2%	1.28 ± 0.10	2.06 ± 0.16	2.98
Cheddar – 3%	1.86 ± 0.14	2.91 ± 0.22	-3.00
Cheddar – 3%	1.91 ± 0.33	2.99 ± 0.56	-0.32
Cheddar – 3.5%	2.16 ± 0.02	3.47 ± 0.31	2.36
Cheddar – 4%	2.36 ± 0.13	3.79 ± 0.21	-0.78
Cheddar – 5%	3.27 ± 0.02	5.26 ± 0.04	-5.14
Cheddar – 6%	3.83 ± 0.09	6.16 ± 0.14	2.63
Cheddar – 8%	5.03 ± 0.04	8.09 ± 0.06	0.06

1: Samples hand blended to percent stated, 2: Flow aid amount calculated based off 62.2% of flow aid being starch, determined through total starch analysis of Free Flow 2032, 3: Percent relative error

Final results (Table 2) showed accurate analysis of Free Flow 2032 flow aid in shredded Cheddar cheese samples. Accuracy was achieved to within 0.26% true value, with the largest percent relative error being -5.15%. The only other method found for the quantitative analysis of starch in cheese was a modified version of the Lane-Eynon method for reducing sugars (Sá Oliveira et al., 2015). This method was adopted by the Ministry of Agriculture, Livestock and Food Supply (MALFS) in Brazil for analyzing the starch content in spreadable cheese (Sá Oliveira et al., 2015). This method involved the addition of a clarification and filtration step, followed by an acid digestion and a second clarification and filtration step before titration with the Fehling reagent (Sá Oliveira et al., 2015). There are many issues associated with this method. The results largely depend on the precise reaction times, temperatures, and reagent concentration used during the assay (McClements, 2003). In addition, this method cannot distinguish among different reducing sugars and is susceptible to interference from other reducing agents in the sample (McClements, 2003). Analysis of this method also showed consistent underestimation due to starch being removed during the first filtration step (Sá Oliveira et al., 2015). For these reasons this method does not serve as a quality choice for the analysis of starch in cheese. The paper *Analysis of spreadable cheese by Raman spectroscopy and chemometric tools* by Kamila de Sá Oliveira et al, states that this method can be found in the Standard Methods for the Examination of Dairy Products; however after thorough review this method could not be located. There is also no indication that this method has been adopted elsewhere other than the Brazilian MALFS.

3.4 Conclusion

With the lack of accuracy provided by the modified Lane-Eynon method, as well as its use of acids and generation of hazardous waste, it does not serve as a quality method of analysis. The results of this research show that not only does the modified Megazyme Total Starch HK assay serve as an accurate method, it also removes the use of acids and reduces hazardous waste production. Through proper manipulation of the sample, the

starch analysis method was able to accurately quantify the starch content in shredded cheese to within 0.26% of its true value, with a total run time of approximately 3 hours. In combination with total starch analysis of the flow aid to quantify its starch content, quantification of the total flow aid on the shredded cheese can be accurately calculated. This method provides a valuable tool for industry to monitor quality control and insure the shelf-life of the product. It also provides a valuable tool for regulators to ensure that the standards set on shredded cheese products are being adhered to, boosting consumer confidence in the process.

4 Analysis of Flow Aid in Shredded Cheddar Cheese through Near-Infrared Spectroscopy

4.1 Introduction

Shredded cheese accounted for 965.7 million pounds of sales in 2014, making it the most sold style of cheese in the U.S (Statista, 2015). To ensure a quality product, flow aids, mainly comprised of starch and cellulose, are added as an anticaking agent to prevent the shreds from binding together, and as a delivery system for antimycotics and oxygen scavengers. With such a large portion of sales coming from shredded cheese, there is high demand for simple, fast, and reliable quality control methods. Fourier Transform Near-Infrared Spectroscopy (NIR) is a method gaining in popularity due to its speed, nondestructive nature, and its non-use of chemicals (Burns and Ciurczak, 2008). NIR uses a light beam to analyze the molecular vibrations present in a sample to analyze its chemical composition (Burns and Ciurczak, 2008). As analytical software continues to become more powerful, more applications for NIR are being developed. One of the most challenging tasks when developing a robust NIR calibration is the collection of the required number of well-characterized samples. Cheese is a difficult product to create an NIR calibration due to the difference in processes, physical and chemical composition, and the high level of moisture and fat (Frankhuizen, 2008). Great care and time must be spent developing the presentation method and calibration. Even with the difficulty of cheese, NIR calibrations have been developed to assess its moisture, fat, and protein content (Frankhuizen, 2008). This research was focused on developing an NIR calibration to quantify the amount of flow aid in shredded Cheddar cheese. With wavelengths of the bond vibrations of starch and cellulose well established, it was believed the machine would be able to accurately determine the quantity applied to the final product (Shenk et al., 2008). The quantity of flow aid typically added to cheese also exceeds the 0.1% threshold for accurate detection, increasing the probability NIR would be a good choice for calibration development (Burns and Ciurczak, 2008, Büchi, 2016).

4.2 Materials and Methods

Method development was completed in two phases. The first phase was to create a preparation and presentation method to consistently provide a quality scanning surface to the NIR instrument. The second phase involved creating a calibration using the Büchi NIRCal Chemometrics software to accurately analyze the data. With correct configurations of the software a calibration was created that can accurate measure the starch and cellulose components, and determine the total content of flow aid in the sample.

4.2.1 Preparation and Presentation

Cheese preparation

Calibration samples were prepared by first shredding block Cheddar cheese with the REGAL La Machine food processor with shredding disk. To mimic the manufacturing process, a tumbler was fabricated used a one gallon food grade bucket attached to a rotation device. The shredded cheese and flow aid were added to the bucket in calculated ratios for each concentration. The mixture was tumbled for sixty seconds at medium speed until even distribution was determined visually.

Cheese preparation for scanning

Temperature, grinding method, and pressure were evaluated for their ability to present a homogenous sample to the NIR. The final process used for calibration measurements is as follows. Two hundred grams of shredded cheese was ground for 60 sec in a REGAL La Machine food processor with S blade insert. Seventy-five g of the homogenized sample was formed into a ball by hand. Temperature of the sample was determined by inserting a Vernier thermocouple probe into the center of the formed ball and ensuring sample was at room temperature (20ºC). The ball was then placed into the center of a 15 mm x 100 mm glass petri-dish. A 9.5 cm circular disc of high density polyethylene (press plate) was placed on the cheese, and pressed by hand until the disc

was below the lip of the petri dish. The petri dish contain the sample and press plate was placed on a Vernier force plate, and a 6.35 cm C-clamp was clamped around the press plate and the force plate. A 300 N force was applied to the cheese through the circular press plate by tightening the clamp. The increase to reach 300 N was done in a slow, consistent manner, while maintaining a level press plate. The sample was allowed to extrude around the press plate for 60 seconds. The pressure was released, and the cheese in the petri dish was scanned on the NIR. Triplicate preparations of each cheese were scanned. Detailed procedure can be found in Appendix 4.

4.2.2 Calibration

The initial calibration development was created with flow aid concentrations ranging from 0 to 10%. These concentrations were blended with a Cheddar cheese produced at the University of Minnesota (UMN) by Dr. Tonya Schoenfuss. Each concentration was prepared as noted in section 4.2.1 and scanned into the NIR. Initial calibration consisted of 60 samples, 10 samples of each concentration, 0, 2, 4, 6, 8, and 10%. Samples were scanned in triplicate for a total of 180 spectra. After assignment of spectra as calibration or validation spectra, a PLS calibration was created. In sequence, 6 spectra were assigned to calibration, and 3 were assigned to validation until all spectra were assigned. The NIRCal 5.2 Chemometrics software (BUCHI Labortechnik AG) analyzed 48 calibration scenarios, where the strongest one was selected for further refinement. Wavelengths consisting of "noise", ranging from 10,000 to 8,750 were removed to increase the accuracy in analyzing for variations in the spectra. Principal components were reduced from 18 to 6 to reduce the possibility of over-fitting the calibration. No pretreatment needed to be applied to the spectra.

After confirmation of the accuracy in quantifying the flow aid, additional samples were scanned at 1, 3, and 5% flow aid, and Cheddar samples of different ages from 5 commercial manufacturers (Kraft, Bongards, Minnesota Creamery, Crystal Farms, and Kroger) were scanned to increase the robustness of the calibration in the range of interest.

