ABSTRACT

FOX, AARON J. The Influence of Bleaching Agent and Temperature on Bleaching Efficacy of Fluid Whey and Whey Retentate and Norbixin Interactions in Whey. (Under the direction of Dr. MaryAnne Drake).

The demand and applications of whey ingredients are increasing. It is necessary to produce consistent and uniform whey products to meet the demand. Harsh chemical bleaching agents are detrimental to the flavor and functionality of whey protein. The two currently approved bleaching agents in the US are hydrogen peroxide and benzoyl peroxide. These bleaching agents cause off flavors in the final whey product. It is important to investigate bleaching at various points during processing in order to find the optimal bleaching parameters that cause the least off flavor.

The objective of the first study was to compare bleaching efficacy of hydrogen peroxide and benzoyl peroxide at elevated solids and protein concentrations in liquid whey and whey retentate. Cheddar cheese whey was produced in triplicate. Liquid whey and liquid retentate (80% protein [w/w]) was bleached with benzoyl peroxide (100mg/kg) or hydrogen peroxide (250mg/kg) at 5^{o}C or 50^{o}C. Norbixin extractions and volatile analyses were conducted on all samples. Bleaching of whey retentate with hydrogen peroxide was higher than liquid whey at both 5^{o}C and 50^{o}C ($p<0.05$). Temperature did not affect benzoyl peroxide bleaching in liquid whey ($p>0.05$). In liquid whey retentate, there was no difference between hydrogen peroxide and benzoyl peroxide bleaching at 5^{o}C or 50^{o}C ($p>0.05$). Hydrogen peroxide bleached whey retentates had higher concentrations of pentanal, hexanal, heptanal, octanal, nonanal, E-E-2,4-nonadienal, 1-octen-3-one, and 2-pentylfuran than liquid wheys

and other retentates ($p<0.05$). These results suggest that hydrogen peroxide can provide a comparably neutral colored final product to benzoyl peroxide bleached product when applied to retentate.

The objective of the second study was to evaluate norbixin binding behavior in liquid whey. Fresh liquid whey was concentrated to whey protein concentrate at 34 or 80% protein (w/w) at 8^{o}C or 50^{o}C. Retentates were held or recirculated through an ultrafiltration membrane system for 3 h at 8^{o}C or 50^{o}C. Norbixin extractions were performed on all samples, permeates, and membrane cleaning solutions. In a separate experiment, liquid whey was concentrated with 0, 1, 4, or 8% (w/v) NaCl or $CaCl_2$ added to the whey and the diafiltration water. Norbixin extractions were performed on all retentates and permeates. A final experiment investigated norbixin binding to individual protein solutions (alpha-lactalbumin and beta-lactoglobulin) through fluorescence quenching of surface tryptophan residues. Increased temperature, shear and/or ionic strength decreased norbixin concentrations in whey ($p<0.05$). Fluorescence quenching results suggested that norbixin was not binding whey proteins through hydrophobic interactions. These results demonstrate that increasing the ionic strength, temperature, and shear during ultrafiltration can remove more than 50% of norbixin from liquid whey retentate.

The Influence of Bleaching Agent and Temperature on Bleaching Efficacy and Volatile of Fluid Whey and Whey Retentate and Norbixin Interactions in Whey

by
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Committee Chair

DEDICATION

To my parents, Ed and Evelyn, who have always supported me. I wouldn't have made it this far without you. And to my father, Joe, who couldn't be here to see this day but I hope he would be proud.

BIOGRAPHY

Aaron James Fox was born in Houston, TX on January 21st, 1982 to Joseph and Evelyn Fox. He has a brother, Jory Fox, and a sister, Kelsey Walker. Aaron spent every summer in New Mexico with his extended family, the Foxes and the Gabaldons. It was in New Mexico where he learned the meaning of hard work doing manual labor around the house and farm. It was this experience that let him know he needed to go to college. Aaron lived in The Woodlands, TX until he was 15 years old and was reluctant to move to Holly Springs, NC in 1997. Aaron finished high school in Fuquay-Varina, NC and began his college career at Wake Technical Community College in Raleigh, NC. While at Wake Tech, he discovered he had a passion for chemistry. During this time, Aaron worked in a sports nutrition store inside of a gym and became interested in weight-training and nutrition. He quickly learned everything he could about whey protein and other athletic performance enhancers. Aaron got into North Carolina State University in 2004 and received his Bachelor's in Food Science in 2006. After taking a few odd jobs, Aaron got a job in Dr. MaryAnne Drake's food science lab at NC State. In 2010, after two years assisting the Drake lab in producing and processing whey protein, Dr. Drake offered Aaron a great opportunity to become a graduate student in her lab. In his free time, Aaron enjoys playing music, making balloon animals, and camping.

ACKNOWLEDGEMENTS

I would like to acknowledge my parent's hard work in keeping me in-line and encouraging me throughout my entire education and life. Mom, you inspire me to be more. As a single mother, raising three kids on your own without once giving up on us proves that you are the bravest and strongest person I've ever met. Ed, you entered this family without hesitation and supported me with open arms. You saw me at my worst and selflessly loved me like a son, for that, I am forever grateful and will always be there for you as you were for me.

To my siblings, Jory and Kelsey, who have taught me to be understanding and patient. I hope we are always close.

To my Drake lab peers, who stuck together under pressure and helped me through my 18 hour pilot plant days. Your help and knowledge in this field has made this journey much smoother.

To Dr. MaryAnne Drake, who took me under her wing and gave me a chance when no one else would. I can't thank you enough for the opportunities you have given me.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Cheese and, subsequently, whey have been produced for roughly 3000 years. It was discovered that storing milk in the stomachs of calves caused the separation of two layers; curds and whey. The milk coagulated due to the naturally occurring enzyme, chymosin, found in calf stomachs (Smithers, 2008). The curds, of course, were made into various cheeses while the whey had limited uses. Whey was believed to have therapeutic properties and was prescribed for various ailments as early as 460BC by Hippocrates and continuing through the middle ages and as late as World War II in European spas (Susli, 1956; Holsinger et al., 1974). In Europe, whey was made into whey-borse (a broth), whey-butter, whey-porridge, and whey-whig (a drink with herbs) (Trelogan, 1970). In modern times, whey has been viewed as a waste product which has been sold as animal feed or discarded into rivers, lakes, oceans, fields, and sewage systems. Whey has a biological oxygen demand (BOD) of >35,000ppm and is considered one of the most polluting byproducts of the food industry (Smithers, 2008). The U.S., the leading producer of cheese, has steadily increased cheese production every year with a forecasted yield of 4,920,000 metric tons in 2012 (USDA 2012).

Currently, in 2012, whey is not a waste product. Whey is an opaque, yellow-green liquid comprised of lactose (44-52g/L), proteins (6-10g/L), minerals (2.5-7.2g/L), and water (Jelen, 2003). Whey represents approximately 90% of the original volume of milk used in cheese production. The majority of the protein fraction (80% of proteins) consists of beta-lactoglobulin (~3g/L) and alpha-lactalbumin (~1.2g/L) (Schmidt et al., 1984; Etzel, 1995).

The remaining protein fraction contains immunoglobulins, bovine serum albumin, lactoferrin, and lactoperoxidase. Since liquid whey is mostly water with a high lactose concentration, the proteins are usually concentrated to increase value and to optimize application as a food ingredient. Liquid whey can be spray-dried into powder or it can be filtered to remove less desirable components (i.e., fat, lactose, minerals) to make a powder with increased protein content and higher value (Onwulata, 2008). The two most common forms of whey protein are powdered whey protein concentrate (WPC) and whey protein isolate (WPI). Whey protein concentrates range from 20-89% protein [w/w] and whey protein isolates must have greater than 90% protein [w/w] content (Tunick, 2008). In recent years, whey protein has become popularized as a functional food ingredient. Whey protein addition is an inexpensive way to enhance protein content and general nutritive value in foods (Tunick, 2008). Consumption of these proteins has been shown to provide health benefits such as: exercise recovery, weight management, cardiovascular health, anti-cancer effects, anti-infection activity, wound repair, and infant nutrition (Smithers, 2008). These proteins can also have other functions in foods such as gelation, thermal stability, foam formation, and emulsification (Foegeding et al., 2002).

Whey proteins are commonly used in breads, cakes, pastries, candy, chocolate/fudge, coffee whiteners, crackers/snack foods, diet supplements, fruit beverages, gravies/sauces, infant formula/baby food, mayonnaise, pasta, pie filling, ice cream, processed fruits/vegetables, soup, meat/sausage, and sports drinks (Cryan, 2001). Whey proteins have been implemented into protein-rich nutritional beverages due to the high-quality and

functional proteins they provide. Acidified whey protein beverages have gained consumer and processor interest due to their increased solubility at lower pH (Beecher et al., 2008). The low pH allows for a wider variety of flavors such as grape, lemon-lime, and fruit punch. The low pH also allows the product to have a lower pasteurization temperature or time as well as an extended storage life due to a more hostile environment for microbial growth (Beecher et al., 2008).

Whey proteins stabilize emulsions by creating a barrier between hydrophilic and hydrophobic food components (Burrington, 2005; Haines, 2005). Food foams such as ice cream, soufflés, and frothed drinks are stabilized by surface-active agents such as whey proteins (Foegeding et al., 2002). Whey proteins are also used in processed cheeses to improve melting, slicing, and spreading (Foegeding et al., 2002; Foreign Agricultural Service, 2003; Kapoor and Metzger, 2004). Upon denaturation, whey proteins bind large amounts of water and improve moisture retention in foods such as muscle proteins (McCord, 1998). The primary protein in whey, beta-lactoglobulin, does not denature until 78^{o}C, which makes it an excellent heat induced gelation agent (Paulsson and Dejmek, 1990). Beta-lactoglobulin is ideal for enhancing protein in acidified beverages due to its high solubility at low pH (Smithers, 1996). Whey powder is an excellent browning agent/flavor enhancer in baked goods. The lactose in whey powder aids in browning via maillard reactions and the natural yellow tinge of whey gives baked goods a golden surface (Sithole et al., 2005; Dattatreya et al., 2010; Sharma et al., 2012).

Despite the many functional and unparalleled uses of dried whey ingredients, several factors have inhibited the widespread use of whey-based ingredients. These factors include: inconsistent performance/functionality in food systems, lack of practical technologies for cost-effective isolation, poor promotion versus competitor products, low-quality ingredient perception, and limited understanding of protein properties (Smithers, 2008). In addition, flavor has been a major hindrance for the usage of whey products in processed foods.

A desirable dried whey product is very bland and imparts little to no flavor to the food item it is applied. This is characteristic of fresh liquid whey. When the whey is concentrated, spray-dried, and stored, the flavor changes due to prolonged exposure to heat and oxygen (Carunchia Whetstine et al., 2005; Wright et al., 2009). A fundamental understanding of off-flavor formation in whey and whey ingredients is essential in the prevention of these flavors in the final product and ultimately validating whey products as a viable, high-quality alternative to conventional ingredients.

ANNATTO

Annatto is a yellow/orange carotenoid that is widely used in the food industry as a colorant. The colorant is naturally found in the seeds of the *Bixa Orellana* tree which was named after Fransisco de Orellana, a scientist and explorer of the upper Amazon (Guiliano et al., 2003). Annatto was used as a colorant and spice by the Aztecs and is still commonly used as a spice in certain Latin American recipes (Gerlach and Gerlach, 2002). The primary use of

annatto in the U.S. is in the coloring of Cheddar cheese (Emerton, 2008). Bixin is fat-soluble and is the major pigment in the seeds. The water-soluble fraction, norbixin, is the pigment of interest for the coloring of Cheddar cheese. To increase yield of the pigment of interest, bixin is saponified under alkaline conditions to produce norbixin (Smith, 2004; Giuliano et al., 2003). Though bixin and norbixin have different properties, they both have a polyene chain backbone which is responsible for their instability and susceptibility to oxidation (heat, light, oxygen, peroxides, acids, etc.) (Scotter, 2009). Bixin and norbixin naturally occur in the cis form but are readily converted to trans by light and heat (Figures 1.1-1.4) (Smith, 2004).

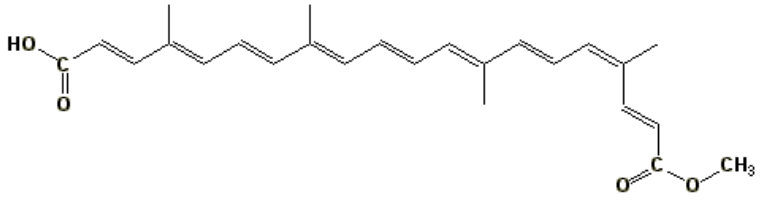

Figure 1.1 Cis-Bixin

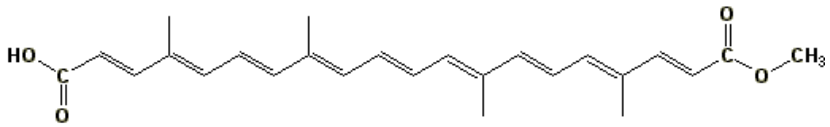

Figure 1.2 trans bixin

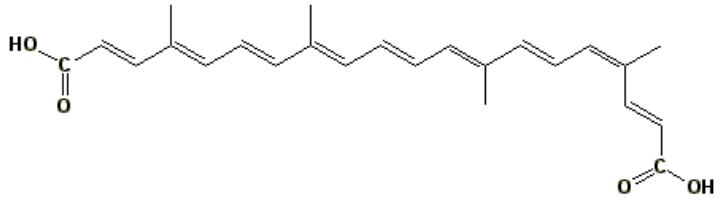

Figure 1.3 Cis-Norbixin

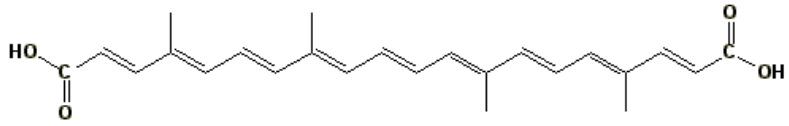

Figure 1.4 Trans-Norbixin

A pink color has also been known to form in cheese with annatto as well as products containing bleached whey powders. The cause of this defect is unknown, however, hypotheses of protein interactions with norbixin as well as heat treatment, pH, and brand of annatto have been suggested (Smith, 2004). Hong et al. (1995a) demonstrated that type of lighting, storage conditions, composition, and source of colorant affected pink discoloration in annatto-colored cheese under fluorescent lighting. Hong et al. (1995b) concluded that pink discoloration in colored cheeses was caused by light-induced lipid and colorant oxidation.

Other carotenoids such as beta-carotenes, alpha-carotene, neoxanthin, lutein, violaxanthin, antheraxanthin, and zeaxanthin are introduced into the milk through the cow's forage diet. These pigments can cause a yellow color in the milk that also carries into the whey (Croissant et al., 2007; Smith, 2004).

BLEACHING

Approximately 10% of the norbixin added to milk to color Cheddar cheese is found in the subsequent whey (Smith, 2012). The yellow color is undesirable in the finished whey product as it imparts color to the product it is added, thus the color must be removed or destroyed to produce a high-quality ingredient (Kang et al., 2010).

As previously stated, norbixin has an unstable polyene backbone which is susceptible to oxidation. Thus oxidizing agents may be used to destroy the colorant. The two chemical

bleaching agents permitted in the United States for bleaching whey are benzoyl peroxide (Figure 1.5) and hydrogen peroxide (Figure 1.6).

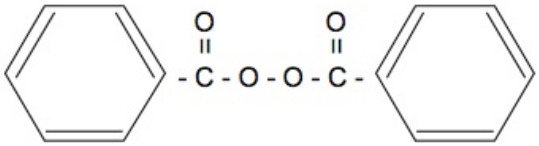

Figure 1.5 Chemical Structure of Benzoyl Peroxide

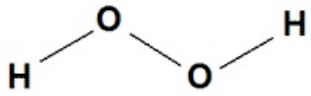

Figure 1.6 Chemical Structure of Hydrogen Peroxide

Benzoyl peroxide ($C_{14}H_{10}O_4$) is a white, crystalline solid that has no limitations on usage rates in foods other than current good manufacturing practices (GMPs) rules (US FDA, 2009k). In 2005, Codex Alimentarius approved the maximum level (100mg/kg) of benzoyl

peroxide for bleaching liquid whey and whey products. In 2007, Codex approved the same maximum (100mg/kg) for dried whey and whey products (Codex, 2008). Until recently, benzoyl peroxide was considered the gold standard for bleaching whey. It has been the popular choice among whey processors for several reasons. Benzoyl peroxide is able to destroy more norbixin with less off-flavor production than hydrogen peroxide in liquid whey and dried whey ingredients (Listiyani et al., 2011; Jervis et al., 2012). Listiyani et al. (2011) observed 73-80% norbixin destruction in liquid whey bleached with benzoyl peroxide at 68^{o}C for 30min compared to 43% norbixin destruction in liquid whey bleached with hydrogen peroxide at the same parameters. Jervis et al. (2012) reported 92% norbixin destruction in whey bleached with benzoyl peroxide at 66^{o}C for 30min compared to 44% norbixin destruction in whey bleached with hydrogen peroxide at the same parameters. In addition, unlike hydrogen peroxide, catalase does not have to be added after bleaching with benzoyl peroxide, and bleaching efficacy is not affected by temperature (US FDA 2009a; Listiyani et al., 2011, Listiyani et al., 2012).