Scanning these samples added an additional 78 samples, creating an additional 234 spectra that were incorporated into the calibration. After removal of outliers, the total spectra incorporated into the calibration totaled 463.

With the incorporation of these additional spectra, the calibration was further modified to provide the best results when predicting the external calibration samples. Wavelengths ranging from 10,000 to 8,750 were removed from the calibration as this range of wavelengths did not add relevant information to the analysis. The pretreatment of Standard Normal Variate (SNV) was added to reduce baseline variation in the original spectra. Principal components (PC) were adjusted based on the information provided in the secondary factor selection plots to include 10 PC.

4.3 Results and Discussion

4.3.1 Preparation and Presentation

In order to collect a sufficient quantity of quality spectra, preparation and presentation development was critical. If a consistent scanning surface was not presented to the NIR, too much variation in reflectance would have created inconsistent spectra and the chemometric software would not have been able to detect the differences that account for the known sample variation. Additionally, one of the goals in method development was to make the preparation and presentation procedure as simple and robust as possible. Minimal training and simplicity of equipment use was of key importance. Fortunately, an outline for sample preparation and presentation was laid out by Rod Frankhuizen in chapter 20 of the Handbook of Near-Infrared Analysis. For homogenization, a food processor with a 6 cup capacity and an S blade insert was used. The 6 cup container was an adequate size to fit the 200 gram sample and achieve thorough homogenization. Homogenization technique also need to be considered to minimize any heat addition to the sample. A time of 60 second was found to be long enough to thoroughly homogenize the sample without adding unnecessary heat to the system. This allowed continuation of sample manipulation without having to re-cooling the sample.

Temperature was one of the significant factors when developing the preparation method. Cheese samples have been known to be sensitive to temperature differences, in part due to their high fat content (Frankhuizen, 2008). Temperature fluctuation of 5 to 10ºC can increase the standard error of a calibration by as much a 50% (Frankhuizen, 2008). Temperature of scanning temperature and the hold time were evaluated for their effect on the spectra. Samples were scanned at varying temperatures to determine the optimal scanning temperature. After homogenization and loading the sample into an NIR petri dish, it was placed in cold storage at 4ºC for 3 hours to equilibrate. Samples were scanned immediately after being removed for cold storage and scanned every 10 minutes until the sample reached room temperature (21ºC). A separate sample was loaded and melted on a hot plate to examine the effects heat would have on the spectra. Figure 8 shows the effects of these different temperature states.

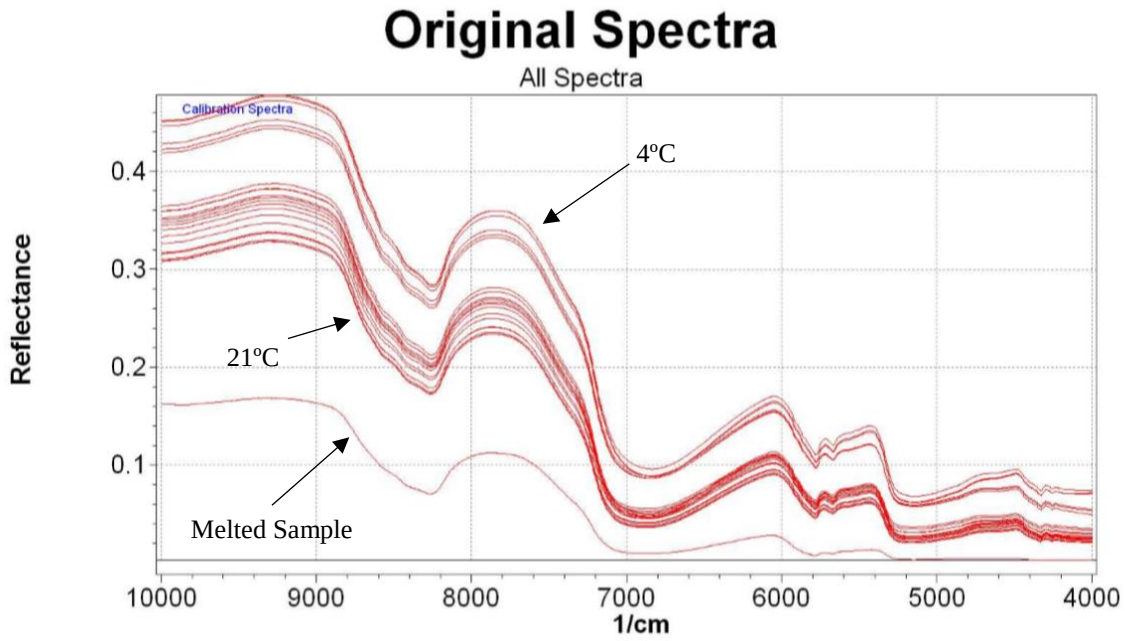

Figure 8: Temperature effects on spectra

When the sample was removed from the cooler and scanned, the changing temperature affected the spectra immediately. With the room temperature being 21ºC,

and the addition of any heat radiating off the NIR equipment, the sample was unable to maintain the 4ºC temperature long enough to complete the scan in triplicate. As the sample temperature warmed to room temperature, the reflectance started decreasing. This is consistent with other studies that found as temperature increased, the band height and area generally decreased (Zhang et al., 2007). Under ideal conditions, all three of the triplicate scans would lay on top of one another. This would indicate the instrument is seeing the sample the same way during each scan. As the interval scan continued, the spectra continued to show variation in the reflectance pattern in a decreasing manner. When the sample reach the 21ºC room temperature the variation in reflectance patterns were minimized. The melted sample lost much of the definition that allowed the instrument to analyze sample variation. For these reason it was determined that running the analysis at 21ºC (room temperature of equipment room) would provide the best constancy, while maintaining definition in the spectra. These results were consistent with the findings of Rob Frankhuizen that found scanning at 20±2ºC with no active heating was the optimal temperature for running analyses. In addition, Frankhuizen made a note of only having the sample out of cold storage long enough to allow the sample to achieve room temperature. This led us to the question of how a sample would be affected if it was left at a room temperature longer than required to achieve room temperature. Two sample sets were tested to explore this question. The first set was allowed to equilibrate to room temperature and was left at that temperature for 2 hours. The second set was only aloud to equilibrate to room temperature and was held at that temperature for less than 1 hour. The samples held at room temperature for more than two hours showed wide variation in the consistence of their reflectance, shown in Figure 9a. Samples held less than one hour after reaching temperature registered more consistent spectra, as show in Figure 9b. This indicates that the most consistent scans are achieved when the sample is allowed to come to room temperature, without active heating, and held at that temperature for the minimum amount of time to be prepared and analyzed by the instrument. Differences observed could be due to syneresis or melting of crystalline fat taking place as the cheese

is held at room temperature, causing the moisture and fat to separate and changing the structural properties of the cheese. Structural changes will cause a shift in reflectance and will result in variable readings by the instrument.