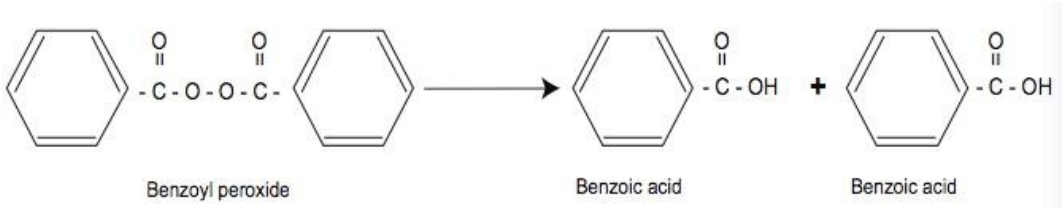

Figure 1.7 Reaction of Benzoyl Peroxide Breakdown

Upon oxidation, benzoyl peroxide breaks down into benzoic acid (Figure 1.7). The residual breakdown product, benzoic acid, has recently been banned in imported food products by China, Japan, and some European countries (Kang et al., 2010). Government regulators of these countries do not like the use of benzoyl peroxide and consider benzoic acid as well as other breakdown products of benzoyl peroxide harmful (Kang et al., 2010). In China, the maximum concentrations of benzoic acid permitted are 200 mg/kg in carbonated drinks, and 1000 mg/kg in juice drinks, sauces, and jams (Listiyani et al., 2011). In China, there is no established legal limit for benzoic acid in dairy products at this time. Health complications to benzoic acid-related compounds are uncommon, and life-threatening reactions are extremely rare (Joint FAO/WHO Expert Committee on Food Additives, 2004)..

Modern methods for detecting benzoic acid involve reconstitution of whey powder, precipitation of fats and protein using potassium hexacyanoferrate(II) trihydrate and zinc acetate, filtration of the sample, and separation using a C-18 HPLC column with UV-VIS

detection (ISO, 2008; Qi et al., 2009; Listiyani et al., 2011). The most common separation technique utilizes high performance chromatography because it has high sensitivity (low level of detection), requires minimum sample preparation, and no sample derivatization is needed (WHO, 2000; Listiyani et al., 2011). Benzoyl peroxide is also used to bleach other foods such as flour (Listiyani et al., 2011). Benzoic acid is naturally found in milk at low concentrations and in fermented dairy products at concentrations up to 50mg/kg (Sieber et al., 1995). Subsequently, benzoic acid is naturally found in whey and whey products (Kang et al., 2010). Listiyani et al. (2011) reported <12.5mg/kg of benzoic acid in unbleached, spray-dried Cheddar WPC34. The same authors reported 272 and 634mg/kg of benzoic acid in spray-dried Cheddar WPC34 bleached with 50 and 100mg/kg benzoic acid, respectively. Benzoic acid is a common and natural compound also found in nuts, fruits, and vegetables (Sieber et al., 1995). It is also added to foods as a preservative. As a preservative, it is added to foods with an acidic pH range such as fruit juices and soft drinks as well as cosmetics such as toothpastes and mouthwashes (WHO, 2000). As a preservative, benzoic acid concentrations should not exceed 2000 mg/kg of food and in foods in which it naturally occurs, it does not exceed 40 mg/kg (WHO, 2000). In the U.S., benzoyl peroxide and hydrogen peroxide remain generally recognized as safe (GRAS) for the purpose of bleaching whey (US FDA, 2008). Concern for benzoic acid in foods is on the rise, however due to natural occurrences, there is some tolerance.

Hydrogen peroxide (H_2O_2) is a clear, colorless liquid which is typically sold at concentrations of 30-35% (v/v) in water. Hydrogen peroxide may be used to bleach whey at

concentrations of no more than 500ppm and must be destroyed/deactivated by the addition of catalase at no more than 20ppm following bleaching (US FDA 2009b; US FDA 2009c). The oxidation reaction breaks hydrogen peroxide down into oxygen and water (Figure 1.8).

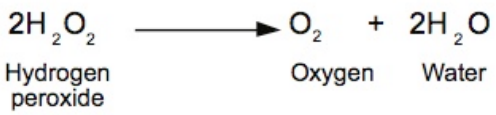

Figure 1.8 Reaction of Hydrogen Peroxide Breakdown

Bleaching efficacy of hydrogen peroxide is affected by temperature and it is generally more effective at higher temperatures (<74^{o}C) (Listiyani et al., 2012). Campbell et al. (2012) reported 32 and 47% norbixin destruction in liquid whey bleached for 60min with hydrogen peroxide (250ppm) at 35 and 50^{o}C, respectively which was consistent with 26 - 43% norbixin destruction in liquid whey at 50, 60 and 68^{o}C for 30min (250-500 ppm hydrogen peroxide) reported by Listiyani et al. (2012), Kang et al. (2012), and Li et al. (2012). Similarly, Jervis et al. (2012) reported 44% norbixin destruction in liquid whey at 66^{o}C for 30min with hydrogen peroxide (500ppm). Though widely used in the industry, hydrogen peroxide causes significantly higher off-flavor formation than benzoyl peroxide in fluid whey

and dried whey ingredients (Croissant et al., 2009; Listiyani et al., 2011; Jervis et al., 2012). This effect may be caused by a combination of a less stable free radical and less steric hindrance making the hydrogen peroxide free radical more reactive and more available for oxidation of compact compounds such as proteins (Benassi et al., 1993; Bach et al., 1996). Jervis and Drake (In press) hypothesized that the hydroxyl radical (from hydrogen peroxide) is less selective to attacks on norbixin double bonds than the benzoyl radical and/or that the hydroxyl radical is more polar than the benzoyl radical. Table 1.1 lists the pros and cons of hydrogen and benzoyl peroxide for bleaching whey.

Table 1.1 Summary of Hyrdrogen Peroxide and Benzoyl Peroxide Characteristics

	Hydrogen Peroxide	Benzoyl Peroxide
Pros	• little to no effect on the nutrients present • more acceptable for usage in other countries	• effective at lower usage levels than hydrogen peroxide • does not require a catalase addition to remove residues • does not pit stainless steel; therefore, is less corrosive to equipment • effective across a wide range of temperatures
Cons	• must be inactivated with catalase • could possibly cause oxidized flavors • corrosive to equipment • less economical to use because it requires much more peroxide for satisfactory bleaching	• possible formation of oxidized flavors • possibility that the carrier used may be considered an allergen • concerns from other countries because it has just been recently approved by Codex

While benzoyl peroxide and hydrogen peroxide are the only two chemical agents approved for bleaching whey in the U.S., alternative bleaching agents have been investigated and implemented. Lactoperoxidase is an oxidoreductase enzyme belonging to the peroxidase family and is found in a wide range of mammalian milks, including bovine milk (Seifu et al., 2005). It is the second most abundant enzyme in bovine milk and constitutes about 0.5% of the total whey proteins (Kussendrager and van Hooijdonk, 2000; Fox and Kelly, 2006). Lactoperoxidase, in conjunction with thiocyanate and hydrogen peroxide, has been used for antimicrobial purposes to preserve raw milk where refrigeration is not feasible (Fweja et al., 2007). The lactoperoxidase system is only active in the presence of 3 components:

lactoperoxidase, thiocyanate (SCN^-), and hydrogen peroxide (Seifu et al., 2005; Campbell et al., 2012). Lactoperoxidase survives conventional pasteurization temperatures (<70^oC) and largely remains active in liquid whey products (Kussendrager and van Hooijdonk, 2000). The lactoperoxidase system has also been applied for the decolorization of whey and whey products (Bottomley et al., 1989; Campbell et al., 2012).

To utilize the LP system for bleaching of whey, hydrogen peroxide must be added at low concentrations (approximately 10ppm) to avoid inactivation of lactoperoxidase (Bottomley et al., 1989). Hydrogen peroxide reacts with lactoperoxidase which metabolizes thiocyanate (SCN^-) into hypothiocyanate ($OSCN^-$). Hypothiocyanate is a strong oxidizing agent which is capable of reacting with carotenoids, such as norbixin, as well as other compounds prone to oxidation (Campbell et al., 2012). Additional enzyme and thiocyanate are not necessary for this bleaching application as these compounds are adequately abundant in whey. Furthermore, despite the addition of hydrogen peroxide, catalase is not necessary since hydrogen peroxide is consumed. Utilizing this system has been shown to destroy more than 99% of norbixin in fluid whey, however, off-flavor production was also significantly higher than chemical bleaching with hydrogen peroxide alone (Campbell et al., 2012). An alternative to lactoperoxidase is the application of an exogenous fungal peroxidase to whey. An exogenous peroxidase, Maxibright is available commercially for the purpose of bleaching whey (DSM Food Specialties, Delft, the Netherlands).

Kang et al. (2012) recently investigated other alternative bleaching agents for fluid whey including bentonite, UV light, and ozone. Both UV light and ozone bleached whey displayed unique off-flavors and destroyed only 39% and 15% norbixin, respectively. An acid-activated clay, bentonite showed the most promise with a 79% reduction in norbixin and the least off-flavor production compared to hydrogen peroxide, UV light, and ozone. Smith et al. (2012) recently reported the application of bixin as an alternative cheese colorant. This study found that <1% of bixin added to cheddar cheese milk was found in the subsequent whey. Smith et al. (2012) observed a lack of solubility with bixin in milk which was not surprising since bixin is more hydrophobic than norbixin. Bixin was dispersed in the milk with a homogenizer prior to cheese-making, but optimal Cheddar cheese is manufactured using unhomogenized milk. These results suggest that bixin or a similar nonpolar carotenoid may be a suitable alternative colorant if stably suspended in cheesemilk.

FLAVOR OF WHEY AND WHEY INGREDIENTS

Whey and whey products are expected to have a bland or delicate flavor for the use of food ingredients (Laye et al., 1995). The flavor of fluid whey is complex with many different chemicals contributing to the flavor (Carunchia-Whetstine et al., 2003). Typical dairy flavors such as cooked/milky and sweet aromatic are expected in these types of products. However, heat-generated and lipid oxidation compounds can create undesirable, nondairy flavors such as cardboard and cabbage flavors (Whetstine et al., 2005; Wright et al., 2006). Milk quality,

type of cheese manufactured, processing, and storage can all contribute to off-flavors in dried whey products. Contarini et al. (1997) demonstrated the link between milk pasteurized at high temperatures and increased concentrations of dimethyl sulfide, a chemical known to contribute to cabbage off-flavor in milk. Tomaino et al. (2004) demonstrated that lipid oxidation in fresh liquid whey increased during storage.

Two general reactions are responsible for off flavor in dairy products; lipolysis and proteolysis (Ramshaw and Dunstone, 1969; Ferretti and Flanagan 1972; Min et al., 1990; Lee and Morr, 1994; Whitson et al., 2011). Hydroperoxides are formed during lipid oxidation, which are susceptible to further decomposition, producing aldehydes, ketones, acids, and alcohols (Whitfield, 1992). These compounds have been shown to negatively affect flavor as well as nutritional and overall quality (Carunchia Whetstine et al., 2005; Wright et al., 2009; Whitson et al., 2010). Cardboard, the main off flavor in whey and dried whey ingredients, is caused by lipid oxidation products (Drake and Civille, 2003; Liaw et al., 2010; Whitson et al., 2010). Proteolytic enzymes, such as chymosin (the key enzyme responsible for milk coagulation in cheese production), in the whey may also promote off-flavors through protein degradation (Mabbit, 1961; Holmes et al., 1977; Amundson, 1984). Whey ingredients are generally added to products with heavy flavors and off flavor is the main obstacle preventing widespread use in the bland/delicately flavored foods market.

PROCESSING

Milk/liquid whey is usually processed in order to concentrate or fractionate into smaller volumes to increase value, reduce transport cost, and lengthen shelf-life. Depending on how the whey is processed, many products can be achieved. Some of these products include: whey protein concentrate (WPC), whey protein isolate (WPI), milk protein concentrate (MPC), micellar casein concentrate (MCC), whey protein hydrolysate (WPH), serum protein concentrate (SPC), and sweet whey powder.

For whey products, milk must first go through the cheese manufacture process. A standard Cheddar cheese-make process includes: pasteurization of milk, addition of calcium chloride, addition of starter culture (or acidification), addition of annatto, addition of rennet, cutting the curd, draining the whey, salting the curd, and pressing (Farkye, 2004). The whey, when drained, should have a pH of about 6.4 (Croissant et al., 2009). The whey is then fat separated and pasteurized to inactivate the culture (to prevent further pH decrease). Liquid whey is rarely used in the industry (since it is mostly water and lactose) and is usually further processed/fractionated to create more valuable products.

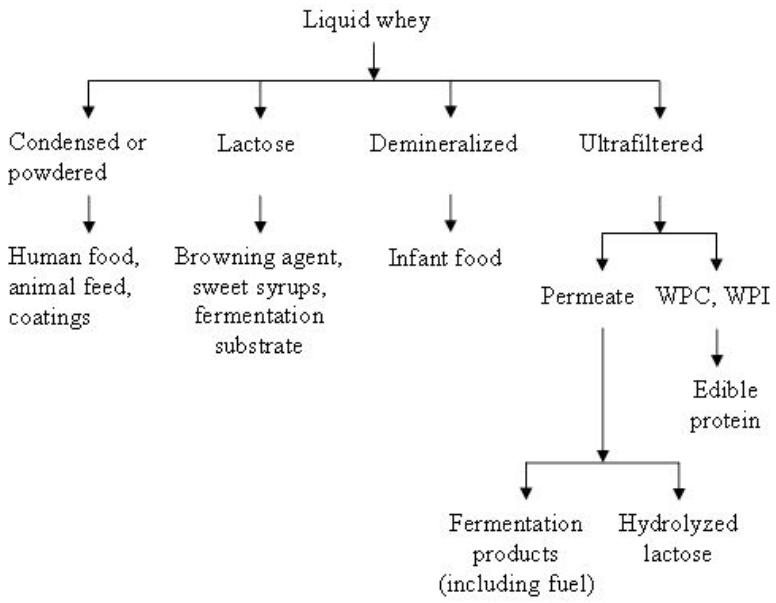

Figure 1.9 Liquid Whey Processing

A popular method to commercially concentrate milk and whey to various end products is membrane filtration. Filtration techniques include: mircrofiltration, ultrafiltration, nanofiltration, and reverse osmosis (Figure 1.10) (Marcello and Rizvi, 2008). Microfiltration separates particles smaller than 30kDa such as lactose, minerals, most proteins, and water from larger particles such as fat globules and large proteins. Ultrafiltration separates lactose, minerals, vitamins, peptide fragments (such as glycomacropeptides which are an enzymatic product of rennet cleaving kappa-casein), and water from macromolecules (>10kDa) such as

proteins and fat globules. Nanofiltration separates minerals and water from whey. Reverse osmosis retains everything except solvent such as water (Cheryan, 1998).

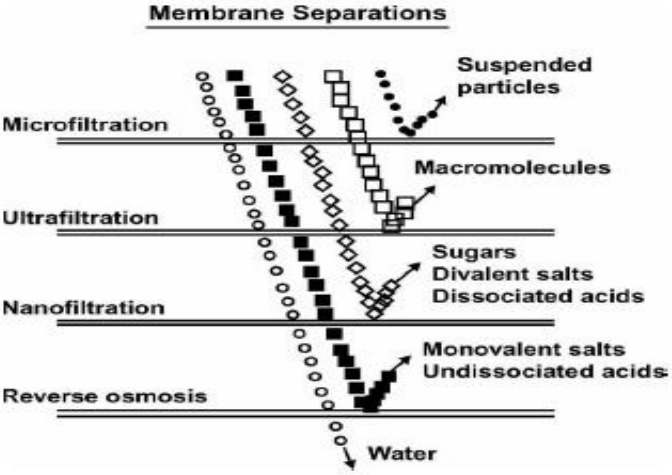

Figure 1.10 Membrane Separation Technologies

Whey protein concentrate (WPC) is generally produced by filtering liquid whey through an ultrafiltration system. Ultrafiltration is a process that uses membranes with small pores (~10kDa) to separate whey protein from lactose and other small molecular weight molecules (Huffman, 1996). Whey protein concentrates can range from 20-90% protein (w/w), however, to achieve a protein concentration above 50%, the concentrated whey (retentate) must be diafiltered (Huffman, 1996; Onwulata, 2008). The diafiltration process is simply a wash step which dilutes the whey to reduce the viscosity of the thickening product and remove more lactose, water, and minerals (Johnson and Lucey, 2006; Huffman, 1996;

Onwulata, 2008). Once the desired protein/solids level is achieved, the concentrate (called retentate) is spray-dried.

Whey protein isolate (WPI) is whey protein concentrate with a protein composition greater than 90% (w/w). To achieve protein levels over 90%, the whey is microfiltered (to remove fat) and then UF and DF are applied (to remove minerals and lactose). Microfiltration removes fat through a membrane with larger pores (~30kDa) than ultrafiltration membranes. Lactose hydrolysis can also be used to further reduce lactose. Ion-exchange chromatography is another way to produce WPI. Ion exchange is typically performed before ultrafiltration and involves lowering the pH below the pI of whey proteins to give them an overall positive charge. The whey is then run through a tank containing negatively charged resin beads to which the whey proteins now have an affinity (Huffman, 1996). The fat, lactose, and minerals are washed away. The pH in the tank is increased and the proteins detach from the resin (Huffman, 1996). Ultrafiltration is then used to remove the remaining water until reaching the desired protein levels and then spray-dried.