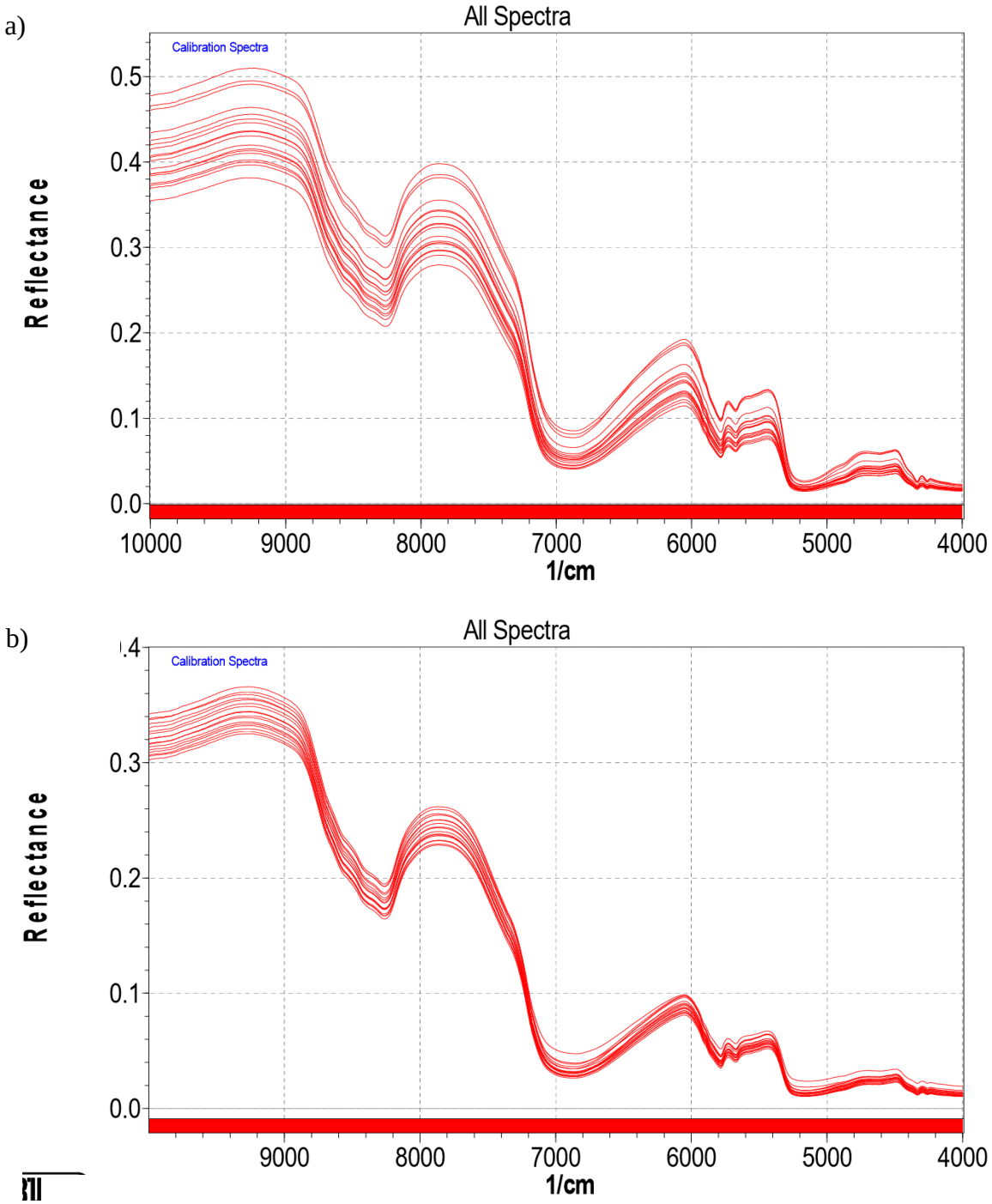

Figure 9: Effects of holding time at room temperature (20ºC) on sample spectra after samples have reached room temperature; a) samples held at room temperature for 2 hours; b) samples held at room temperature for under 1 hour

With the goal to make preparation and presentation as simple as possible, presentation methods were tested providing the lowest chance for operator error. Before sample grinding was evaluated, the feasibility of using un-manipulated shreds was tested. Shreds were loaded into a petri dish and compressed with 300N of force. The scanning surface still contained pits and fissures, as shown in Figure 10, and did not produce repeatable spectra when scanned. This method also does not address the issue of uneven flow aid distribution in the packaged product, which could have led to inaccurate analysis of the total quantity of flow aid.

Figure 10: Sample cell loaded with un-manipulated cheese shreds and pressed

A melting method was also tested for feasibility. A sample was homogenized to achieve an accurate representation of the sample and loaded into the petri dish. The sample was heated on a hot plate at the low setting until completely melted. Analysis of the scanning surface showed separation of the fat from the sample and an inconsistent scanning surface, shown in Figure 11. This produced unrepeatable results. Melting also reduced definition of reflectance pattern, as shown in Figure 8, making this preparation method inadequate for accurate analysis of the sample.

Figure 11: Sample cell loaded with homogenized cheese sample and melted

Homogenization not only provided a representative sample, it also provided a better medium to work with during sample loading. After the homogenization process was established, sample loading was tested based on the method referenced by Frankhuizen. The homogenized sample was transferred to a petri dish until the cup was “amply filled” and compressed by hand (Frankhuizen, 2008). Contrary to Frankhuizens results, this method did not produce consistent results as expected. While the results were more consistent than the previous two method, accurate repeatability was not achieved. As Figure 12 shows, air pocket and fissure remained in the scanning surface. The inconsistent pressure and nonsystematic loading are believed to have caused these results. With the inability to create repeatable results, the Frankhuizen method was disregarded.

Figure 12: Sample homogenized, loaded in sample cell, and pressed by hand

To control for the inconsistent pressure and alleviate the remaining gaps, the cell loading and pressure application were modified. A 75 g portion of the homogenized sample was weighed out and formed into a ball by hand. The ball was placed in the center of the petri dish and pressed using a press plate and clamp. Force was applied to the press plate by hand until it was below the lip and the petri dish. A C-clamp was used to apply 300 N of force. This created an even spread of the sample in the dish alleviating the air pocket issue and created a consistent and repeatable scanning surface for analysis (Figure 13).

Figure 13: Sample homogenized, rolled into a ball, and pressed with controlled pressure using a press plate and clamp

This finalized sample presentation method was contradictory to the findings of Frankhuizen. Frankhuizen stated that it was important to avoid “spreading” during sample loading (Frankhuizen, 2008). It was believed this would create structural changes similar to those that occur during temperature fluctuation resulting in fluctuations in the reflectance patterns (Frankhuizen, 2008). However, this was not the result we observed. The most accurate results were achieved when a small amount of spreading was created during pressure application. The spreading alleviated the fissure issue, and delivered repeatability in the spectral analysis without compromising precision or accuracy, as would happen if structural changes were taking place. This indicated that spreading did not have the negative structural effects observed by Frankhuizen.

4.3.2 Calibration

To alleviate inconsistencies present in the sample preparation and presentation step, a pretreatment of Standard Normal Variation (SNV) was added to the spectra. Normalization has been established as a good pretreatment for solids as it will reduce the effects of varying particle size and pressure differences that occur during sample preparation (Büchi, 2007). SNV will center each spectra and bring it to scale using its standard deviation, and correct the y-axis shift (Büchi, 2007). Figure 14a shows the original spectra collected with the established method. Even with a tightly controlled method, variation in the spectra still exists due to the natural variations, and difficulties associated with cheese. With the SNV pretreatment applied, shown in Figure 14b, those variations are minimized. The addition of this pretreatment allows the instrumentation to better analyze the differences associated with the changing variable of the sample. Without the addition of a pretreatment, some of the natural variations could be interpreted as important information in the determination of flow aid content, reducing calibration accuracy.

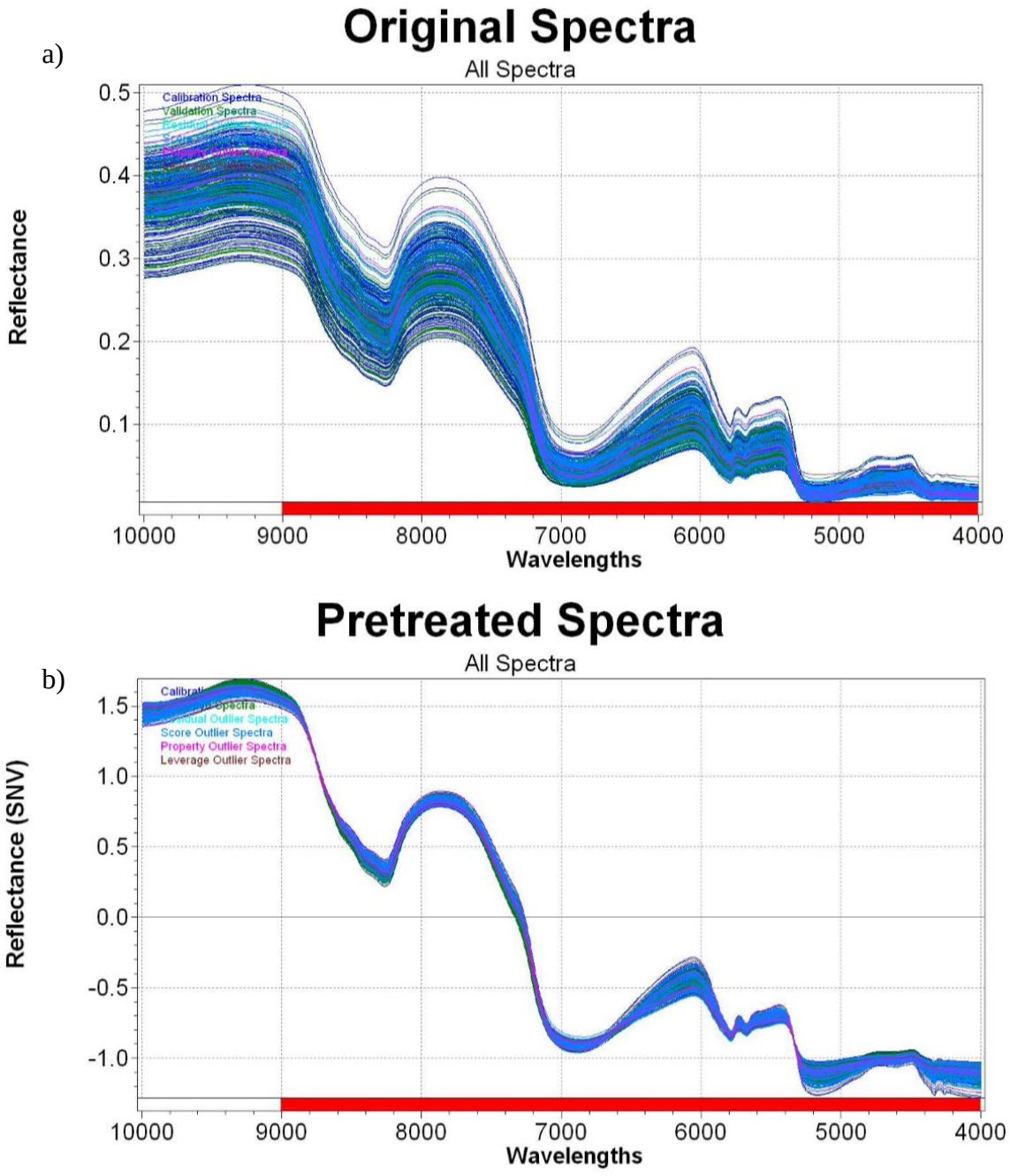

Figure 14: a) Collected spectra without SNV pretreatment; b) Collected spectra with SNV pretreatment

Wavelength selection was done to isolate the important sections of wavelengths relevant to the calibration. To accurately determine the wavelengths of interest the property wavelength regression plot was used. When analyzing the property wavelength regression plot, shown in Figure 15, the higher the regression coefficient, the more the wavelength was associated with the changes in the sample (Büchi, 2007). A maximum value of 1 shows the strongest association (Büchi, 2016). The peak with the highest regression coefficient, 0.735, was found at 8776 1/cm. This peak does not have an established association, however the additional peaks at 6836, 6320, 5618, and 5304 1/cm exceeded a regression coefficient of 0.4, and showed valuable information. These peaks coincided with the known wavenumbers of starch at 6993, 6897, 6545, 6494, 6329, 5263, and 5000 1/cm, and cellulose at 5618 and 5495 (Büchi, 2007). An additional graph examined was the regression coefficients plot, shown in Figure 16. This plot was used to remove "noise" that did not add valuable information to the analysis of the sample. Defined peaks and valleys are seen when the instrument is able to ascertain valuable information from the data collected. These defined peaks and valleys can be seen in Figure 16 in the wavelengths between 9,000 and 4,000 1/cm. A region of noise can be seen in the region of 10,000 to 9,000 1/cm. This region provides little to no spectral information and is not valuable in the analysis of the sample (Palermo, 2016). Removal of the region allowed the instrument to disregard the noise. This increased both the accuracy and consistence of the calibration. Analysis of these plots showed that the most valuable information accounting for flow aid was between the wavenumbers of 9,000 and 4,000 1/cm. The region above 9,000 1/cm was therefore removed from the calibration.

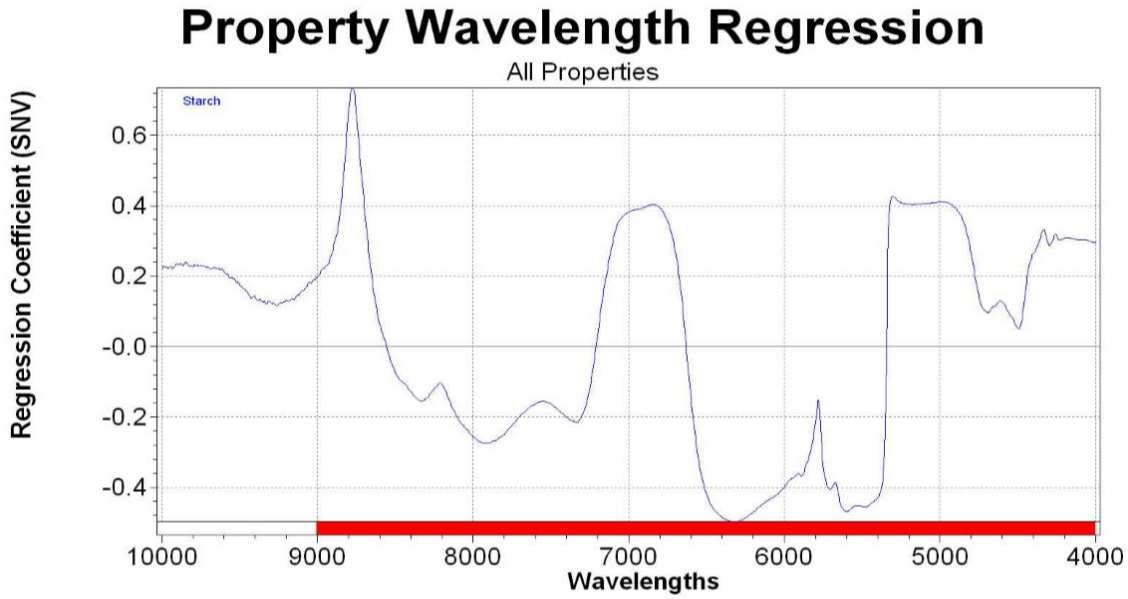

Figure 15: Property wavelength regression plot showing wavelength characteristics of Cheddar cheese samples with flow aid

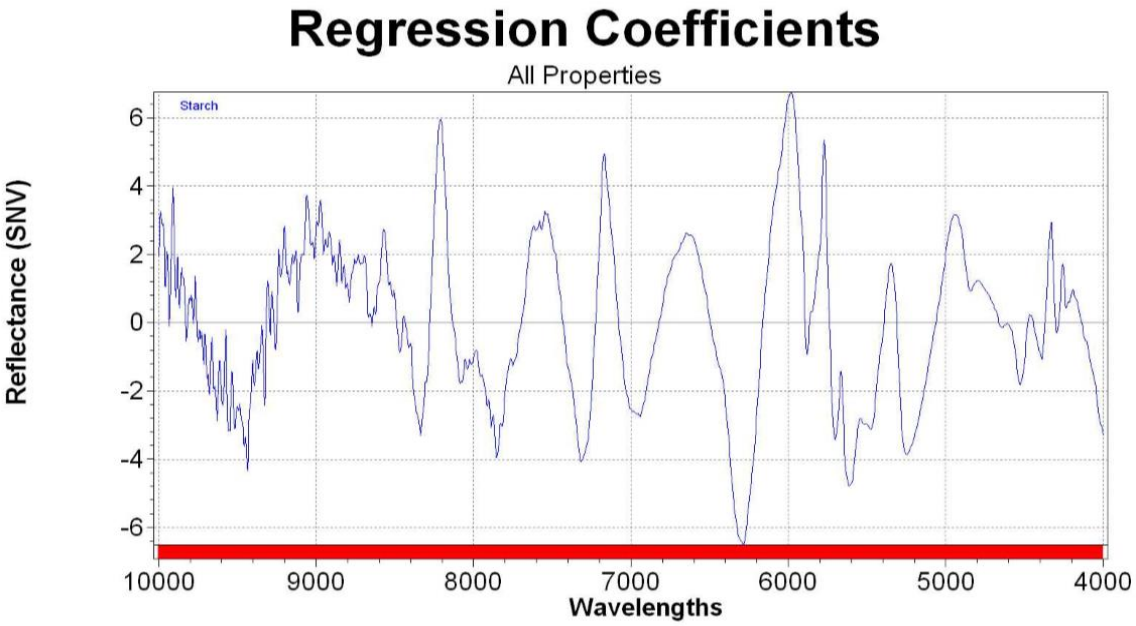

Figure 16: Regression coefficients plot showing wavelength characteristics of Cheddar cheese samples with flow aid

Selection of principal components (PC) established the components of the spectra responsible for the parameter calculations. PC are one of the key features that determine the quality of the calibration (Büchi, 2007). Based on the information presented in the secondary factor selection plots, it was determined that 10 PC were ideal for this calibration. To determine the optimal number of PC, several graphs were examined. The first graphs considered were the Q-value and consistency, shown in Figure 17. The Q-value, Figure 17a, is considered a general quality marker for PC selection. For a quantitative calibration such as this, a minimum Q-value of 0.6 was required, but a Q-value higher than 0.8 was preferable (Büchi, 2007). With the selection of 10 PC, a Q-value of 0.867 was achieved. It was possible to achieve Q-value of 0.885 at 15 PC, however consistency and regression coefficients were negatively affected, and there was also a concern of over fitting. Consistency, Figure 17b, looks at the relationship between the standard errors of the calibration and validation set. Ideally, consistency will be around 100%, indicating the standard errors of the calibration and validation sets are equal, with the acceptable range being 80-110% (Büchi, 2007). At 10 PC a consistency of 102.9% was achieved. As PC selection moved beyond 10 PC, the consistency began to drop. A low level of consistency is an indicator that many PC have been selected and there is overfitting of the calibration (Büchi, 2007).