Milk protein concentrate (MPC) is a milk product that has gained popularity in recent years. However, MPC may not be considered to be as valuable as whey products since it is not a byproduct of an industrial process such as cheese-making. MPC is the product of ultrafiltering skim milk. It is essentially milk that has had fat, minerals, and lactose removed. MPC, unlike WPC and WPI, retains all of the proteins found in milk. This means that the protein in MPC is approximately 80% casein. Many studies have shown that these caseins

are the main culprit of low solubility of MPC (Anema et al., 2006; Fang et al., 2012; Fang et al., 2011; Havea et al., 2006; Sikand et al., 2011; Mimouni et al., 2010). One study has shown that the casein micelles were not altered during the manufacture of MPC (Martin et al., 2010). However, it has been demonstrated that a lack of minerals (e.g., magnesium, calcium, phosphorus, and sodium) may cause the caseins to aggregate (Sikand et al., 2011). Other studies have found a relationship between storage time and MPC/casein solubility (Haque et al., 2010; Anema et al., 2006; Fang et al., 2011; Kher et al., 2007; Havea et al., 2006; Mimouni et al., 2010). This effect was amplified when storage temperature was elevated or when water activity was increased (Haque et al., 2010). Anema et al. (2006) observed that the casein proteins became lactosylated over time at elevated storage temperatures. Fang et al. (2012) showed that during spray drying, the solubility of MPC deteriorated with increased inlet air temperature due to increasing protein denaturation. One study concluded that changes in solubility were due to changes in the beta-sheet structure of the casein proteins (Kher et al., 2007). Havea et al. (2006) stated that the majority of the insoluble MPC was comprised of alpha- and beta-caseins that aggregated via weak non-covalent (hydrophobic) protein-protein interactions. These findings agree that casein is the cause of insolubility in MPC but do not necessarily agree on which interactions are causing the insolubility. It is more than likely due to a combination of protein denaturation and a lack of minerals/lactose to stabilize the charges on caseins. Very little research has addressed flavor of this emerging dairy ingredient (Drake et al., 2009). MPC is used in products such as frozen dairy foods, fluid cultured products, nutritional/protein beverages, and cheesecake

filling to increase viscosity, protein content, gel strength, improve texture/mouth-feel, and contribute mild dairy flavor.

Serum protein concentrate (SPC) is produced through the microfiltration (MF) of skim milk. Essentially serum proteins (SP) are whey proteins that have not been subjected to the cheese manufacture process (Evans et al., 2009). Serum proteins are also referred to as "native, virgin, and ideal" whey proteins. Two major differences are that SPC does not contain glycomacropeptide (GMP), a small peptide cleaved from k-casein by rennet during the cheese-making process, and SPC contains very little fat compared to WPC (Evans et al., 2009). SPC34 and SPC80 have similar sensory profiles as WPC34 and WPC80, however, SPC34 and SPC80 had significantly lower lipid oxidation compounds and lower flavor intensities than corresponding whey proteins (Evans et al., 2009; Evans et al., 2010). In addition, SPC80 is clear while WPC80 is opaque (Evans et al., 2010). These differences may be due to the increased fat content of WPC80. SPC generally have a higher pH than WPC since acid producing bacteria are not used in the manufacture of SPC (Evans et al., 2010). SPC is used in the same food products as WPC since SPC has a similar composition. During production, SPC is exposed to less processing techniques than WPC and thus may offer, as an ingredient, less off-flavors than WPC. In addition, diacetyl, which has a buttery flavor, is found in WPC34 and WPC80 but not in SPC34 or SPC80 (Evans et al., 2009). Removal of SP from milk before the cheese-making process has been shown to have no effect on quality of Cheddar cheese (Nelson and Barbano, 2005).

Micellar casein concentrate (MCC) is the product that remains once SP are removed via MF from skim milk. This separation occurs because of the approximately 10- to 100-fold difference between casein micelles and SP (Walstra et al., 1999). MCC consists mainly of casein in miccellar form, lactose, minerals, and a minor amount of serum proteins (Beckman et al., 2010). MCC could be used to increase cheese yields or possibly as an ingredient in food applications where caseinates are currently used (Papadatos et al., 2003). MCC could potentially be utilized to standardize cheese-milk before cheese-making (Beckman et al., 2010). There is an increasing interest in using MCC in the manufacture of shelf-stable high-protein beverages. The manufacture of shelf-stable beverages involves a sterilization step which affects the colloidal stability, viscosity, and flow behavior of MCC (Sauer and Moraru, 2011; Beliciu et al., 2012). There is currently no published work, to our knowledge, on flavor of MCC.

Whey processing technology continues to grow more sophisticated and advances in protein fractionation/isolation have lead to higher-valued products. Technologies such as continuous separation (CSEP) chromatography have enabled the manufacture of beta-lactoglobulin rich WPI which is more desirable in many products that demand consistent and reliable functionality. The ever-advancing membrane technologies, as well as ion-exchange chromatography, have made whey protein more cost effective and viable as a functional ingredient (Onwulata, 2008; Smithers, 2008). This is a far cry from a product that was discarded only decades ago.

BLEACHING PARAMETER VARIATIONS

Liquid whey processing may not occur at the same location as cheese-making. Whey processing is a relatively new industry and has not yet been fully incorporated into a linear system with the older cheese-making industry. Often liquid whey and retentate are stored/transported prior to bleaching and further processing (Whitson et al., 2011). Bleaching of whey can be carried out at various parameters and stages during processing. Temperature, time, atmosphere, and shear during storage/transport/processing can have a negative impact on the flavor of the final product. In addition, bleaching whey also has a negative impact on the flavor.

Generally, whey is bleached as liquid whey or liquid retentate with hydrogen or benzoyl peroxide (Li et al., 2012). In order to minimize off-flavor formation during bleaching, various bleaching parameters have been investigated. Croissant et al. (2009) demonstrated that liquid whey that was hot bleached with HP or BP and then concentrated to WPC70 had higher relative abundances of lipid oxidation products and higher off flavor intensities compared to unbleached whey products. Listiyani et al. (2011) and Jervis et al. (2012) confirmed these findings with liquid whey bleached with HP or BP, concentrated and spray-dried to WPC34 and WPC80, respectively. Listiyani et al. (2012) reported that bleaching at higher temperatures in fluid whey caused higher concentrations of lipid oxidation products (i.e., hexanal, Z-4-heptenal, heptanal, 2-pentylfuran, and nonanal) compared to cold bleached fluid whey. These findings confirmed that oxidation reactions increase with increased heat and can be expected when using oxidizing bleaching agents that

are not target specific. Listiyani et al. (2012) demonstrated that decreased temperature also decreased HP bleaching efficacy, but had no effect on BP. Studies have also investigated the sources of off flavors that occur during whey processing.

Campbell et al. (2011) demonstrated that annatto did not affect bleaching or flavor of fluid whey or spray dried whey protein. This result demonstrates that off-flavor formation is not directly caused by norbixin or norbixin degradation products. Approved chemical bleaching agents are not norbixin specific and lipid and protein oxidation occur while norbixin bleaching occurs. Listiyani et al. (2011) reported no significant effect (in bleaching efficacy or flavor of fluid whey) between bleaching before or after fat separation of fluid whey. This finding suggests that there is no connection between fat available for oxidation reactions and oxidized flavor intensity although oxidized flavors are clearly associated with lipid and protein oxidation compounds. Li et al. (2012) showed a decrease in bleaching efficacy from fresh whey to whey that was stored cold or frozen for 1, 2, and 10 days. This may be due to a reduction in viable enzymes, such as lactoperoxidase, that aid in bleaching. In the same study, the authors documented that bleaching after spray drying increased bleaching efficacy in WPC at 34% protein (w/w) (WPC34) while a decrease in bleaching efficacy was documented in WPC at 80% protein (w/w) (WPC80). While spray drying decreased bleaching efficacy of WPC80, bleaching efficacy was increased in liquid WPC80 retentate compared to fresh liquid whey (Li et al., 2012). It is important to note that lactoperoxidase is concentrated (like other whey proteins) during ultrafiltration. The authors hypothesized that the higher concentrations of lactoperoxidase in whey retentate were not

deactivated by the large amounts of hydrogen peroxide (250ppm) used and enzymatic as well as chemical bleaching occurred. It should be noted that this study investigated bleaching using only hydrogen peroxide and did not compare bleaching efficacy of benzoyl peroxide with fluid whey and whey protein retentate. To date, there are no known publications comparing hydrogen peroxide and benzoyl peroxide bleaching efficacy in whey with different solids/protein concentrations.

NORBIXIN BINDING

Norbixin is a small molecule (~380g/mol), and like lactose (~342g/mol), would be expected to filter into permeate during ultrafiltration. Some research has suggested that norbixin may have the ability to bind with whey proteins (Govindarajan and Morris, 1973; Hammond et al., 1975). Few studies have investigated the binding properties of norbixin to whey components/proteins. Cho et al. (1994) demonstrated the binding of retinol, a carotenoid, in an interior hydrophobic cavity of beta-lactoglobulin. Also a carotenoid, norbixin has similar properties to retinol. Given the hydrophilic nature of norbixin (two polar, carboxyl ends), it does not necessarily share the property which binds retinol (one polar end and one hydrophobic end) to beta-lactoglobulin (Figure 1.4 and 1.10). It should also be noted that Cho et al. (1994) investigated binding at pH 8. At this pH, the protein is in the form of a monomer (with an overall negative charge, the same as in liquid whey pH 6.3)

and may not represent the binding behavior of the beta-lactoglobulin dimer that exists in liquid whey at pH 6.3.

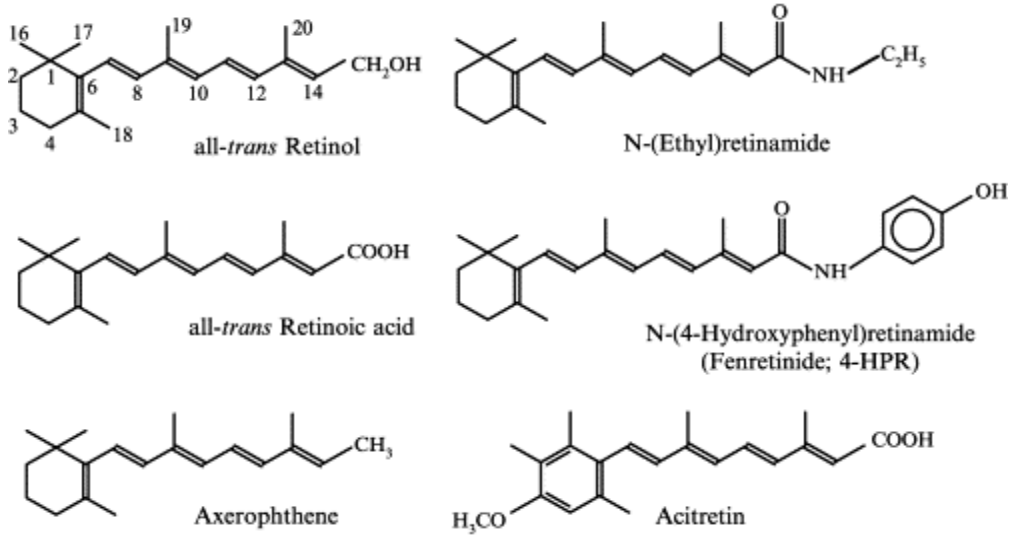

Figure 1.11 Structural Formulas of Natural and Synthetic Retinoids

Zhang and Zhong (2012) investigated binding between bixin, a hydrophobic carotenoid (Figure 1.2), and whey proteins. Bixin (only slightly structurally different than norbixin) favored hydrophobic binding with alpha-lactalbumin, beta-lactoglobulin, and bovine serum albumin at lower temperature and higher ionic strength. This is evidence that the three major whey proteins bind hydrophobic compounds in a similar fashion and could all have a role in binding hydrophilically as well. Though norbixin is hydrophilic and may not behave similarly, this research gives further insight into whey protein binding behavior.

A recent study suggested that norbixin, given its hydrophilic and hydrophobic characteristics, may exist in the form of a micelle dispersed in whey rather than a "soluble" state (Zhu and Damodaran, 2012). Micellar norbixin would be more likely associated with the milk fat globule membrane (MFGM) particles than globular proteins. Zhu and Damodaran (2012) demonstrated binding of 60% of the norbixin in whey isolated in the MFGM fraction. The MFGM fraction represented >97% of the lipids in whey, however, this fraction also contained 65-70% protein (w/w) (Damodaran, 2011). This was due to an average protein loss of 13.9% from the concentrated whey (Damodaran, 2011). It is not known which proteins were extracted in the MFGM fraction but up to 7% of the protein loss could be minor proteins that are typically MFGM-bound (Zhu and Damodaran, 2012). Nevertheless, this research suggests (due to 60% norbixin in MFGM fraction) norbixin may not bind specifically to only one component of whey.

As the demand for whey increases, processing of whey must be increasingly sophisticated to ensure a desirable, high quality product. Maintaining a bland flavor, while removing color to achieve a versatile and cheap ingredient is of the utmost importance. Many studies have addressed flavor defects caused by bleaching agents. Few studies have investigated bleaching parameter variations during processing to optimize bleaching efficacy. In addition, there has been a dearth of publications investigating norbixin binding in fluid whey. To our knowledge, no studies have compared fluid whey and fluid whey protein retentate bleaching with HP and BP, furthermore, few studies have investigated norbixin binding in fluid whey. The objective of this research was to determine optimal bleaching

parameters using HP and BP in fluid whey and fluid retentate and to investigate norbixin binding behavior.

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CHAPTER 2: THE INFLUENCE OF BLEACHING AGENT AND TEMPERATURE ON BLEACHING EFFICACY AND VOLATILE COMPONENTS OF FLUID WHEY AND WHEY RETENTATE

The influence of bleaching agent and temperature on bleaching efficacy and volatile components of fluid whey and whey retentate

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ABSTRACT

Whey protein is desirable as a neutral flavored, uncolored powder. Fluid whey or retentate are often bleached to remove residual annatto Cheddar cheese colorant and previous research has demonstrated that this process causes off-flavors in dried whey proteins. The objective of this research was to determine the impact of temperature and bleaching agent on bleaching efficacy and volatile components in fluid whey and fluid whey retentate. A standard Cheddar cheese make-procedure was used to manufacture liquid whey at 6.7% solids. The whey was concentrated to 12% solids (w/v) and 80% protein (w/w) by ultrafiltration and diafiltration. Liquid whey or concentrated whey protein (retentate) were subsequently bleached using benzoyl peroxide (BP) at 100 mg/kg (w/w) or hydrogen peroxide (HP) at 250 mg/kg (w/w) at 5^{o}C for 16 h or at 50^{o}C for 1 h. Unbleached controls were subjected to a similar temperature profile. The experiment was replicated three times. Annatto destruction (bleaching efficacy) among treatments was compared by solvent extraction and quantitation of norbixin of each treatment compared to an unbleached control. Volatile compounds were extracted and separated using solid phase microextraction gas chromatography mass spectrometry (SPME GC-MS). Bleaching efficacy of BP was higher than HP ($p < 0.05$) for fluid whey at both 5^{o}C and 50^{o}C. HP bleaching efficacy was increased in retentate compared to liquid whey ($p < 0.05$). In whey retentate, there was no difference between bleaching with HP or BP at 50^{o}C or 5^{o}C ($p > 0.05$). Retentate bleached with HP at either temperature had higher relative abundances of pentanal, hexanal, heptanal, and octanal than BP bleached retentate ($p<0.05$). Liquid wheys generally had lower concentrations of

selected volatiles compared to retentates. These results suggest that optimal bleaching of liquid whey is achieved using BP at 5^{o}C or 50^{o}C and that optimal bleaching of whey protein retentate is achieved at 50^{o}C with HP or BP. These results also confirm that bleaching with BP is less detrimental to flavor than bleaching with HP.

Keywords: whey, bleaching, annatto

INTRODUCTION

Whey is a value-added byproduct of the cheese-making process. Cheddar cheese colored with annatto (norbixin is the primary carotenoid component of annatto) has become widely popular since the initial use of annatto to mask color differences due to seasonal changes in milk (Giuliano et al., 2003). Due to a wide variety of food applications, dried whey ingredients are expected to be colorless with a bland/neutral flavor. Annatto colorant added to Cheddar cheese is also present in the whey and is removed by bleaching (Kang et al., 2010). Bleaching whey consists of adding an oxidizing agent which is not specific to norbixin. Benzoyl peroxide (BP) and hydrogen peroxide (HP) are the two currently approved chemical bleaching agents used in the industry (21CFR184.1157 and 21CFR184.1366, respectively). These bleaching agents contribute to lipid oxidation, and lipid oxidation products are a primary source of off-flavors in dried whey products (Whitson et al., 2010; Whitson et al., 2011; Liaw et al., 2011). Lipid oxidation products such as pentanal, hexanal, heptanal, octanal, nonanal, dimethyl trisulfide, 1-octen-3-one, and 2-pentylfuran are key contributors to whey protein off-flavors (Carunchia-Whetstine et al., 2005; Croissant et al., 2009; Liaw et al., 2010; Whitson et al., 2010). Processing parameters and bleaching conditions vary widely among manufacturers (Kang et al., 2010; Whitson et al., 2011; Listiyani et al., 2012). Bleaching of whey (whey or fluid retentate) can occur at any step in whey protein manufacture and varies among manufacturers.

Several studies have addressed the effects of bleaching parameters on volatile off-flavors and annatto destruction in whey proteins. Croissant et al. (2009) first demonstrated

increased volatile lipid oxidation compounds in liquid whey bleached with HP (500mg/kg) or BP (10 and 20mg/kg) and increased off-flavors associated with lipid oxidation in bleached fluid whey and spray-dried whey protein concentrate 66% (WPC66) compared to unbleached whey and WPC66. Another recent study demonstrated that fat content in liquid whey did not impact bleaching efficacy using HP (250 or 500mg/kg) or BP (50 or 100mg/kg) but that temperature of bleaching had a large influence on HP bleaching (but not BP) activity in fluid whey (Listiyani et al., 2011). These authors also demonstrated that temperature impacted volatile compound composition of bleached wheys; higher temperatures increased lipid oxidation. Li et al. (2012) demonstrated increased bleaching efficacy for 60min at 50^{o}C with HP (250ppm) in WPC80 retentate compared to WPC34 retentate but did not investigate or compare the influence of solids on BP bleaching efficacy. Similarly, Jervis et al. (2012) compared color, flavor, and functional properties of WPC80 from liquid whey bleached with HP or BP, but the effect of bleaching liquid whey versus whey protein retentate was not evaluated. Since bleaching occurs industrially with either fluid whey or retentate, an understanding of bleaching effects with both media and chemical bleaching agents is needed. To our knowledge, there is no published information comparing BP and HP bleaching in fluid whey and fluid whey protein retentate. The objectives of this study were to compare bleaching efficacy and volatile compound formation in fluid whey and fluid whey retentate (12% solids, 80% protein [w/w]). Both bleaching agent and temperature of bleaching were investigated in these two matrices.

MATERIALS AND METHODS

Experimental Design

This study was separated into two experiments. Controls with no bleaching agent were included in all experiments. Experiment 1 investigated bleaching efficacy of hydrogen peroxide (HP) and benzoyl peroxide (BP) in fluid whey (6.7% solids) and fluid whey protein retentate (12% solids, 80% protein). Three bleaching temperatures (5, 50, and 60^{o}C) and a range of bleaching agent concentrations (BP: 25, 50, and 100mg/kg; HP: 250 and 500mg/kg) were evaluated. From this experiment, treatments were selected for subsequent experiments. For experiment 2, selected treatments (BP 100mg/kg; HP 250mg/kg; 5 and 50^{o}C) were applied to fluid whey and whey protein retentate. Bleaching efficacy and volatile compound analysis were conducted and compared to controls.

Liquid Whey Production

Raw whole milk was obtained from the North Carolina State University Dairy Research and Education Farm. Milk was batch pasteurized (model MPD1050, Micro Process Design, D&F Equipment Co, McLeansville, NC) at 63^{o}C for 30 min. The milk was cooled and refrigerated overnight at 4^{o}C. The next day the milk was heated to 31^{o}C in a 250kg cheese vat (model MX4, Kusel Equipment Company, Watertown, WI) and then inoculated with a freeze-dried lactic acid starter culture (Choozit MA 11, Danisco, New Century, NJ) at a rate of 41mg/kg of milk. A calcium chloride solution (50% w/v, Dairy Connections Inc., Madison, WI) was added at a rate of 0.39ml/kg. The milk was agitated and allowed to ripen

for 60 min. Double strength annatto colorant (3% norbixin w/v, Danisco, New Century, NJ) was added 30 min into ripening at a rate of 0.033ml/kg milk and diluted 20 times in deionized (DI) water. The milk was coagulated with double strength recombinant rennet (Dairy Connections Inc., Madison, WI) for 30 min at a rate of 0.09ml/kg of milk diluted 80 times in DI water. The curd was cut with 0.95cm wire knives, and both the curd and whey were allowed to rest for 5 min followed by gentle stirring for 10 min without added heat. The temperature was increased gradually from 31 to 39^{o}C over 30 min. During this time the pH and titratable acidity were closely monitored until target pH of 6.35 was reached. At this point the whey was drained and fines were removed with a nylon cloth. The whey was immediately pasteurized at 63^{o}C for 30 min and processed with a hot-bowl cream separator (Westfalia Separator, C.A. De Fehr & Sons Ltd., Winnipeg, Manitoba, Canada) to reduce the fat content.

For bleaching experiments, the whey (5.4L) was divided into 300ml aliquots and placed into 500ml sanitized amber glass jars and submerged in a 5, 50, or 60^{o}C water bath. All samples were covered to minimize light exposure. At each temperature, benzoyl peroxide powder (32% w/w, Oxylite Type XX, Nelson Jameson, Inc., Marshfield, WI) was added at 25, 50, or 100mg/kg. Hydrogen peroxide (35% w/v, VWR International, West Chester, PA) was added at 250 or 500mg/kg. These concentrations represented the legal range of HP (>500mg/kg) for bleaching of whey as well as the range covering good manufacturing practices for the bleaching of whey using BP (U.S. FDA, 2010a and U.S. FDA 2010b, respectively). An unbleached control was also included for each temperature (total of 6

treatments/temperature). All treatments were manually agitated every 10 min for 60 min. After 60 min, the HP samples at 50 and 60^{o}C were deactivated by catalase addition (20mg/kg)(Food Pro CAT, Danisco). Inactivation of HP was confirmed when EM Quant peroxide testing strips (EMD Chemical Inc., Gibbstown, NJ) confirmed the absence of HP. No deactivation step was necessary for the use of BP. The samples were submersed in an ice bath and cooled to 5^{o}C (30 min). For samples bleached at 5^{o}C, samples remained at 5^{o}C for 16h. After 16h, the HP samples were deactivated by catalase addition at 20mg/kg of whey. All samples were frozen at -20^{o}C and analyzed within five days of production. This experiment was replicated three times.

Concentrated Whey Protein Retentate Production

The same cheese make procedure was followed as described previously. After fat separation, the whey was then placed into a 102L stainless steel container (F3-27, Tri-clamp models, Blichmann Engineering, LLC). The whey was concentrated using an ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) equipped with five polyethersulfone cartridge membrane filters (Model P2B010V05, nominal separation cutoff: 10,000kDa, surface area: 0.5m^{2}). A variable speed peristaltic pump (Model 77410-10), equipped with a pumphead (Model 77601-00), and silicone tubing (Model 96440-73) was used to circulate the product. The pump, pumphead, and tubing were obtained from Cole-Parmer (Vernon Hills, IL). The whey was kept at 50^{o}C using a stainless steel submerged-coil heat exchanger (PAC Stainless LTD, Seattle, WA) while processed on the UF system. Deionized (DI) water was added to the whey at 50% of the weight of the initial whey (diafiltration) in order to

reach a protein content of 80%. After a solids content of 12% and a protein content of 80% (WPC80)(CEM Rapid Protein Analyzer, Mathews, NC) was reached, the weight of the permeate and retentate was recorded. The retentate (5.4L) was collected and bleached as the liquid whey previously described.

Composition Analysis

Total percent solids and fat content of fluid milk and whey were analyzed using the Smart System 5 moisture-solids analyzer with SmartTrac rapid fat analysis (CEM, Matthews, NC). The total solids of liquid whey and retentate were determined by an oven drying method (AOAC, 2000; method number 990.20; 33.2.44). Fat in liquid whey was measured using the Modified Pennsylvania babcock method. Fat in retentate was determined by ether extraction (AOAC, 2000; method number 989.05; 33.2.26). Protein was determined using the Kjeldahl method (AOAC, 2000; method number 991.20; 33.2.11). Mineral analysis (phosphorous, calcium, potassium, magnesium, sulfur, sodium and iron) was performed by the NCSU Soil Science Analytical Services Laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009). All samples were measured in duplicate.

Norbixin Extraction and Quantification

The norbixin extraction method described by Croissant et al. (2009) was performed for liquid wheys. For retentate, annatto extractions were modified from Campbell et al. (2011). First, 3ml of retentate was added to a 50ml centrifuge tube (Nalgene, Rochester, NY). Next, 6ml

ethanol (EMD Chemicals Inc.) was added, vortexed for 30 s and allowed to stand for 30 min. 3ml chloroform (EMD Chemicals Inc.) was added and vortexed. The solution was then centrifuged at 16,500 x g for 10 min at 4^{o}C (model RC5B, Thermo Scientific). The supernatant was removed and added to a separate clean centrifuge tube. To the remaining solids, 3ml of chloroform was added, the sample was vortexed, and centrifuged again at 16,500 x g for 10 min at 4^{o}C. The bottom yellow liquid layer was removed and added to supernatant previously collected. Two ml of a 1% acetic acid (w/v) (EMD Chemicals Inc.) solution was added to the collected supernatant, vortexed, and centrifuged at 16,500 x g for 10 min. The bottom chloroform layer (yellow) containing the norbixin was collected and the volume was measured. The extraction procedure and measurements were performed with premium full spectrum F885 flat sheet filters covering all lights (Ergomart, Dallas, TX) to minimize norbixin isomerization and degradation (Mercadante, 2008). Solid-phase extraction (SPE) was applied to filter and purify the norbixin-containing chloroform extract. The Strata-NH2 SPE column (500mg/3ml, Phenomenex) was conditioned with 7ml of n-hexane (VWR International). A 1ml aliquot of the chloroform extract was transferred onto the SPE column. The column was rinsed with 5ml of a 1:1(v/v) of n-hexane:diethyl ether solution followed by 1ml of acetone (VWR International). The remaining norbixin was eluted with 3ml of a 7:3(v/v) of methanol:glacial acetic acid solution. The volume of the eluent was measured and recorded.

Norbixin was quantified from the SPE eluent by UV-visible spectrophotometry (Cary 300 Bio, Varian, Cary, NC) (Croissant et al., 2009). A 0.7ml aliquot of each sample was

measured in a 28Q10 Spectrosil quartz cuvette (Starna Cells Inc., Atascadero, CA). A standard curve for norbixin was created within the concentration range of 50µg/kg to 3mg/kg norbixin. Norbixin powder (45%w/w, Chr Hansen, Milwaukee, WI) was rehydrated in 2.5% potassium hydroxide then diluted in a 7:3(v/v) methanol:glacial acetic acid solution. Carotenoids generally have three peaks of absorption maxima (Levy and Rivadeneira, 2000). The maxima used for calculation was 458nm. The carotenoid concentration of the SPE extract was calculated using a standard curve. Norbixin concentration was calculated by total solids and correction for dilution during the extraction and SPE processes. Bleaching efficacy was determined by the percentage of norbixin recovered compared to the unbleached control. Norbixin measurements were conducted in triplicate.

Color Analysis

Color of liquid whey and retentate (WPC80) was measured using a Minolta Chroma meter (CR-410, Ramsey, NJ). First, a factory-supplied calibration plate was used to calibrate the instrument. Then a 10ml aliquot was placed into the bottom of a 60mm x 15mm polystyrene petri dish in duplicate (Beckton Dickinson, Franklin Lakes, NJ). Each petri dish was measured in duplicate. The Hunter CIE Lab color scale was used, where L* is lightness, a* is green/red, and b* is blue/yellow. Measurements were made in triplicate.

Solid-Phase Microextraction of Volatile Compounds with Gas Chromatography-Mass Spectrometry

Volatile compounds in fluid whey and retentate were extracted using solid-phase microextraction (SPME) (Wright et al., 2006). Volatiles were then separated and identified

by GC-MS as described by Campbell et al. (2011). Sodium chloride (VWR International) at 10%(w/w), 20µl of internal standard solution (2-methyl-3-heptanone in methanol at 8.1mg/kg (Sigma Aldrich, Milwaukee, WI)), and 5g of sample were added to 20ml autosampler vials with steel screw-tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA). Samples were injected using a Combipal autosampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890N GC with 5973 inert mass selective detector (Agilent Technologies Inc., Cary NC) equipped with a DB-5 column of 30m, 0.25mm I.D., 0.25µm film thickness (Restek US, Bellefonte, PA). Purge time was set at 1 min. The MS transfer line was maintained at 250^{o}C with the quad at 150^{o}C and source at 250^{o}C. Samples were maintained at 5^{o}C before fiber exposure. Samples were equilibrated to 40^{o}C for 25 min before 30 min fiber exposure of a 1cm DVB/Carboxen/PDMS Stableflex SPME fiber (Supelco, Bellefonte, PA) at 31mm with 4 s of pulsed of pulsed agitation at 250rpm. Fibers were inserted into an injector held at 250^{o}C for 5min at a depth of 50mm. The GC method used an initial temperature of 40^{o}C for 3 min with a ramp rate of 10^{o}C/min to 250^{o}C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250^{o}C. Compounds were identified using the NIST 2005 library of spectra and comparison of spectra of authentic standards injected under identical conditions. Relative abundance for each volatile compound was calculated using the known concentration of internal standard and the relative concentrations of the volatiles based on peak areas. Retention indices were calculated using an alkane series (Sigma Aldrich) (Van den Dool and Kratz, 1963).

Statistical Analysis

All data was analyzed using a 3-way analysis of variance (ANOVA) using a general linear model (PROC MIXED) in SAS (SAS Statistical Analysis Software, version 9.1, SAS Institute, Cary, NC). Sample effects (bleaching agent, bleaching agent concentration, temperature, and solids content) and interactions [for experiment I (bleaching agent*bleaching agent concentration*temperature) and for experiment II (bleaching agent*temperature*solids content)] were analyzed.

RESULTS

Composition

Solids, protein, and fat of liquid wheys were not different (5.29%[w/w]±0.58; 11.38%[w/w]±1.48; 0.01%[w/w]±0.01; respectively) ($p>0.05$). Solids, protein, and fat of whey protein retentates were also not different (12.44%[w/w]±0.95; 83.15%[w/w]±1.2; 0.76%[w/w]±0.07; respectively) ($p>0.05$). Mineral content [P, Mg, Fe, Ca, K, S, and Na] were not different, respectively, and were within previously reported ranges (Jervis et al., 2012).

Bleaching Efficacy

<u>Experiment 1:</u>

Fluid Whey:

Benzoyl peroxide was a more effective bleaching agent in fluid whey compared to hydrogen peroxide ($p<0.05$) (Fig. 2.1). Temperature and bleaching agent concentration had little to no effect on bleaching efficacy of BP (Fig. 2.1). HP at 500ppm was less efficient at bleaching than 250ppm at 50^{o}C and 60^{o}C ($p<0.05$). HP bleaching at 250ppm was significantly reduced at 5^{o}C compared to 50 and 60^{o}C ($p<0.05$). HP at 250ppm was most effective at 50^{o}C.

Retentate:

HP at 250 or 500ppm was a more effective bleaching agent in retentate at 50^{o}C and 60^{o}C compared to BP at 25 or 50ppm (Fig. 2.2). The highest concentration of BP evaluated (100ppm) was not distinct in bleaching efficacy from 500ppm HP at 50 or 60^{o}C. At 5^{o}C, HP and BP at 100ppm were not distinct in bleaching efficacy. BP at 25ppm was significantly less effective at norbixin destruction than any other treatment at 5^{o}C ($p<0.05$). At 50^{o}C and 60^{o}C there were no differences in HP bleaching at 250ppm or 500ppm ($p>0.05$).

Experiment 2:

Experiment 2 allowed direct comparison of whey and retentate bleaching as samples were manufactured from the same lot of whey (Fig. 2.3)(Table 2.1). Bleaching results were consistent with experiment 1. HP was more effective at bleaching retentate than fluid whey ($p<0.05$). BP bleaching was a more effective bleaching agent than HP in fluid whey and was not affected by temperature in fluid whey ($p<0.05$). HP and BP bleaching were comparable in retentate at 5^{o}C and 50^{o}C at 100mg/kg or 250mg/kg, respectively ($p>0.05$).

Volatile Compounds

Experiment 2:

Higher concentrations of pentanal, hexanal, heptanal, octanal, nonanal, 1-octen-3-one, 2-pentylfuran, and E-E-2,4-nonadienal were detected in the HP bleached whey protein retentates regardless of temperature compared to benzoyl peroxide bleached retentates (Table 2.2) ($p<0.05$). However, the most notable volatile compound difference between bleached retentates was hexanal. In whey protein retentate bleached at 50^{o}C, hexanal had an average relative abundance of 8.82ppb in HP and 0.239ppb in BP bleached samples. In whey protein retentate bleached at 5^{o}C, hexanal averaged 7.21ppb in HP compared to 0.493ppb in BP bleached retentates ($p<0.05$). Fluid whey contained lower abundances of most volatiles than fluid whey protein retentate ($p<0.05$). Whey retentates were characterized by higher concentrations of lipid oxidation volatiles than fluid wheys (Fig. 2.4). The HP bleached retentates were characterized by the highest concentrations of the selected volatile compounds (Fig. 2.4).