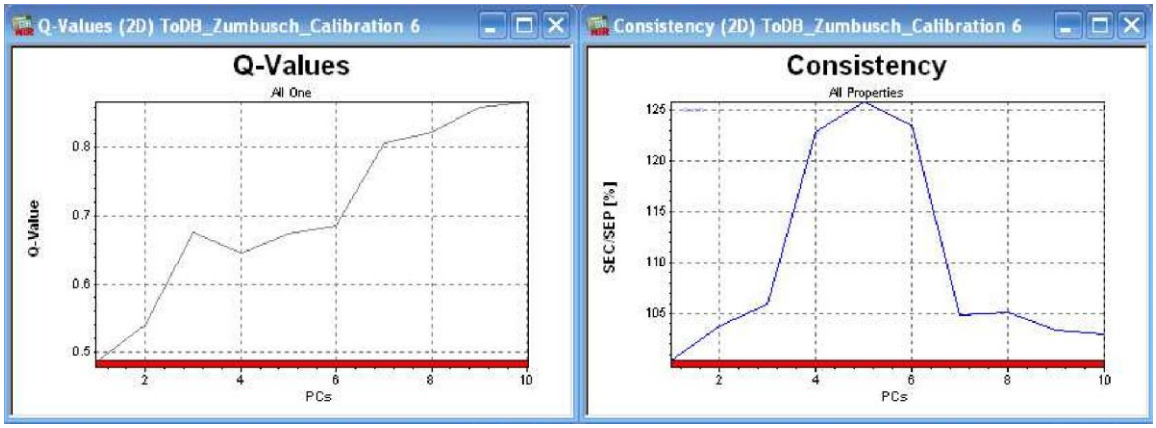

Figure 17: a) Q-values based on principle component selection; b) Consistency based on principle component selection

With the range of PC narrowed down with the Q-value and consistency percent, refinement of PC selection was done with the remaining graphs. Validation set (V-set) and Calibration set (C-set) Predicted Residual Error Sum Square (PRESS) graphs, Figures 18a and 18b, respectfully, were looked at in concert. When looking at these graphs, the fewest number of PC were selected where both graphs were minimal while approximately being equal. At 10 PC V-set PRESS was 15.0 and C-set PRESS was 32.8. Similarly, the V-set standard error of prediction (SEP) and C-set standard error of calibration (SEC) were minimized while maintaining approximate equality. At 10 PC V-set SEP was 0.31 (Figure 18c) and C-set SEC was 0.32 (Figure 18d).

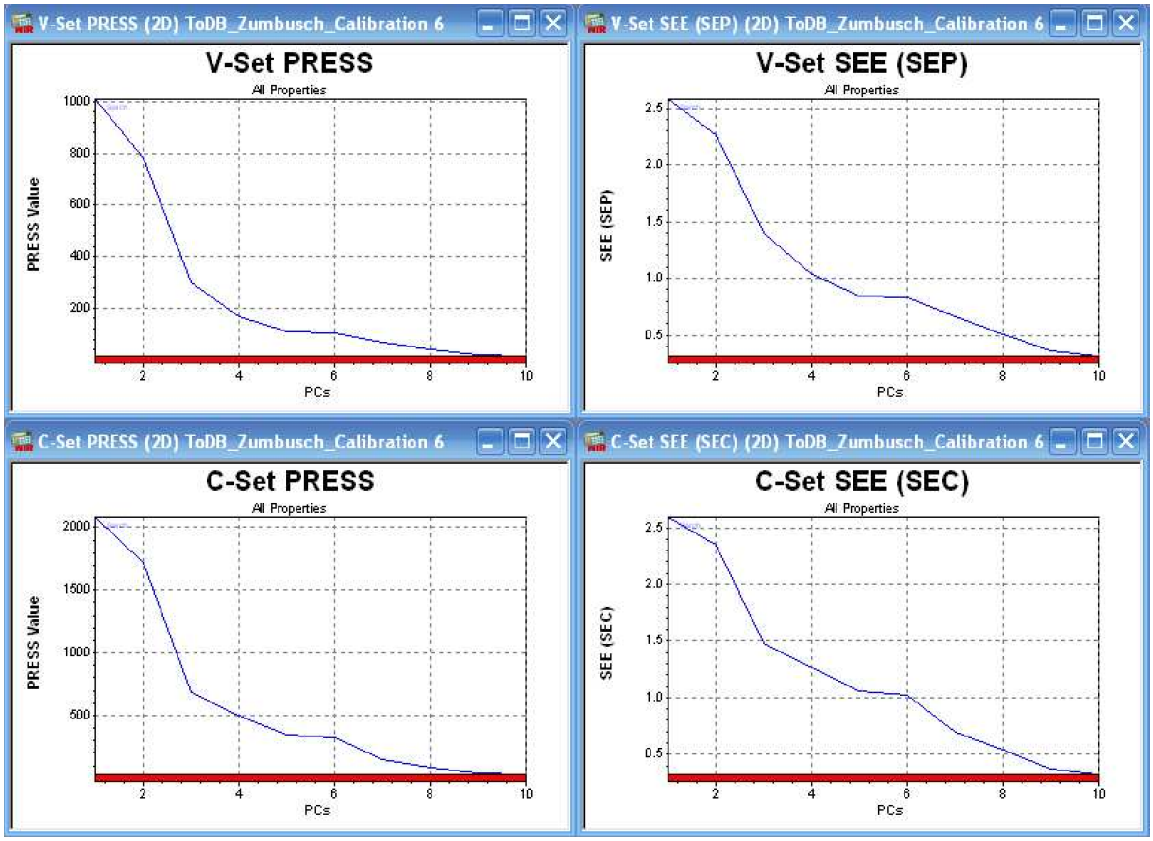

Figure 18: a) Validation set predicted residual error sum square based on principle component selection; b) Calibration set predicted residual error sum square based on principle component selection; c) Validation set standard error of estimation based on principle component selection; d) Calibration set standard error of estimation based on principle component selection

The V-set and C-set regression coefficients represent how well the predicted value matched with the reference value (Büchi, 2007). A value as close as possible to 1 indicates strong correlation between the values, with a minimum value of 0.9 needing to be achieved to deem a calibration acceptable (Büchi, 2007). A V-set regression coefficient of 0.994 and a C-set regression coefficient of 0.993 was achieved with the selection of 10 PCs, as shown in Figure 19.