DISCUSSION

Bleaching with BP destroyed more norbixin in liquid whey than HP. These results are consistent with previous research (Jervis et al., 2012; Listiyani et al., 2012). In contrast, and a particular objective of this study, the opposite effect was observed in retentate where HP destroyed more norbixin than in fluid whey and was comparable in bleaching efficacy to that

of BP (Table 2.1). BP bleaching capacity was not as affected by temperature or increased solids concentrations (liquid whey compared to retentate) compared to HP.These results are consistent with previous research (Listiyani et al., 2012). This observation suggests that BP and HP destroy norbixin through different bleaching mechanisms. HP was most effective at destroying norbixin at higher protein concentrations. These results are also consistent with previous research (Li et al., 2012). Li et al. (2012) demonstrated increased HP bleaching in fluid whey protein retentate (WPC80) at 50^{o}C for 60min versus fluid whey, although, BP bleaching was not evaluated.

Increased HP bleaching efficacy in whey protein retentate may be due to higher concentrations of lactoperoxidase (LP) (a native enzyme in fluid milk that is present in whey). Campbell et al. (2012) recently demonstrated that LP in whey could be activated by low concentrations of HP (10-20ppm) and applied to enzymatically bleach fluid whey. Lactoperoxidase is inactivated in fluid whey at HP concentrations greater than 100ppm (Campbell et al., 2012; Jervis and Drake, 2013). When bleaching with HP in fluid whey, HP concentrations of >100ppm inactivate lactoperoxidase and HP chemical bleaching occurs. However, processing liquid whey into whey protein retentate concentrates proteins including lactoperoxidase and higher concentrations of LP will be more tolerant to a higher concentration of hydrogen peroxide (Campbell et al., 2012). The LP enzyme would still be destroyed as HP concentration increased beyond a certain concentration. This situation would explain why 250ppm HP destroyed more norbixin than 500ppm in retentate.

Temperature did not affect the bleaching capacity of BP in liquid whey (Fig 2.1, 2.3, Table 2.1). This is consistent with previous research (Listiyani et al., 2012; Kang et al., 2012). Increased bleaching at 50^{o}C compared to 5^{o}C was observed with both BP and HP in whey protein retentate. Reduced bleaching ability at 5^{o}C in retentate could be caused by a lack of water activity due to lower temperatures coupled with reduced water content (6.7-12% solids) leading to slower chemical reactions (bleaching/oxidation). Additionally, in the case of HP, bleaching at 50^{o}C in retentate could also be due to lactoperoxidase (as with most enzymes) having a faster reaction rate at higher temperatures. Temperature does not affect BP bleaching in liquid whey but does in liquid retentate. This could be due to the loss of protein buffering ability with increasing temperature through the loss of buffering agents such as salts and lactose. Further research would be needed to theorize the cause of this phenomenon; however, the purpose of this study was to investigate parameters that optimize HP bleaching to compete with the industry standard, BP.

Higher concentrations of aldehydes (as well as other compounds known to cause off-flavor in whey products) were present in retentate bleached with HP at 5^{o}C and 50^{o}C compared to BP (Table 2.2). This implies that HP is more prone to oxidize proteins and lipids when they are more concentrated in solution compared to BP. Regardless of bleaching agent, bleached fluid wheys contained lower relative abundances of nonanal, 2-methylbutanal, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), benzaldehyde, 2-pentylfuran, E-2-octenal, E-2-nonenal, E-Z-2,6-nonadienal, E-E-2,4-nonadienal, diacetyl, acetic acid, and limonene than bleached whey protein retentates. This may be due to decreased oxidation reactions during

bleaching and/or a concentrating effect from ultrafiltration due to the higher solids concentration of retentate. Reduced oxidation reactions in fluid whey could be attributed to increased water activity or higher concentrations of charge-quenching minerals (i.e., Na, Ca, Mg, K, Cl, etc.). In fluid whey, BP and HP bleaching produced comparable lipid oxidation volatiles. In contrast, whey protein retentates bleached with HP had higher concentrations of pentanal, hexanal, heptanal, octanal, nonanal, 1-octen-3-one, 2-pentylfuran, and E-E-2,4-nonadienal than the control or BP bleached samples. The high concentrations of aldehydes are indicators of lipid oxidation (Frankel, 2005). BP bleaching of whey protein retentate formed the same aldehydes as HP, however, these results suggest that HP has a higher reactivity to lipids than BP. Croissant et al. (2009) reported higher concentrations of hexanal, DMTS, heptanal, and octanal in WPC70 from HP-bleached whey than BP. Listiyani et al. (2011) reported higher concentrations of pentanal, hexanal, heptanal, and octanal in HP bleached WPC34 (dried) than unbleached and BP bleached WPC34 (dried). This agrees with the current experiment.

Overall, these results suggest that HP bleaching causes increased lipid oxidation volatile production compared to BP. Lipid oxidation of whey protein retentate can be minimized with HP by decreasing the bleaching temperature (50 to 5^{o}C) with minimal decreases in norbixin destruction. In contrast, temperature and solids concentration have little effect on BP bleaching and lipid oxidation volatile production. HP bleaching is comparable to BP bleaching at 5 and 50^{o}C in liquid retentate.

CONCLUSION

Neutral color and bland flavor are desired in whey products. Bleaching affects both color and flavor of the final product. BP bleaching in fluid whey or retentate was not affected by temperature at 100ppm BP ($p>0.05$). BP (100ppm) destroyed more norbixin in fluid whey than HP (250ppm) ($p<0.05$). BP bleaching produced fewer lipid oxidation volatiles than HP in retentate ($p<0.05$), but there was no significant difference between HP and BP bleaching efficacy ($p>0.05$). Benzoyl peroxide is not currently an approved bleaching agent for whey products in China or Japan. These results demonstrate that comparable bleaching efficacy between HP and BP is achieved with bleaching of whey protein retentate at 5 or 50^{o}C.

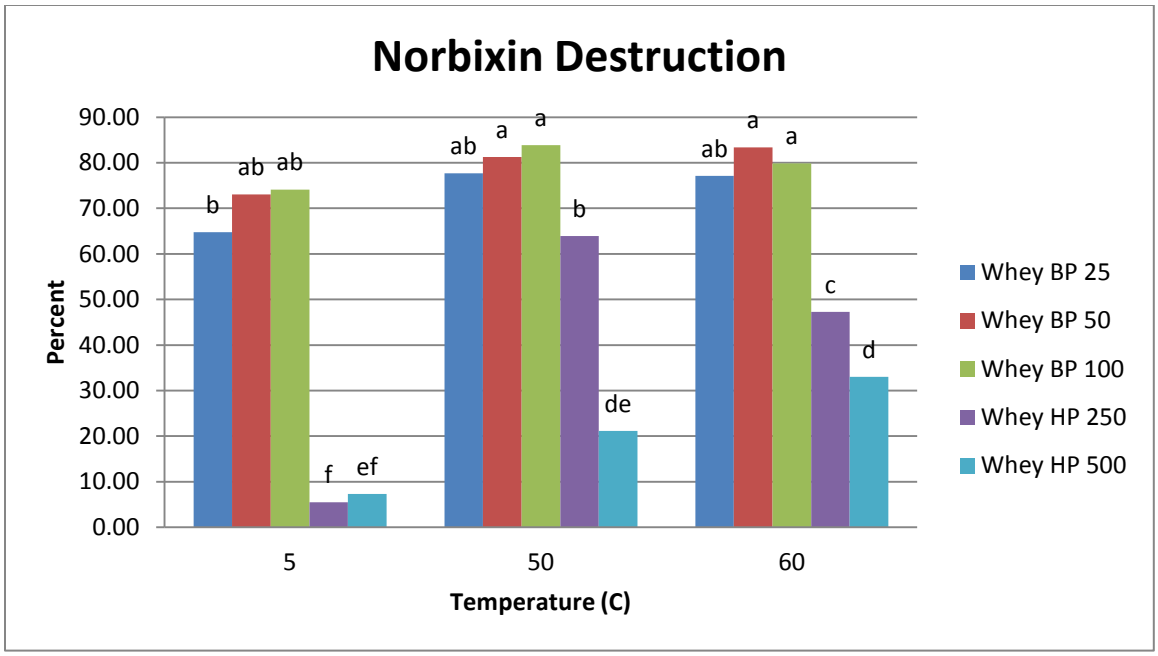

Figure 2.1 Norbixin Destruction in Liquid Whey

Norbixin percent destruction in liquid whey (6.7% solids) following bleaching with BP at 25, 50, and 100ug/kg or HP at 250 and 500ug/kg at 5, 50, or 60^{o}C (Experiment 1). Note: statistical lettering is across all three temperatures.

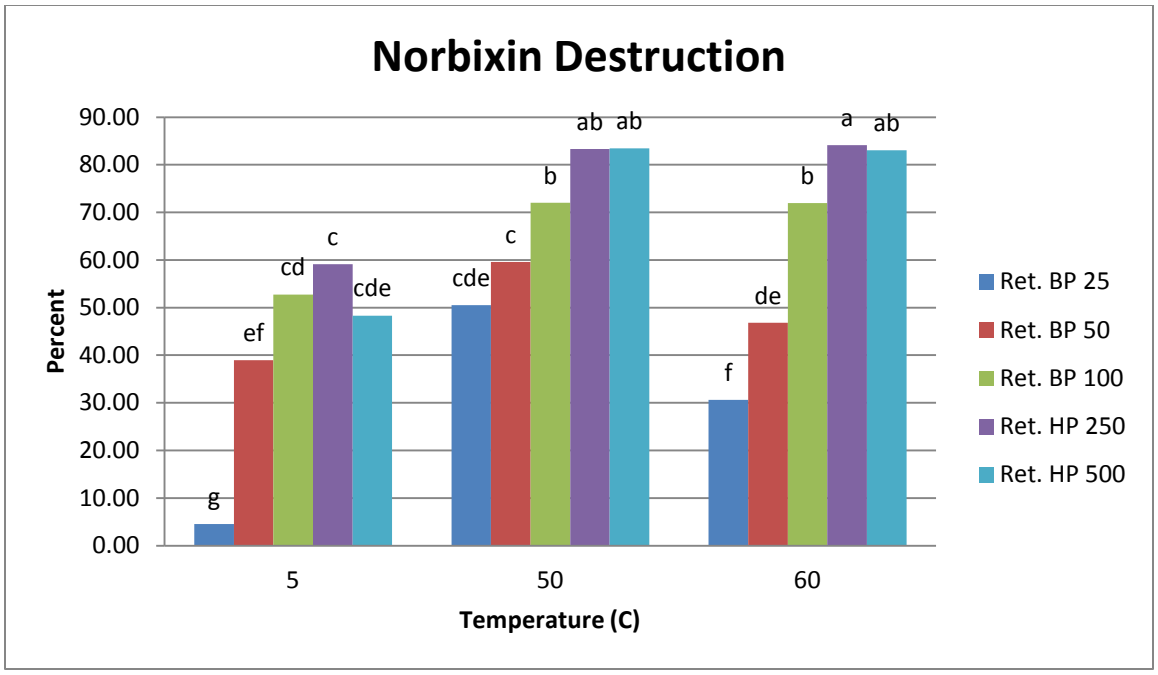

Figure 2.2 Norbixin Destruction in Retentate

Norbixin percent destruction in retentate (12% solids, 80% protein[w/w]) following bleaching with BP at 25, 50, and 100ug/kg or HP at 250 and 500ug/kg at 5, 50, or 60^{o}C (Experiment 1). Note: statistical lettering is across all three temperatures.

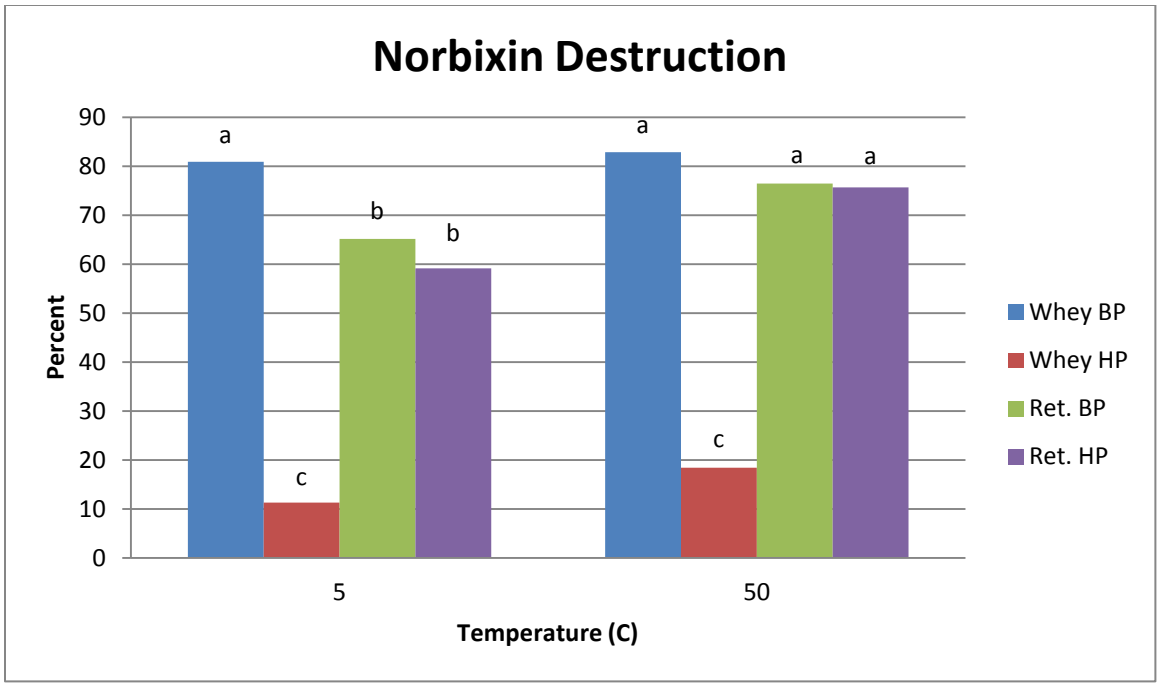

Figure 2.3 Norbixin Destruction in Liquid Whey and Retentate

Norbixin percent destruction in liquid whey and retentate (80% protein[w/w]) bleached using BP at 100ug/kg or HP at 250ug/kg at 5 or 50^{o}C (Experiment 2).

Table 2.1

Least squares means values of norbixin concentrations (mg/kg) by solids concentration, bleaching temperature, and bleaching agent and their interactions

Solids x Temperature x Treatment	Norbixin (mg/kg)
Whey x 5C x Control	27.54a
Whey x 5C x BP	5.22e
Whey x 5C x HP	24.31ab
Whey x 50C x Control	27.77a
Whey x 50C x BP	4.76e
Whey x 50C x HP	22.64bc
Ret x 5C x Control	26.77ab
Ret x 5C x BP	7.16e
Ret x 5C x HP	11.38d
Ret x 50C x Control	22.05c
Ret x 50C x BP	6.04e
Ret x 50C x HP	6.26e
Solids x Temperature	
Whey x 5C	19.02a
Whey x 50C	18.39a
Ret x 5C	15.10ab
Ret x 50C	11.45b
Solids x Treatment	
Whey x Control	27.66a
Whey x BP	4.99c
Whey x HP	23.48b
Ret x Control	24.41ab
Ret x BP	6.60c
Ret x HP	8.82c
Temperature x Treatment	
5C x Control	27.15a
5C x BP	6.19d
5C x HP	17.84b
50C x Control	24.91a
50C x BP	5.40d
50C x HP	14.45c
Solids	
Whey	18.71a
Ret	13.27b
Temperature	
5C	17.06a
50C	14.92a
Treatment	
Control	26.03a
BP	5.79c
HP	16.15b

a-eMeans in the same column that do not share a common superscript are different ($p < 0.01$).
HP = hydrogen peroxide; BP = benzoyl peroxide.

Table 2.2

Least squares means values of volatile compound concentrations (µg/kg) by solids concentration, bleaching temperature, and bleaching agent and their interactions (Experiment 2)

Solids x Temperature x Treatment	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Decanal	E-2-octenal
Whey x 5C x Control	0.010b	0.047b	0.068d	0.032c	0.527d	0.007ab	0.025c
Whey x 5C x BP	0.016b	0.506b	0.114cd	0.043bc	0.745d	0.015a	0.029c
Whey x 5C x HP	0.011b	0.085b	0.054d	0.026c	0.222d	0.002b	0.021c
Whey x 50C x Control	0.009b	0.034b	0.061d	0.021c	0.448d	0.004b	0.031c
Whey x 50C x BP	0.006b	0.303b	0.162cd	0.034c	0.701d	0.007ab	0.029c
Whey x 50C x HP	0.008b	0.062b	0.063d	0.023c	0.389d	0.006ab	0.026c
Ret x 5C x Control	0.017b	0.170b	0.153cd	0.065b	2.773c	0.010ab	0.075ab
Ret x 5C x BP	0.041b	0.493b	0.217c	0.063b	3.111c	0.008ab	0.069ab
Ret x 5C x HP	0.136a	7.210a	0.781b	0.126a	4.688ab	0.011ab	0.066ab
Ret x 50C x Control	0.023b	0.113b	0.147cd	0.063b	3.344c	0.009ab	0.081a
Ret x 50C x BP	0.037b	0.239b	0.214c	0.060b	3.859bc	0.004b	0.071ab
Ret x 50C x HP	0.157a	8.820a	0.982a	0.148a	5.512a	0.011ab	0.061b
Solids x Temperature							
Whey x 5C	0.012b	0.212b	0.079b	0.034b	0.498b	0.008a	0.025b
Whey x 50C	0.008b	0.133b	0.095b	0.026b	0.513b	0.006a	0.028b
Ret x 5C	0.064a	2.624a	0.384a	0.085a	3.524a	0.010a	0.070a
Ret x 50C	0.072a	3.057a	0.448a	0.091a	4.239a	0.008a	0.071a
Solids x Treatment							
Whey x Control	0.009c	0.041b	0.065c	0.027c	0.488c	0.006ab	0.028c
Whey x BP	0.011c	0.405b	0.138bc	0.039c	0.723c	0.010a	0.029c
Whey x HP	0.010c	0.074b	0.059c	0.025c	0.305c	0.004b	0.024c
Ret x Control	0.020bc	0.141b	0.150bc	0.064b	3.059b	0.10ab	0.078a
Ret x BP	0.039b	0.366b	0.215b	0.062b	3.485b	0.006ab	0.070ab
Ret x HP	0.146a	8.015a	0.882a	0.137a	5.100a	0.011a	0.064b
Temperature x Treatment							
5C x Control	0.013b	0.108b	0.111c	0.049b	1.650c	0.009a	0.050ab
5C x BP	0.029b	0.499b	0.166c	0.053b	1.928bc	0.011a	0.049ab
5C x HP	0.074a	3.647a	0.417b	0.076a	2.455ab	0.007a	0.044b
50C x Control	0.016b	0.074b	0.104c	0.042b	1.896bc	0.007a	0.056a
50C x BP	0.022b	0.271b	0.188c	0.047b	2.280bc	0.006a	0.050ab
50C x HP	0.082a	4.441a	0.523a	0.086a	2.951a	0.008a	0.043b
Solids							
Whey	0.010b	0.173b	0.087b	0.030b	0.510a	0.007a	0.027b
Ret	0.068a	2.841a	0.416a	0.088a	3.881a	0.009a	0.071a
Temperature							
5C	0.038a	1.418a	0.231a	0.060a	2.011a	0.009a	0.048a
50C	0.040a	1.595a	0.272a	0.058a	2.376a	0.007a	0.050a
Treatment							
Control	0.015b	0.091b	0.107c	0.045b	1.773b	0.008a	0.0528a
BP	0.025b	0.385b	0.177b	0.050b	2.104b	0.008a	0.050ab
HP	0.078a	4.044a	0.470a	0.081a	2.703a	0.008a	0.044b

a-eMeans in the same column that do not share a common superscript are different ($p < 0.01$).
HP = hydrogen peroxide; BP = benzoyl peroxide

Table 2.2 Continued

Solids x Temperature x Treatment	E-2-nonenal	E-Z-2,6-nonadienal	E-E-2,4-nonadienal	DMDS	DMTS	1-octen-3-one	2-pentylfuran
Whey x 5C x Control	0.004c	0.024c	0.003e	0.002de	0.228cd	0.004b	0.032e
Whey x 5C x BP	0.006bc	0.024c	0.010de	0.002cde	0.252cd	0.022b	0.115de
Whey x 5C x HP	0.003c	0.019c	0.004e	0.001e	0.184d	0.005b	0.036e
Whey x 50C x Control	0.005c	0.024c	0.003e	0.002de	0.281c	0.002b	0.035e
Whey x 50C x BP	0.007bc	0.025c	0.011de	0.002de	0.247cd	0.016b	0.150cde
Whey x 50C x HP	0.003c	0.019c	0.004e	0.003bcd	0.231cd	0.003b	0.040e
Ret x 5C x Control	0.021a	0.077a	0.014cd	0.003abc	0.631ab	0.006b	0.209cd
Ret x 5C x BP	0.021a	0.061b	0.022c	0.003abc	0.588b	0.015b	0.268cd
Ret x 5C x HP	0.019ab	0.078a	0.078a	0.004ab	0.643ab	0.075a	0.684b
Ret x 50C x Control	0.022a	0.080a	0.011de	0.004ab	0.706a	0.000b	0.262cd
Ret x 50C x BP	0.022a	0.078a	0.022c	0.004ab	0.586b	0.004b	0.295c
Ret x 50C x HP	0.019ab	0.085a	0.066b	0.005a	0.589b	0.071a	0.834a
Solids x Temperature							
Whey x 5C	0.004b	0.022b	0.006b	0.002b	0.221b	0.010b	0.061b
Whey x 50C	0.005ab	0.023b	0.006b	0.002b	0.253b	0.007b	0.075b
Ret x 5C	0.020ab	0.072a	0.038a	0.004a	0.620a	0.032a	0.387a
Ret x 50C	0.021a	0.081a	0.033a	0.004a	0.627a	0.025ab	0.463a
Solids x Treatment							
Whey x Control	0.004cd	0.024c	0.003d	0.002b	0.255c	0.003c	0.034d
Whey x BP	0.007bc	0.025c	0.011c	0.002b	0.250c	0.019b	0.132c
Whey x HP	0.003d	0.019c	0.004d	0.002b	0.208c	0.004bc	0.038d
Ret x Control	0.021a	0.078ab	0.013c	0.004a	0.668a	0.003bc	0.235bc
Ret x BP	0.022a	0.069b	0.022b	0.004a	0.587b	0.010bc	0.282b
Ret x HP	0.019ab	0.081a	0.072a	0.005a	0.616b	0.073a	0.759a
Temperature x Treatment							
5C x Control	0.012ab	0.050a	0.009c	0.003b	0.430ab	0.005bc	0.120c
5C x BP	0.014ab	0.043a	0.016b	0.003ab	0.420b	0.019b	0.192bc
5C x HP	0.011b	0.048a	0.041a	0.003ab	0.414b	0.040a	0.360a
50C x Control	0.013ab	0.052a	0.007c	0.003ab	0.494a	0.001c	0.149bc
50C x BP	0.014a	0.051a	0.017b	0.003ab	0.416b	0.010bc	0.222b
50C x HP	0.011b	0.052a	0.035a	0.004a	0.410b	0.037a	0.437a
Solids							
Whey	0.005a	0.023b	0.006b	0.002b	0.237a	0.009a	0.068b
Ret	0.021a	0.076a	0.036a	0.004a	0.624a	0.029a	0.425a
Temperature							
5C	0.012a	0.047a	0.022a	0.003a	0.421a	0.021a	0.224a
50C	0.013a	0.052a	0.020a	0.003a	0.440a	0.016a	0.269a
Treatment							
Control	0.013ab	0.051a	0.008c	0.003a	0.462a	0.003c	0.134c
BP	0.014a	0.047a	0.016b	0.003a	0.418b	0.015b	0.207b
HP	0.011b	0.050a	0.038a	0.003a	0.412b	0.039a	0.398a

a-eMeans in the same column that do not share a common superscript are different ($p < 0.01$).
HP = hydrogen peroxide; BP = benzoyl peroxide

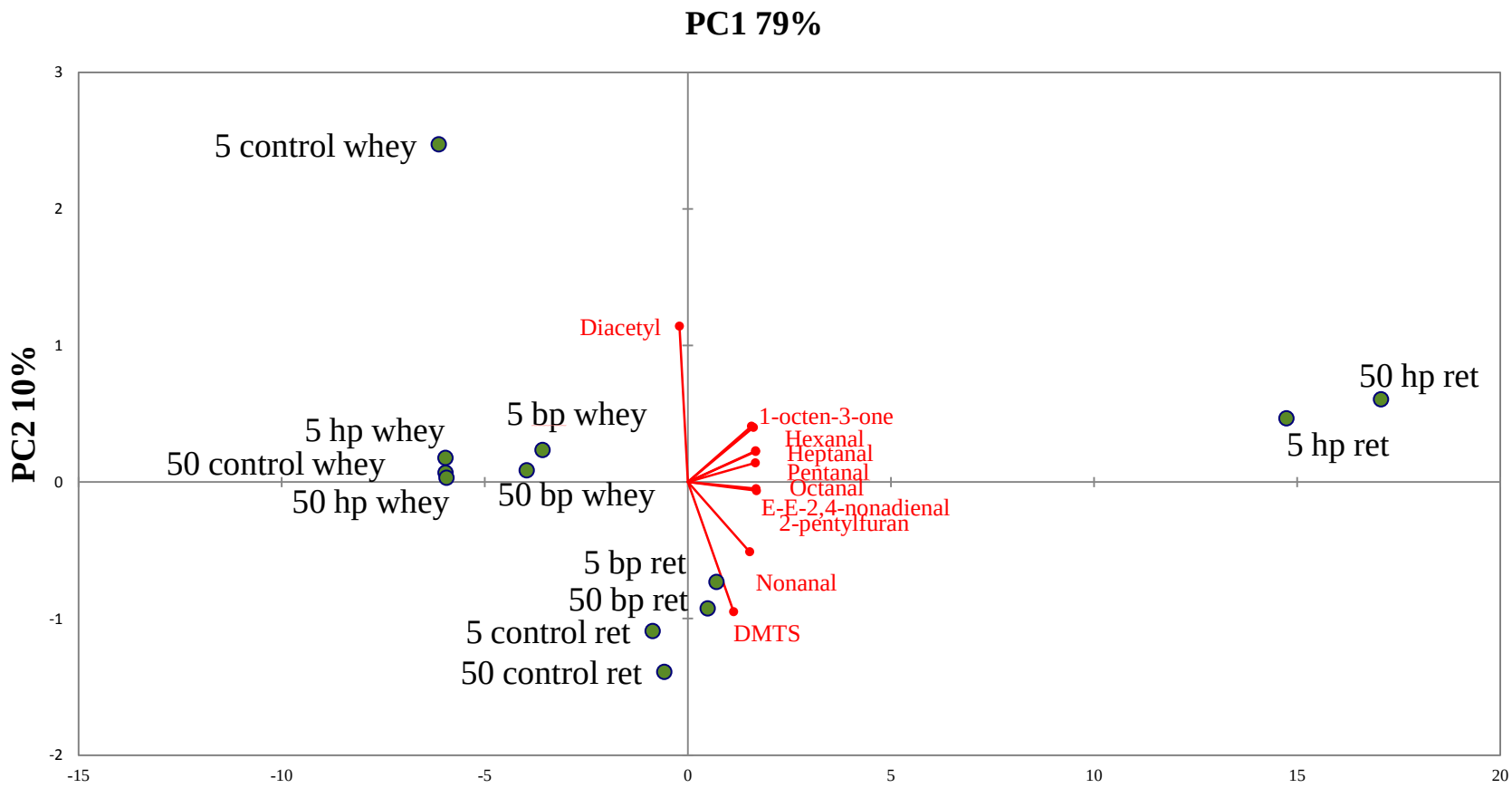

Figure 2.4 Principal Component Analysis Biplot of Volatile Flavor Compounds

Principal component analysis biplot of volatile flavor compounds from all bleaching treatments (experiment 2). Whey = fluid whey; ret = whey protein retentate; HP = hydrogen peroxide (250mg/kg); BP = benzoyl peroxide (100mg/kg)

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CHAPTER 3: NORBIXIN INTERACTIONS IN WHEY

ABSTRACT

Norbixin is the primary carotenoid responsible for the color of Cheddar cheese and the subsequent Cheddar whey. Previous studies have investigated chemical bleaching of norbixin in whey. However, few have evaluated interactions of norbixin with other whey components. The objective of this study was to investigate interactions between norbixin and whey components. Pasteurized, fat-separated Cheddar cheese whey was manufactured in triplicate. One half of the whey was heated to 50^{o}C and the other cooled to 8^{o}C. Wheys were ultrafiltered (UF) to a protein concentration of 34% [w/w](WPC34). The liquid WPC34 and permeate were then sampled and the remaining WPC34 was diafiltered to 80% protein [w/w](WPC80). Norbixin content of the WPC34, WPC80, permeate, diafiltered permeate, and the UF cleaning solutions were determined by high performance liquid chromatography. Hot UF reduced norbixin concentration compared to cold UF in both WPC34 and WPC80 ($p<0.05$). In WPC80 retentate, amounts of norbixin recovered from the UF membranes following hot or cold UF were not different ($p>0.05$), suggesting that heat was a source of norbixin loss from hot UF. Heat alone (hot hold) reduced norbixin by as much as 13.3% while shear alone (cold recirculation) reduced norbixin by as much as 21.9%. Combining heat and shear resulted in as much as 48.5% norbixin loss. Hot or cold whey ultrafiltered with 1 or 4% NaCl reduced norbixin more than no salt addition or 8% NaCl addition ($p<0.05$). A solution of beta-lactoglobulin (10uM) and a solution of alpha-lactalbumin (10uM) spiked with 0 (control), 10, 50, 100, and 200ppb norbixin showed no difference in fluorescence spectra. These results suggest that norbixin does not bind to whey proteins

(beta-lactoglobulin and alpha-lactalbumin) hydrophobically. The results also suggest that norbixin has a higher affinity for a charged matrix and may bind with whey components through polar interactions. The binding is weak enough to be significantly removed/destroyed by a combination of increased heat, shear, and charge.

Keywords: norbixin, whey, protein

INTRODUCTION

Whey protein is an increasingly used functional ingredient in many processed foods. As an ideal functional ingredient, whey protein should be bland and colorless. In the United States, milk that is used to make Cheddar cheese is colored with annatto, giving the cheese an orange color. Annatto colorant added to Cheddar cheese is also present in the whey and is removed by bleaching (Kang and others 2010). Norbixin is the primary carotenoid pigment in annatto. Bleaching whey consists of adding an oxidizing agent which is not specific to norbixin. Benzoyl peroxide and hydrogen peroxide are the two approved chemical bleaching agents used in the U.S. (21CFR184.1157 and 21CFR184.1366, respectively). These bleaching agents contribute to lipid oxidation, and lipid oxidation products are a primary source of off-flavors in dried whey products (Croissant and others 2009; Whitson and others 2010; Whitson and others 2011; Liaw and others 2011; Jervis and others 2012). In order to facilitate bleaching with minimal or no off-flavors, it is beneficial to understand the interactions between norbixin and whey.

Norbixin is a polar water soluble carotenoid, derived from the saponification of bixin, a hydrophobic carotenoid naturally found in the seeds of the *Bixa orellana* tree. Norbixin is a small molecule (~380g/mol), and like lactose (~342g/mol), would be expected to pass through the 10kDa membrane into permeate during ultrafiltration of whey, although to our knowledge, no study had demonstrated this effect. Norbixin has a carboxylic acid group on each end of an eighteen carbon backbone and contains a series of nine conjugated double bonds. There are few publications that have investigated the interactions between fluid whey

and norbixin. Previous research suggested that norbixin may have the ability to bind with whey proteins (Govindarajan and Morris, 1973; Hammond and others 1975). Cho and others (1994) demonstrated the binding of retinol, a carotenoid, in an interior hydrophobic cavity of beta-lactoglobulin. Also a carotenoid, norbixin (MW=380) has some similar properties to retinol (MW=286). However, norbixin has two polar ends which make it more suitable for hydrophilic binding than retinol (one polar end and one hydrophobic end). It was suggested that retinol binds to beta-lactoglobulin hydrophobically and since norbixin doesn't have a hydrophobic end, it is unlikely that norbixin will bind beta-lactoglobulin in the same way. Slight affinity differences, such as the difference between retinol and norbixin, have been known to completely change binding properties. It should also be noted that Cho and others (1994) investigated binding of retinol to beta-lactoglobulin at pH 8. At this pH, beta-lactoglobulin is in the form of a monomer and may not represent the binding behavior of the beta-lactoglobulin dimer that exists in liquid whey at pH 6.3.

Zhang and Zhong (2012) investigated binding between bixin, a hydrophobic carotenoid, and whey proteins at pH 7.4. Bixin, like retinol, has one polar end and one hydrophobic end. They studied binding of bixin with three major whey proteins (alpha-lactalbumin, beta-lactoglobulin, and bovine serum albumin) using UV-VIS absorption spectroscopy, fluorescence spectroscopy, isothermal titration calorimetry, and circular dichroism. Zhang and Zhong (2012) also demonstrated that bixin favored binding with these three whey proteins at a lower temperature (15^{o}C) and higher ionic strength (0.1-0.5M NaCl). They suggested that bixin bound hydrophobically to the three major whey proteins and that

binding of bixin to whey proteins was also dependent on pH and degree of protein denaturation. Zhang and Zhong (2012) studied bixin binding to whey proteins at pH 7.4. This study provided evidence that the three major whey proteins bind hydrophobic compounds in a similar fashion, but did not provide information on how or if norbixin interacts with whey proteins.

Another recent study suggested that norbixin may exist in the form of a micelle dispersed in whey (due to its hydrophilic and hydrophobic characteristics) rather than in a "soluble" state (Zhu and Damodaran, 2012). Micellar norbixin would more likely be associated with the milk fat globule membrane (MFGM) than with globular whey proteins. Zhu and Damodaran (2012) demonstrated binding of 60% of the norbixin in whey in the isolated MFGM fraction. The MFGM fraction was isolated by lowering the pH of fluid whey to 4.2 followed by centrifugation. The MFGM fraction represented >97% of the lipids in the whey, however, the lipids only represented 19% (on a dry weight basis) of this fraction. On a dry weight basis, the majority of the MFGM fraction was protein (65-70%) (Damodaran, 2011). This was due to an average protein loss of 13.9% (of the total whey protein) from the concentrated whey during the process of isolating the MFGM fraction (Damodaran, 2011). It is not known which proteins were extracted in the MFGM fraction but up to 7% of the protein loss could be minor proteins that are typically MFGM-bound (Zhu and Damodaran, 2012). This study did not address the possibility that norbixin could be binding to major proteins within the MFGM fraction or that adjusting the pH may affect binding properties of the norbixin, proteins, and MFGM. This research suggested that norbixin may not bind

specifically to only one component of whey (such as proteins).