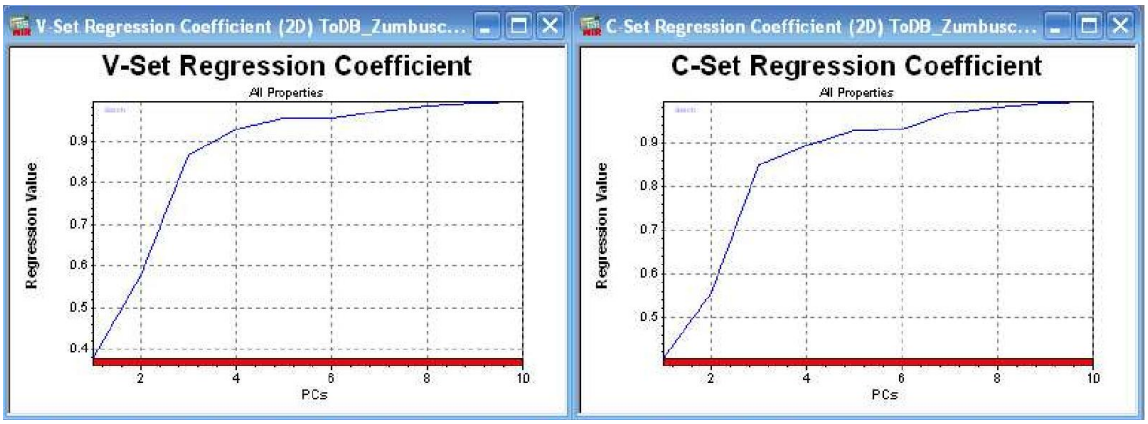

Figure 19: V-set and C-set regression coefficient graphs for principle component selection

The last graph looked at for the selection of PC was the V-set bias graph. V-set bias is an indicator of precision. When the V-set bias is as close to zero as possible, there is the smallest deviation from the predicted to true value (Büchi, 2007). The V-set bias (Figure 20) was minimized to 0.011 with the selection of 10 PC.

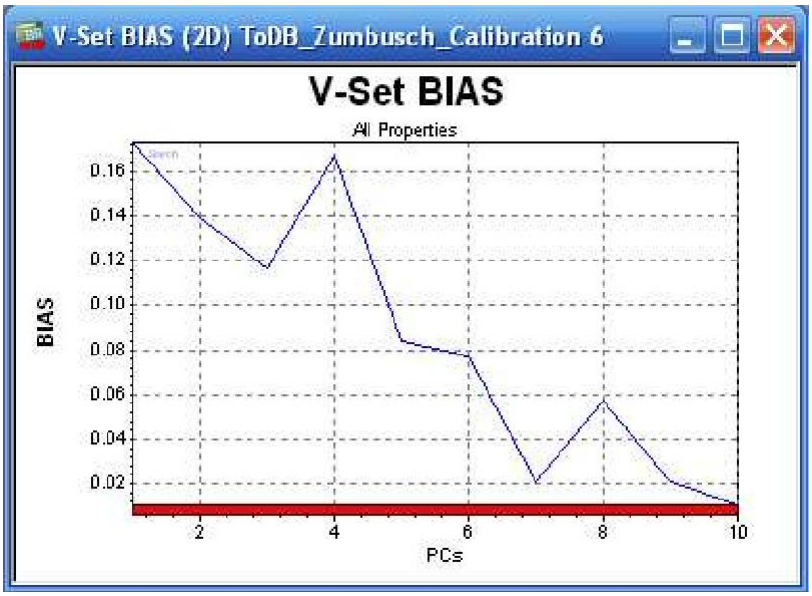

Figure 20: V-set bias for principle component selection

With selections based on the information at hand, the refinement of the calibration was complete. Pretreatment, wavelength, and PCs established the calibration was tested for accuracy on outside samples.

4.3.3 Validation

Initial calibration development was done to confirm that NIR was a valid method for accurately analyzing the flow aid content of a sample. The literature has shown that NIR can be used to measure the fat, moisture, and protein content of cheese, but it has failed to accurately assess its minor constituents and the age of Cheddar, even with a robust calibration (Frankhuizen, 2008). However, because of the specific compositional changes that are produced by the flow aid addition, and there quantities having a direct correlation, NIR should serve as an accurate instrument. After establishment of the initial calibration, an r^2 of 0.9945 with a standard deviation of 0.2158 was achieved, as shown in Figure 21. To test the true accuracy of the calibration, a sample set was created using the same Cheddar cheese and flow aid. Samples ranged from 1.96 to 4.23% flow aid were scanned in as unknown sample using the established preparation and presentation

method. These samples were calculated gravimetrically and mixed by hand. NIR analysis was able to accurately predict the quantity of flow aid to within 0.18% of the true value, as shown in Table 3.

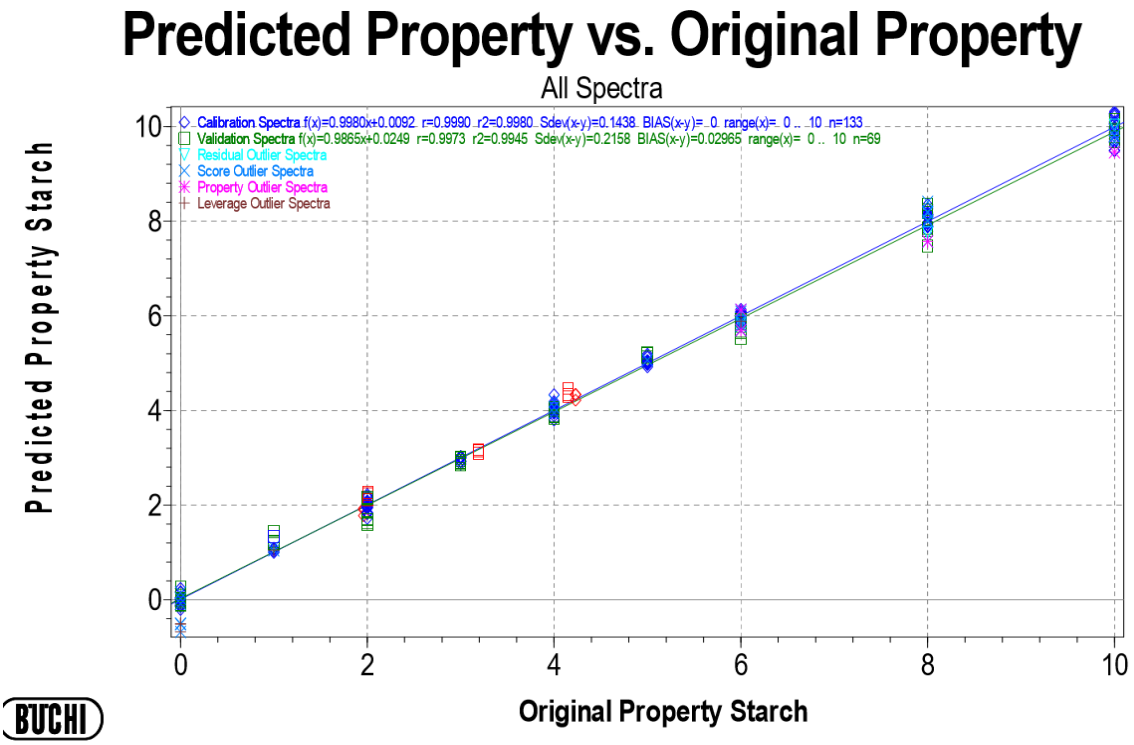

Figure 21: Initial calibration for flow aid analysis

Table 3: Results of NIR prediction of unknown Cheddar cheese samples

Sample	True Value (%)[1]	Predicted Value (%)	Difference
Cheddar 1	1.96	1.78	0.18
Cheddar 2	2.01	2.14	-0.13
Cheddar 3	3.19	3.08	0.11
Cheddar 4	4.15	4.27	-0.12
Cheddar 5	4.23	4.24	-0.01

1: Calculated gravimetrically and mixed by hand

After confirmation of the ability and accuracy of NIR analysis of flow aid, the calibration robustness was increased through the addition of Cheddar cheeses from 5 manufacturers. These Cheddars were mixed with the same flow aid used to create the original calibration in concentrations ranging from 0.5 to 10%. Many manufactures target between the 2 and 4% range for their flow aids, making the range from 0 to 6% the most critical (Bussell, 2016). After incorporation into the calibration, an r^2 of 0.988 with a standard deviation of 0.315 was achieved, as shown in Figure 22. Although the calibration was created with Cheddar cheese, other types of shredded cheese were tested to determine if the calibration would be applicable. Parmesan, Mozzarella, Colby and Monterey Jack (CJ), and Mexican blend were prepared with the established method and analyzed. Parmesan, Mozzarella, and the Mexican blend all tested as outliers. This is likely due to the variation in moisture, fat, and protein content of these cheese deviating from Cheddar. CJ was similar enough in composition to Cheddar that it did not test as an outlier as the other cheeses had. To determine the accuracy of the calibration, commercially produced shredded Cheddar and CJ samples purchased from Byerlys grocery store in St. Louis Park (containing an unknown amount of flow aid) were prepared and analyzed by the NIR. The amount of flow aid was determined through analyzing the cheese with the total dietary fiber assay, and the total starch assay as described in Chapter 3. The results from these assays were combined to calculate total flow aid percentage in the sample. Analysis indicated Cheddar cheese was accurate within 0.37% while CJ was accurate within 1.18%, as shown in Table 4.