A better understanding of norbixin binding will aid in the proper removal/destruction of norbixin from whey with little impact on flavor/functionality of the whey protein. To our knowledge, no current studies have investigated norbixin interactions in fluid whey rather than a model system. The objective of this research was to investigate norbixin binding properties to whey proteins in liquid whey and isolated whey proteins.

MATERIALS AND METHODS

Experimental Design

This study was separated into four experiments. Experiment 1 investigated the effects of hot and cold ultrafiltration on norbixin concentration in liquid whey protein concentrate at 34 and 80% protein (WPC34 and WPC80, respectively). Experiment 2 investigated the effects of hot and cold hold effects compared to hot and cold ultrafiltration on norbixin concentration in liquid WPC34 and WPC80. Experiment 3 investigated ionic strength effects on norbixin concentration in liquid whey protein concentrate by the addition of NaCl and $CaCl_2$ prior to hot or cold ultrafiltration. Experiment 4 investigated norbixin binding to major whey proteins (beta-lactoglobulin and alpha-lactalbumin) via fluorescence quenching of hydrophobic surface tryptophan residues. For experiments 1, 2, and 3; raw whole milk was obtained from the North Carolina State University Dairy Research and Education Farm, batch pasteurized and liquid whey obtained from a standard Cheddar cheese-make procedure. The liquid whey

was pasteurized and fat-separated. Liquid WPC34 and WPC80 retentates for experiments were then manufactured by ultrafiltration and diafiltration. All experiments were conducted in triplicate.

Liquid Whey Production (Experiments 1, 2, 3)

Raw whole milk was obtained from the North Carolina State University Dairy Research and Education Farm. Milk was batch pasteurized (model MPD1050, Micro Process Design, D&F Equipment Co, McLeansville, NC) at 63^{o}C for 30 min. The milk was cooled and refrigerated overnight at 4^{o}C. The next day the milk was heated to 31^{o}C in a 250kg cheese vat (model MX4, Kusel Equipment Company, Watertown, WI) and then inoculated with a freeze-dried lactic acid starter culture (Choozit MA 11, Danisco, New Century, NJ) at a rate of 41mg/kg of milk. A calcium chloride solution (50% w/v, Dairy Connections Inc., Madison, WI) was added at a rate of 0.39ml/kg. The milk was agitated and allowed to ripen for 60 min. Double strength annatto colorant (3% norbixin w/v, Danisco, New Century, NJ) was added 30 min into ripening at a rate of 0.033ml/kg milk and diluted 20 times in deionized (DI) water. The milk was coagulated with double strength recombinant rennet (Dairy Connections Inc., Madison, WI) for 30 min at a rate of 0.09ml/kg of milk diluted 80 times in DI water. The curd was cut with 0.95cm wire knives, and both the curd and whey were allowed to rest for 5 min followed by gentle stirring for 10 min without added heat. The temperature was increased gradually from 31 to 39^{o}C over 30 min. During this time the pH and titratable acidity were closely monitored until target pH of 6.35 was reached. At this point the whey was drained and fines were removed with a nylon cloth. The whey was

immediately pasteurized at 63^{o}C for 30 min and processed with a hot-bowl cream separator (Westfalia Separator, C.A. De Fehr & Sons Ltd., Winnipeg, Manitoba, Canada) to reduce the fat content.

Concentrated Whey Protein Retentate Production (Experiment 1, 2)

The same cheese make procedure was followed as described previously. After fat separation, the whey was then placed into a 102L stainless steel container (F3-27, Tri-clamp models, Blichmann Engineering, LLC). The whey was concentrated using an ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) equipped with five polyethersulfone cartridge membrane filters (Model P2B010V05, nominal separation cutoff: 10,000kDa, surface area: 0.5m^{2}). A variable speed peristaltic pump (Model 77410-10), equipped with a pumphead (Model 77601-00), and silicone tubing (Model 96440-73) was used to circulate the product. The pump, pumphead, and tubing were obtained from Cole-Parmer (Vernon Hills, IL). The whey was kept at 50^{o}C or cooled to 8^{o}C using a stainless steel submerged-coil heat exchanger (PAC Stainless LTD, Seattle, WA) while processed on the UF system. Whey was concentrated to 34% protein (w/w) (WPC34) and sampled for analysis. The remaining WPC34 was further concentrated via diafiltration. Deionized (DI) water was added to the whey at 50% of the weight of the initial whey (diafiltration) in order to reach a protein content of 80%. After a solids content of 12% and a protein content of 80% (WPC80)(CEM Rapid Protein Analyzer, Mathews, NC) was reached, the weight of the permeate and retentate was recorded.

Experiment 1: Effect of hot and cold UF

Pasteurized and fat-separated liquid whey (94L) was split and cooled to 8^{o}C or heated to 50^{o}C. Each part was concentrated via ultrafiltration (UF) at 8^{o}C or 50^{o}C to whey protein concentrate at 34% protein[w/w] (WPC34) and sampled. Cold whey concentrated to WPC34 and WPC80 was a slower process than hot ultrafiltration due to a lower flux caused by a higher viscosity (170 vs 120 min in WPC34; 360 vs 240 min in WPC80). The cold treatment was processed longer than the hot treatment and therefore introduced another variable (2 variables: hot/cold and different processing times). This variable (different processing times) was removed and addressed in experiment 2. It is important to note that the cold UF flux was initially lower than the hot UF flux but fouled slower than the hot UF thus the ending flux of the hot UF was comparable to the ending flux of the cold UF. The remaining WPC34 was further concentrated via ultrafiltration/diafiltration to a target composition of 12% solids [w/w] and 80% protein [w/w] (WPC80). The resulting retentates and permeates were sampled for norbixin analysis.

Experiment 2: Effect of hot or cold hold time versus hot or cold UF

Pasteurized and fat-separated whey (94L) was cooled to 8^{o}C and ultrafiltered to WPC34. Two-300mL samples were taken. One aliquot of retentate was held at 8^{o}C in an ice bath and the other was heated to 50^{o}C in a water bath. Once temperature was reached, aliquots were held at these temperatures for 3 h and gently agitated every 10 min. The remaining liquid WPC34 was split into two parts, one part remained at 8^{o}C and the other was heated to 50^{o}C. Once temperature was reached, whey retentates were recirculated through the UF apparatuses

for 3 h at 206kPa. This same process was repeated with a subsequent batch of fluid whey. Parameters remained the same except that the whey was subjected to ultrafiltration and diafiltration to liquid WPC80 before splitting into aliquots for hot or cold hold time for 3 h or hot or cold UF recirculation for 3 h at 206kPa.

Experiment 3: Effect of ionic strength

The purpose of this experiment was to determine whether increasing the ionic strength (addition of NaCl or $CaCl_2$) of the matrix caused an increase in norbixin concentration in the permeate. We hypothesized that if norbixin was associated via ionic bonding with a protein constituent, increasing ionic strength may decrease the affinity for a binding constituent. If norbixin favored the charged matrix (over the binding constituent), more of the norbixin would be filtered into the permeate with the compounds of similar molecular weight. Pasteurized and fat-separated whey (2L) was separated into aliquots for each salt (NaCl or $CaCl_2$) addition: 8^oC or 50^oC, with eight treatments per temperature; control, 1% NaCl (or $CaCl_2$), 4% NaCl (or $CaCl_2$), and 8% NaCl (or $CaCl_2$). Each treatment (18mL) was ultrafiltered 2-fold (2x) using an ultrafiltration centrifugal device (Pall Corporation; Ann Arbor, MI) with the respective NaCl or $CaCl_2$ concentration. The hot and cold treatments were centrifuged/ultrafiltered for 75min and 120min at 2683 x g, respectively. The cold treatments were centrifuged longer than hot treatments due to decreased flux rate. The permeate for all samples was removed and collected. DI water (9mL) spiked with the respective NaCl or $CaCl_2$ concentration was added to each sample and centrifuged again (hot for 90min at 2683 x g and cold for 120min at 2683 x g). The permeate was collected again.

This step was repeated a second time (total DI water added = 18mL). The final liquid WPC retentates (3-4.78mL) were at a protein concentration of 3-4% (w/v) (protein content based on volume and not solids since different amounts of solids [NaCl or $CaCl_2$] were added to each sample).

Norbixin extraction and quantitation

Norbixin was extracted from liquid wheys (200µl in 800µl acetonitrile), retentates (100µl in 900 µl acetonitrile), and permeates (200µl in 800µl acetonitrile) using the solvent extraction procedure described by Campbell et al. (2013). The whey, retentate, or permeate solutions were vortexed for 30s and centrifuged for 5min at 14000 x g. The supernatant was injected and quantified at 460nm on an HPLC (Waters 1525 Binary Pump, Waters, Milford, Mass., U.S.A.). The mobile phase consisted of 70%(v/v) acetonitrile (99.9%[w/v], ACROS, Fair Lawn, NJ), 29.9%(v/v) HPLC grade water (Honeywell, Burdick and Jackson, Muskegon, MI), and 0.1% formic acid (98 to 100%[w/v] Sigma-Aldrich, St. Louis, MO). 50µl of sample was injected (Waters 2707 Autosampler) onto a column (Phenomenex Kinetex 2.6µ 100x4.6mm, 40^{o}C) at a rate of 1mL/min. A standard curve was generated using 0 to 200mg/kg norbixin (45%[w/w], Chr. Hansen, Milwaukee, WI). Norbixin was dissolved in a 2.5% KOH solution in order to solubilize the carotenoid. The solution was then diluted to 10, 50, 100, 200, and 500µg/kg in acetonitrile (same solvent as the final extract). The standards were injected onto the HPLC under the same parameters as the samples.

Experiment 4: hydrophobic binding to isolated proteins

Alpha-lactalbumin or beta-lactoglobulin (92.5% [w/w],Davisco Foods International, La Seur, MN) was added to deionized water at a concentration of 10µM (Zhang and Zhong, 2012). The two solutions were heated to 31^{o}C and stirred at 350 rpm for 90 min and aliquots were taken for analysis. Ten ppb norbixin was added to each solution and stirred at 200 rpm for 15 min. Each solution was then sampled. This step was repeated three more times bringing each solution to a norbixin concentration of 50, 100, and 200ppb. These concentrations of norbixin are representative of the concentrations of norbixin found in fresh liquid Cheddar whey (~120ppb). The concentrations of protein in solution evaluated were much lower than what is found in liquid whey (i.e., 0.0183% beta-lactoglobulin in solution compared to 0.28-0.385% beta-lactoglobulin in liquid whey). This provides a higher ratio of norbixin to protein molecules compared to whey and insures that there is at least as much norbixin available for binding as in liquid whey. These samples, as well as fresh liquid whey and a norbixin standard (45ppm) in deionized water, were analyzed on a spectrofluorometer (Tecan, Durham, NC) scanned at 26^{o}C. The excitation and emission slit widths were set to 10nm and the excitation wavelength was set to 285nm (Zhang and Zhong, 2012). The emission spectra was recorded between 300 and 400nm (Zhang and Zhong, 2012).

Composition Analysis

Total percent solids and fat content of fluid milk and whey were analyzed using the Smart System 5 moisture-solids analyzer with SmartTrac rapid fat analysis (CEM, Matthews, NC). The total solids of liquid whey and retentate were determined by an oven drying method

(AOAC, 2000; method number 990.20; 33.2.44). Fat in liquid whey was measured using the Modified Pennsylvania babcock method. Fat in retentate was determined by ether extraction (AOAC, 2000; method number 989.05; 33.2.26). Protein was determined using the Kjeldahl method (AOAC, 2000; method number 991.20; 33.2.11). All samples were measured in duplicate.

Statistical Analysis

Data from experiments 1-3 were analyzed using a 2-way analysis of variance (ANOVA) using a general linear model (PROC GLIMMIX) in SAS (SAS Statistical Analysis Software, version 9.2, SAS Institute, Cary, NC). Sample interactions for experiment 1 (solids content*temperature), experiment 2 (treatment*temperature), and for experiment 3 (ion concentration*temperature) were analyzed.

RESULTS

Experiment 1: Effect of hot and cold UF

Norbixin is significantly smaller than the pore size of the membranes and would be expected to pass through the membranes into the permeate. This was not observed since norbixin was not found in the permeate. In addition, norbixin was concentrated in the retentate as protein, fat, and other solids were increased. This effect suggests norbixin is binding to a larger constituent of whey that does not pass through the UF membrane. Hot UF reduced norbixin

concentration in both WPC34 and WPC80 retentates compared to cold UF (Fig. 3.1) ($p<0.05$). Since hydrophobic interactions increase with increasing temperature this finding suggests that norbixin is not bound by hydrophobic interactions. It is also possible that heat caused increased oxidation/destruction of norbixin, however, heat alone may not be responsible for this phenomenon (see experiment 2).

Concentrating liquid whey via UF reduced norbixin concentration at both temperatures (Fig. 1)($p<0.05$). This result suggests that norbixin may be bound to a constituent loosely enough to be removed by shear during UF or that it was destroyed by shear or bound by UF membranes. The liquid whey had an average of 6.2mg of norbixin (Fig. 3.2). The norbixin retained following hot or cold UF to WPC80 was 4.06 and 2.44mg, respectively (Fig. 3.2). There was a norbixin loss of 2.14mg (34.5% loss) and 3.76mg (60.6% loss) following cold or hot WPC80, respectively. Of the norbixin lost during ultrafiltration to WPC80, 1.21 and 1.88mg was recovered in the wash water of the UF membranes (Fig. 3.2). The remaining 0.85 and 1.65mg norbixin was unrecovered and likely destroyed during cold and hot UF, respectively (Fig. 3.2). Norbixin was not detected in permeates. This suggests (as well as the norbixin in the UF wash water) that norbixin binds to polyethersulphone (PES) ultrafiltration membranes and has an increasing affinity to the PES UF membranes with increased temperature.

Experiment 2: Effect of hot or cold hold time versus hot or cold UF

In WPC34 and WPC80, hot recirculation (heat/shear) resulted in increased norbixin loss compared to cold recirculation (no heat/shear) or hot and cold hold (heat/no shear and no heat/no shear, respectively) treatments (Fig. 3.3 and 3.4). Heat alone (hot hold) reduced norbixin concentration by 13.3% in WPC34 and 10.3% in WPC80 (Fig. 3.3 and 3.4). In WPC80, heat (hot hold) resulted in greater norbixin loss than cold recirculation or cold hold. Overall these findings reveal a detrimental effect of heat and a synergistic effect between heat energy and shear and norbixin loss. Combining heat and shear resulted in 40.1% and 48.5% norbixin destruction in WPC34 and WPC80, respectively (Fig. 3.3 and 3.4).

Experiment 3: Effect of ionic strength

Hot whey ultrafiltered with 1% and 4% NaCl resulted in the highest norbixin loss compared to any other treatment (Fig. 3.5)($p<0.05$). Hot whey ultrafiltered with 0 (control), 1, and 4% NaCl reduced norbixin by 45, 50, and 50.8%, respectively (Fig. 3.5). Adding 4% NaCl resulted in 5.8% more norbixin loss than the hot control ($p<0.05$). Cold whey ultrafiltered with 1 and 4% NaCl resulted in the highest norbixin loss compared to other cold treatments (Fig. 3.5)($p<0.05$). Hot and cold ultrafiltered whey with 8% NaCl was not different from the controls (Fig. 3.5)($p>0.05$). These results suggest that norbixin may have a higher affinity for a charged/polar environment rather than a hydrophobic one. However, there may be a secondary effect due to the lack of norbixin loss in the 8% NaCl sample. The higher ion concentration may have denatured proteins to the point of changing binding potential/properties.

Norbixin loss was also decreased when liquid whey was ultrafiltered/diafiltered hot with $CaCl_2$ addition of 1, 4, or 8% (Fig. 3.6). However, due to significant protein aggregation/precipitation during ultrafiltration (and diafiltration) in all hot and cold $CaCl_2$ retentates, these results cannot necessarily be attributed to norbixin loss caused by increased electrostatic interactions within the matrix. Instead, this effect was likely caused by calcium cross-linking of proteins thus trapping norbixin within the aggregated precipitate (Simons and others, 2002).

Experiment 4: hydrophobic binding to isolated proteins

There were no observed emission peak shifts or peak intensity changes with either alph-lactalbumin or beta-lactoglobulin at any of the four norbixin concentrations evaluated (Fig. 3.7-3.14). A lack of emission peak shifts or intensity change shows that norbixin did not alter the hydrophobic surface tryptophan residues. These results suggest that norbixin does not bind hydrophobically to either of the major proteins in liquid whey.