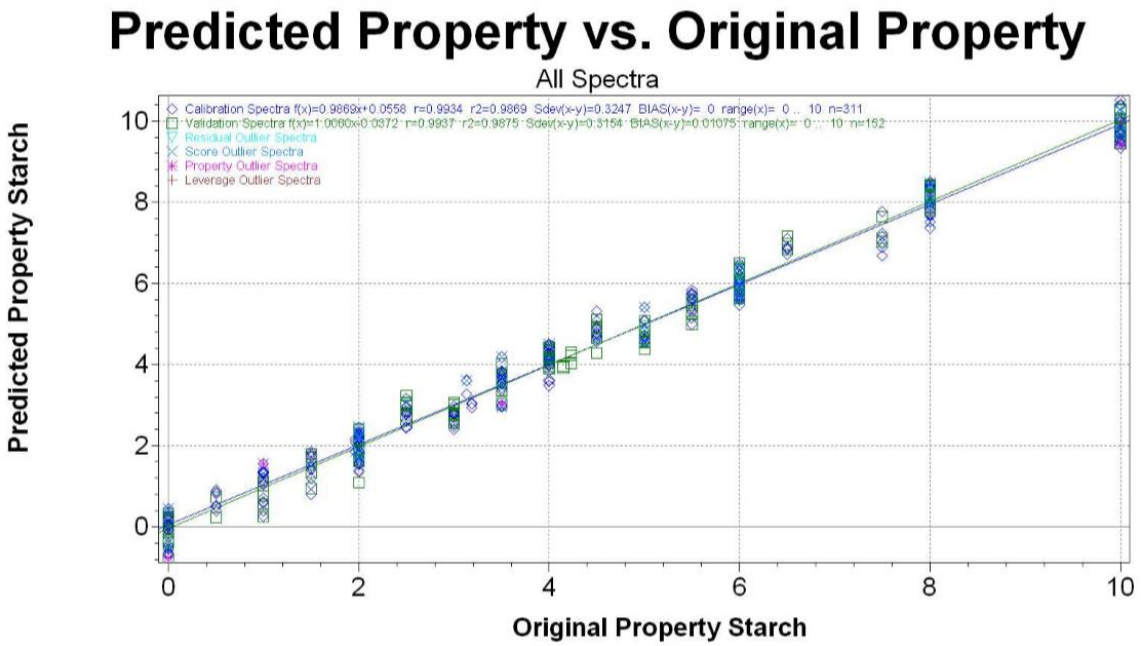

Figure 22: Practiced vs original properties of final calibration

Table 4: Analysis of results of Cheddar and Colby & Monterey Jack samples

Sample	NIR Flow Aid prediction (%)	Reference Method Flow Aid (%)	Difference From NIR Prediction
Cheddar 1	3.17	3.18[1]	0.01
Cheddar 2	4.62	4.29[1]	-0.33
Cheddar 3	2.80	3.17[1]	0.37
Colby & Monterey Jack	2.74	3.92[1]	1.18
Cheddar - 3% hand blended	2.95	3.09[2]/3.45[1]	0.14

1: Total dietary fiber and total starch assay were used as reference methods; 2: Total starch assay was used as reference method in combination with flow aid analysis

The results of NIR analysis may be more accurate than reference values indicate. A control sample of Cheddar cheese with 3% flow aid addition tested at 2.95% through NIR analysis. Total starch analysis of this sample indicated 1.91% starch. Factoring for 62% of the flow aid composition being starch, as confirmed through analysis, the total flow aid was calculated at 3.09%. Combining the results from the total dietary fiber assay, the

total flow aid was calculated at 3.43%. This could indicate the total dietary fiber method may need to go through further refinement to increase its accuracy when analyzing cheese. When testing samples for accuracy it is advised that the total starch assay discussed in Chapter 3 be used to analyze the starch content of the cheese and flow aid to calculate total flow aid percent.

4.4 Conclusion

The NIR calibration was able to successfully determine the content of flow aid accurately to within 0.37% for commercially produced shredded Cheddar cheese samples. Results from analysis of a Colby/Monterey Jack blend was not accurate enough to confirm flow aid content in its current form, but shows the potential for this calibration to be used for this application. Through visual analysis of the scanning surface the method developed for Cheddar is an effective preparation and presentation method for Mozzarella, Colby & Monterey Jack, and Mexican blends. However individual calibrations for these cheese would need to be developed. Results were not clear that this would be an acceptable preparation and presentation method for Parmesan or other hard cheeses. After establishment of future calibrations, combining spectra from different cheese types into one calibration would be worth exploring to determine if this would increase or decrease the accuracy. There is the potential that the compositional changes for each cheese type could diminish their effects on the calibration. However, with the variation in moisture, fat, and protein content it may be difficult to create a single calibration that would be effective for all types of cheese.

5 Concluding Remarks

The cheese industry continues to be a strength in the dairy industry. Even with the declining sales in fluid milk, consumers are continuing to turn to cheese as a natural, high protein product. Shredded cheese holds a large market share in the cheese industry. In order to boost quality controls and consumer confidence in the industry it is important to continue to look for newer, more rapid analytical techniques to control the quality of the product. The flow aid used in shredded cheese product have come under scrutiny after reports of over use for economic gain. Incidences like these can damage the industry and erode consumer confidence. Two analytical techniques were developed during this research to help monitor the use of flow aids. An enzymatic starch analysis method provides an accurate method to determine flow aid concentrations at a low cost in approximately 3 hours. The NIR calibration is a much more rapid method, taking less then ten minutes, but require a more expensive upfront cost. A Megazyme kit to run the enzymatic starch assay will cost $350 for one hundred samples, whereas an NIR can cost $20,000. Ultimately, this research has provided the dairy industry and regulators with two valuable analytical methods to ensure that the standards of the cheese industry are being met. This will ensure the continued strength of the cheese market and rebuilt any consumer confidence that was lost.

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7 Appendix

Appendix 1: Calibration Error Sources with Recommended Solution (Workman, 2008)

Variance Source	Recommended Solution
Nonhomogeneity of sample	• Improve mixing guidelines • Improve grinding procedures • Average replicate repacks • Rotate sample cup • Measure multiple readings of large sample volume
Laboratory Error	• Laboratory audit to correct procedural error • Suggest improvements on analytical procedures • Retrain analysts on procedures • Check and recalibrate reagents, equipment, etc.
Physical Variation in Sample	• Improve sample mixing during sample preparation • Diffuse light before it strikes the sample using a light diffusing plate • Pulverize sample to less the 40-µ partial size • Average multiple repacks • Rotate sample, or average five sample measurements
Chemical variation in sample with time	• Freeze-dry sample for storage and measurement • Immediate data collection and analysis following sample preparation • Identification of kinetics of chemical change and avoidance of rapidly changing spectral regions
Population sampling error	• Review calibration set selection criteria • Use sample selection techniques such as SUBSET or PICKS used for Selection Calibration Set

Non-Beer's law relationship (nonlinearity)	• Use smaller concentration ranges for each calibration • Use baseline correction such as standard normal variate or polynomial baseline correction • Use one or more indicator variables • Try shorter path length • Check dynamic range of instrumentation
Spectroscopy does not equal manual chemistry	• Use different chemical procedures • Redefine analytical requirements in terms of known chemistries
Instrumentation noise	• Check instrument performance • Determine signal-to-noise • Check precision with standard sample replicate measurements
Integrated circuit problems	• Replace faulty components
Optical polarization	• Use depolarizing elements
Sample presentation extremely variable	• Improve sample presentation methods • Investigate wide variety of commercially available sample presentation equipment
Calibration modeling incorrect	• Select and test calibration model carefully • Calculate new equation
Poor calibration transfer	• Select calibration with lowest noise, wavelength shift sensitivity, and offset sensitivity • Identify and transfer actual wavelengths and corresponding regression coefficients
Outlier samples with calibration set	• Cumulative normal plots • Center program by ISI • DISCRIM by Bran and Luebbe
Transcription errors	• Triple-check all hand-scribed data

Appendix 2: Sample-Associated Factors in NIR Analysis (Williams, 2008)

Sampling

1) Type of Sampler
2) Location of Sampler
3) Material to be Sampled
4) Foreign Material
5) Physical Nature of Material
6) Size of Sample
7) Flow Characteristics
8) Sample Transfer Method
9) Blending
10) Storage
11) Identification/ Documentation
12) Variability of Population
13) Frequency of Sampling
14) Subsampling
15) Sample Selection

Samples

1) Type of Material
2) Composition
   Oil
   Moisture
   Fiber
3) Physical Texture
4) Foreign Material
5) Blending
6) Identification/ Documentation

Sample Preparation

1) Type/Model of Instrument
2) Type of test
3) Sample Cell Type
4) Sample Cell Size
5) Particle Size
6) Bulk Density
7) Composition
8) Physical Nature
9) Stratification
10) Static Electricity
11) Cell Loading
12) Cell Cleanup
16) Grinder Types

Appendix 3: Megazyme total starch analysis procedure

Material:

Supplied in Megazyme Total Starch HK kit. See data booklet for preparation instructions.

1) Thermostable a-amylase [12 ml, 8300 U/ml on soluble starch at pH 5.0 and 40ºC]. Stable for > 3 years at 4ºC.
2) Amyloglucosidase [10 ml, 3300 U/ ml on soluble starch] at pH 4.5 and 40ºC]. Stable for > 3 years at 4ºC.
3) Buffer (15 ml, pH 7.6) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4ºC.
4) $NADP^+$ plus ATP. Stable for > 5 year at -20ºC
5) Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 ml. Stable for > 2 years at 4ºC.
6) D-glucose standard solution (5 ml, 1.0 mg/ml). Stable for > 5 years at room temperature.
7) Standardized regular maize starch control. Stable for > 5 years at room temperature.

Reagents not supplied in kit

1) Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM).

   Add 5.8 ml of glacial acetic acid to 900 ml of distilled water. Adjust pH of solution to 5.0 with 1 M sodium hydroxide solution (approximately 30 ml). Dissolve 0.74 g calcium chloride dehydrate in solution and adjust volume to 1 liter. Add 0.2 g of sodium azide and dissolve. Stable for approximately 2 year at room temperature.

Equipment:

1) Glass test tubes (round bottom; 30 ml; 20 x 150 mm)
2) Glass beads
3) 50 ml plastic centrifuge tubes
4) Disposable plastic cuvettes (1 cm light path)
5) Positive displacement pipettor
   a. 5.0 ml combitip to dispense 0.1 ml aliquots of amyloglucosidase solution, imidazole buffer, and $NADP^+$/ATP
   b. 50 ml combitip to dispense 3 ml aliquots of α-amylase solution
6) Bench top centrifuge (required speed 3,000 RPM; approximately 2000 g)
7) Analytical balance
8) Spectrophotometer set at 340 nm

9) Vortex mixer
10) Water bath
11) Whatman Grade 1 filter paper
12) Plastic funnel
13) 100 ml volumetric flask

Procedures:

Cheese:

1) Homogenize 200 g of shredded cheese sample in 10 cup food processor with S blade insert for 60 seconds.
2) In duplicate, add 0.5 g of sample to glass test tube containing approximately 15 glass beads.
3) Add 5.0 ml of 80% v/v aqueous ethanol, and incubate in 80-85ºC water bath for 5 minutes. Mix contents of test tube on a vortex mixer. Add an additional 5.0 ml 80% v/v aqueous ethanol.
4) Centrifuge tube for 10 minutes at 3,000 RPM on a bench top centrifuge. Carefully pour off the supernatant and discard.
5) Add 10.0 ml of 80% aqueous ethanol and place in 80-85ºC water bath for approximately 30 seconds to help soften the pellet. Resuspend the pellet by stirring on a vortex mixer. Centrifuge as above and carefully pour off the supernatant and discard.
6) Add 3.0 ml of thermostable α-amylase. Incubate tube for 6 minutes in a boiling water bath. Stir tube vigorously on vortex mixer after 2, 4, and 6 minutes. (ensures complete homogeneity of slurry and prevents any sample from expelling from tube as alcohol evaporates)
7) Place tube in 50ºC water bath and allow mixture to equilibrate for 3 minutes. Add 0.1 ml amyloglucosidase and stir on vortex mixer. Incubate tube in 50ºC water bath for 30 minutes.
8) Transfer entire contents of the test tube to a 100 ml volumetric flask. Use wash bottle with distilled water to rinse the test tube. Adjust volume with distilled water and mix thoroughly.
9) Transfer approximately 30 ml of solution to 50 ml centrifuge tube and centrifuge for 10 minutes at 3,000 RPM.
10) Using grade 1 filter paper, gravity filter 5 ml of solution. Use this aliquot for analysis.

Flow Aid:

Procedures running analysis of flow aid follows the same procedure with the following exception.

1) As flow aid are in powdered form no homogenization step in needed.
2) Adjust sample size to 0.1 g. Glass beads are optional
10) Transfer 1.0 ml of solution to 10 ml graduated cylinder and dilute to 10 ml with distilled water. Use this 1:10 dilution for analysis.

Analysis:

Wavelength: 340 nm
Cuvette: 1 cm light path, 3 ml (glass of plastic)
Temperature: ~25ºC
Final Volume: 2.27 ml
Sample Solution: 4-80 g of D-Glucose per cuvette

Pipette into Cuvettes	Blank	Cheese Sample	Flow Aid Sample
Distilled Water (~25ºC)	2.05 ml	1.85 ml	1.90 ml
Sample	-	0.20 ml	0.15 ml
Solution 3 (buffer)	0.10 ml	0.10 ml	0.10 ml
Solution 4 ($NADP^+$/ATP)	0.10 ml	0.10 ml	0.10 ml
Read the absorbance of the solution after ~ 3 minutes (A_1). Start reaction with addition of:			
Suspension 5 (HK/G-6-PDH)	0.02 ml	0.02 ml	0.02 ml
Read the absorbance of the solution at the end of the reaction (A_2). May take up to 30 minutes. Read absorbance in 5 minute intervals for the first 20 minutes and 2 minute intervals after that until absorbance remains the same.			

Calculations:

Determine the absorbance difference for both blank and sample (A_2-A_1). To obtain $\Delta A_{D\text{-}glucose}$, subtract the absorbance difference of the blank from the sample. $\Delta A_{D\text{-}glucose}$ needs to be above 0.100 absorbance units to achieve accurate results. The concentration of starch can be calculated from the following formula.

$$c = \frac{V * MX}{\varepsilon * d * v} * \frac{162}{180} * \Delta A_{D-glucose}$$

Where:

V = Final volume (ml)
MX = Molecular weight of D-glucose (g/mol)
ε = Extinction coefficient of NADPH at 340 nm = 6300 ($1*mol^{-1}*cm^{-1}$)
d = Light path (cm)
v = Sample volume (ml)
162/180 = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch)

Creating the equation (for cheese samples):

$$c = \frac{2.27 * 180.26}{6300 * 1 * 0.10} * \frac{162}{180} * \Delta A_{D-glucose}$$

Therefore (for cheese samples):

$$c = 0.5842 * \Delta A_{D-glucose}$$

If the sample has been diluted, as it will be with analysis of flow aids, the result are multiplied by the dilution factor. To convert the content to g/100g use the following calculation.

$$\frac{c_{Starch}(g/L\ sample\ solution)}{Weight_{Sample}(g/L\ sample\ solution)} * 100$$

Appendix 4: NIR sample preparation method

Materials

1) Food processor with 10 cup container and S blade insert
2) 15 x 100 mm NIR glass petri dish
3) 9.5 cm polypropylene press plate
4) Force Plate
5) C-clamp

Equipment

1) Büich N500 NIRFlex with solids attachment
2) NIRCal chemometric software

Procedure

1) 200 grams of sample weighed out and transferred to a food processer.
2) Sample ground for 60 second.
3) 75 g of homogenized sample is weighed out and placed in the NIR petri dishes.
4) The 75 g sample is to be formed into a ball and placed in the center of the NIR petri dish.
5) A press plate is placed on top of the sample and centered on the plate.
6) Using the clamp, apply 300N of pressure in the middle of the sample.
7) Increase to 300N in a smooth and consistent manner, maintaining the levelness of the press plate.
8) The sample should start to flow slightly around the press plate. Leave clamp in place for 60 seconds. Pressure registered on the force plate will decrease during the 60 seconds due to the flow of the cheese. This is a desired result.
9) Release pressure from clamp.
10) Place Petri dish on NIR and scan sample.
11) Remove sample and thoroughly clean petri dish.
12) Repeat steps 4 through 11 in triplicate.