DISCUSSION

Previous research has demonstrated enhanced carotenoid oxidation/destruction with increased oxygen, heat, light, pressure, and organic solvents (i.e., toluene, benzene, etc.) (Jorgensen and Skibsted, 1989; Chen and Tang, 1998; Boon and others, 2010; Choudhari and others, 2011; Pertig and others, 2012). Combinations of these carotenoid oxidation accelerators have been shown to further amplify these oxidation/destruction reactions. This

effect is likely responsible for the destruction/loss of norbixin during whey processing. The remaining norbixin is retained by binding of a constituent larger than 10kDa or possibly by a theoretical property that allows norbixin to form micelles with itself (Zhu and Damodaran, 2012). The results for these experiments suggest that norbixin is not binding hydrophobically to the major whey proteins (alpha-lactalbumin and beta-lactoglobulin) but does not disprove the theory that norbixin is forming a micelle. It is possible that shear/pressure (such as that from a UF) may break a micelle, however, once the micelle is broken it would be expected to filter into the permeate. This effect was not observed and may be due to too little shear/pressure. Lipids also form micelles in aqueous environments. The lipid micelles in whey also prevent lipids/fat from being filtered into the permeate and the same effect may occur with norbixin (Rombaut and others, 2007).

It has been demonstrated that carotenoids are able to bind to proteins (Cho and others, 1994; Zhang and Zhong, 2012; Zhang and others, 2013). Biologically, beta-lactoglobulin is believed to bind/transport hydrophobic carotenoids and vitamins (via a hydrophobic pocket) (Cho and others, 1994; Zhang and Zhong, 2012; Zhang and others, 2013). Our results negate the earlier theory that norbixin behaves like hydrophobic carotenoids and binds to beta-lactoglobulin via the hydrophobic pocket. These results also suggest that norbixin is not binding hydrophobically to any other constituent in whey. Increasing heat energy causes increased hydrophobic interactions. Hot ultrafiltration/diafiltration reduced norbixin concentration, therefore, norbixin is not binding hydrophobically. It is possible that thermal

destruction caused a reduction in norbixin, however, heat alone did not significantly destroy more norbixin than cold in WPC34 or WPC80 retentates.

Proteins are large and complex molecules that contain regions that are polar, non-polar, and ionic. The non-polar, hydrophobic regions are generally tucked in the compact core in an aqueous, polar environment/matrix (such as whey). The surface of proteins in the same environment (such as whey) strongly interact with other compounds (generally ionic and polar interactions) (Dickinson, 1999). The major mechanism preventing protein aggregation is electrostatic repulsion (McClements, 2005). The ionic strength experiment demonstrated that norbixin has a higher affinity for a charged matrix. Increasing the ionic concentration of the matrix (by adding NaCl) caused the norbixin to dissociate from its binding constituent (or possibly disrupted a micellar formation), thus allowing it to oxidize or bind to the UF membrane. This effect was evident due to the reduced concentration of norbixin in the retentate when 1-4% NaCl was added to the whey and diafiltration water yet norbixin was still not found in the permeate. This effect could also be due to ions quenching charges on the surface of proteins and thus quenching charges that loosely bind the polar ends of norbixin.

The fluorescence quenching experiment results suggested that norbixin is not binding hydrophobically; specifically to alpha-lactalbumin or beta-lactoglobulin. More specifically, these proteins are not hydrophobically binding norbixin by surface tryptophan residues. This finding does not prove that norbixin is not binding hydrophobically. This data suggests that norbixin does not bind hydrophobically to surface tryptophan residues in beta-lactoglobulin

or alpha-lactalbumin. Norbixin may still have some affinity to hydrophobic compounds but is not likely due to the hydrophilic (water-soluble) nature of norbixin. This experiment negates earlier theories of norbixin binding in the hydrophobic pocket of beta-lactoglobulin (by tryptophan residues) like other [hydrophobic] carotenoids. Despite the mechanism, the results suggest that norbixin has a higher affinity for charged/polar interactions due to increased norbixin loss/destruction caused by ion addition. The binding interactions of norbixin appear to be weak enough in liquid whey that norbixin can be removed by shear alone. Increasing the heat energy and ionic strength appear to have a synergistic effect along with shear. Combining the three treatments (heat, increased ionic strength, and pressure/shear) can remove more than 50% of norbixin from liquid whey retentate.

CONCLUSION

Our results show no evidence of hydrophobic binding between norbixin and major whey proteins. Utilizing a combination of heat, shear, and salt in the proper proportions significantly reduced norbixin concentration in WPC34 and WPC80. This effect was achieved by increasing the oxidation rate via heat energy while simultaneously reducing binding affinity by adding salts and increasing shear.

ACKNOWLEDGEMENT

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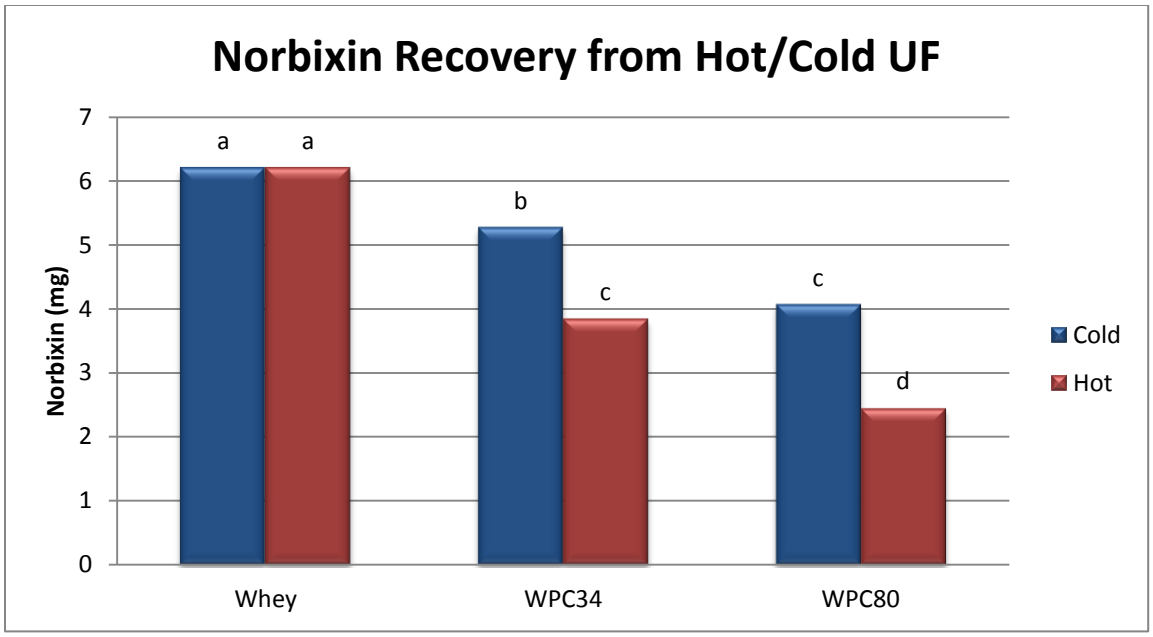

Figure 3.1 Norbixin Recovery from Hot and Cold Ultrafiltration

Norbixin concentration (mg) in hot and cold ultrafiltered whey protein concentrate (WPC). Cold = 8°C; Hot = 50°C; WPC34 = 34% protein(w/w); WPC80 = 80% protein (w/w); Whey hot and cold started at the same norbixin concentration

[a-d]Means that do not share a common letter are significantly different ($p<0.05$)

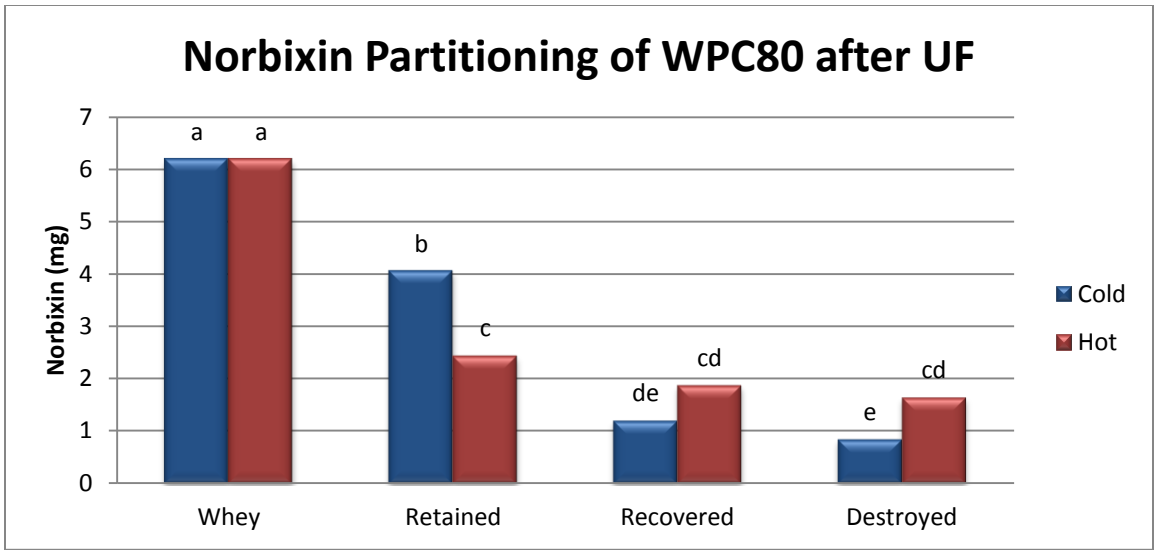

Figure 3.2 Norbixin Partitioning of WPC80 After Ultrafiltration

Norbixin concentration (mg) in WPC80 (retained) and UF wash water/cleaning solution (recovered). Retained = in WPC; recovered = from UF wash water and cleaning solution; destroyed = lost; Whey hot and cold started at the same norbixin concentration

[a-d]Means that do not share a common letter are significantly different ($p<0.05$)

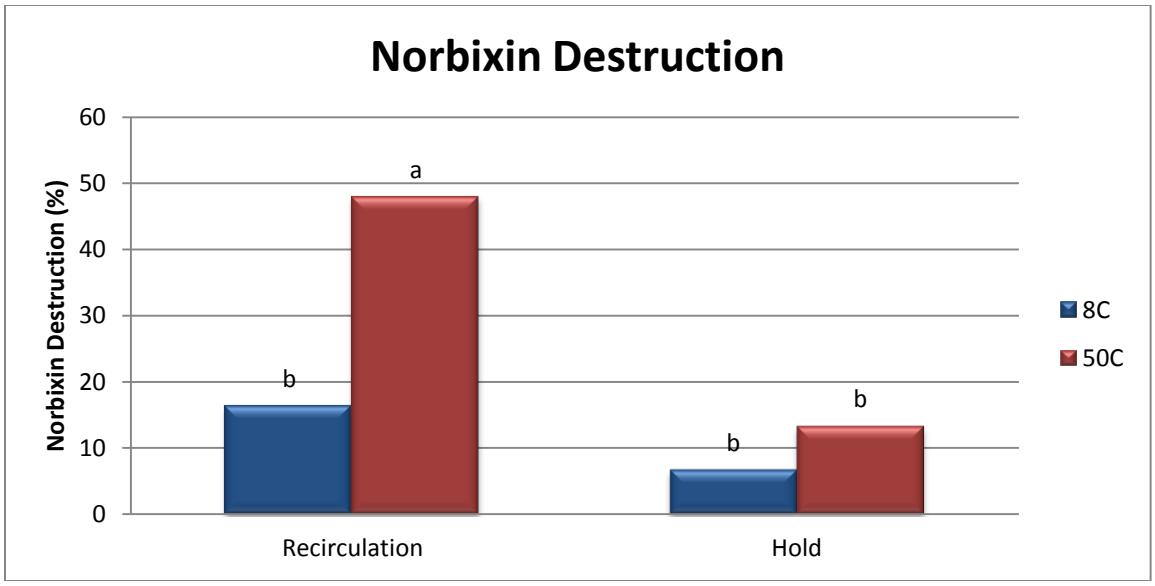

Figure 3.3 Norbixin Destruction in WPC34 Retentate

Norbixin loss in WPC34 retentate recirculated through UF compared to fresh WPC34, or WPC34 retentate held for 3h compared to fresh WPC34. Cold = 8^{o}C; Hot = 50^{o}C; recirculation = through UF for 3h; hold = held (no shear) at temperature for 3h; recirculation and hold loss compared to fresh WPC34

[a-b]Means that do not share a common letter are significantly different ($p<0.05$)

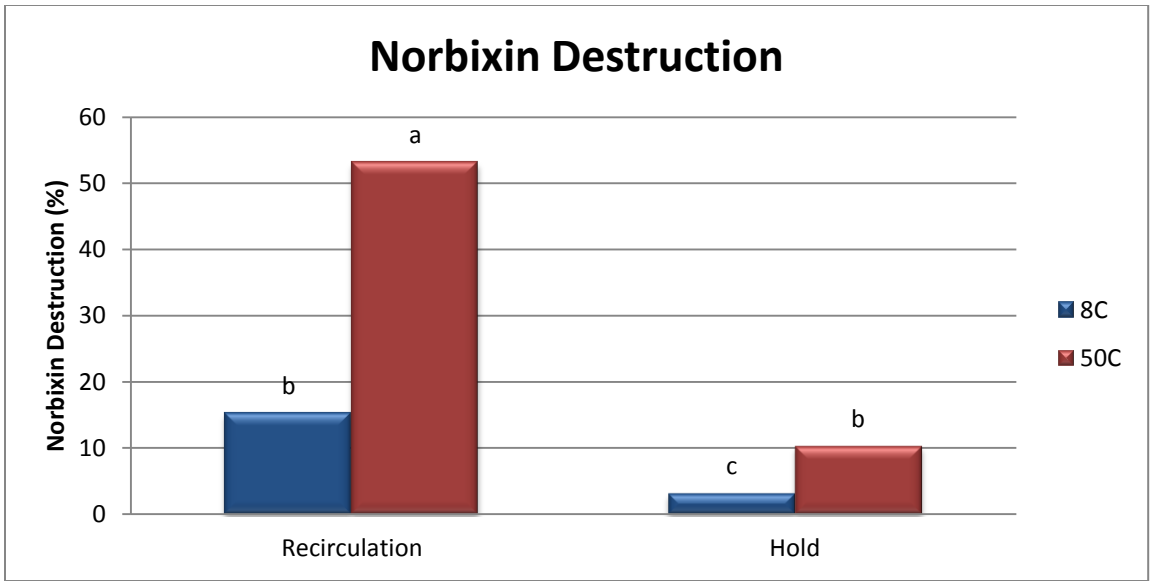

Figure 3.4 Norbixin Destruction in WPC80 Retentate

Norbixin loss in WPC80 retentate recirculated through UF compared to fresh WPC80, or WPC80 retentate held for 3h compared to fresh WPC80. Cold = 8^{o}C; Hot = 50^{o}C; recirculation = through UF for 3h; hold = held (no shear) at temperature for 3h; recirculation and hold loss compared to fresh WPC80

[a-b]Means that do not share a common letter are significantly different ($p<0.05$)

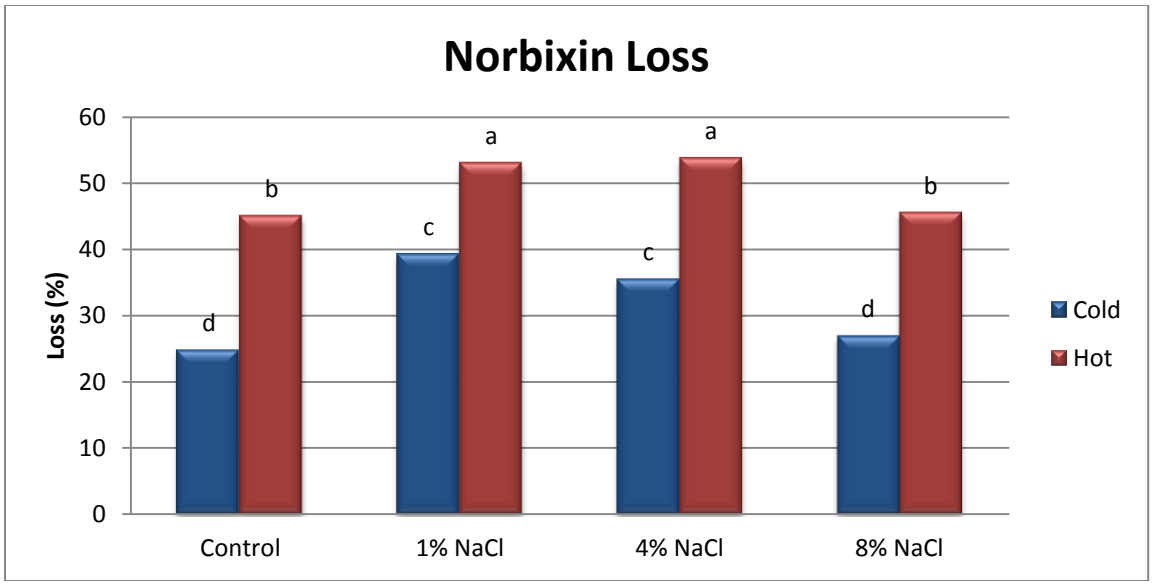

Figure 3.5 Norbixin Loss in Ultrafiltered WPC at Varying NaCl Concentrations

Norbixin loss in ultrafiltered WPC at varying NaCl concentrations. Cold = 8°C; Hot = 50°C; percent loss as compared to liquid whey

[a-d]Means that do not share a common letter are significantly different ($p<0.05$)

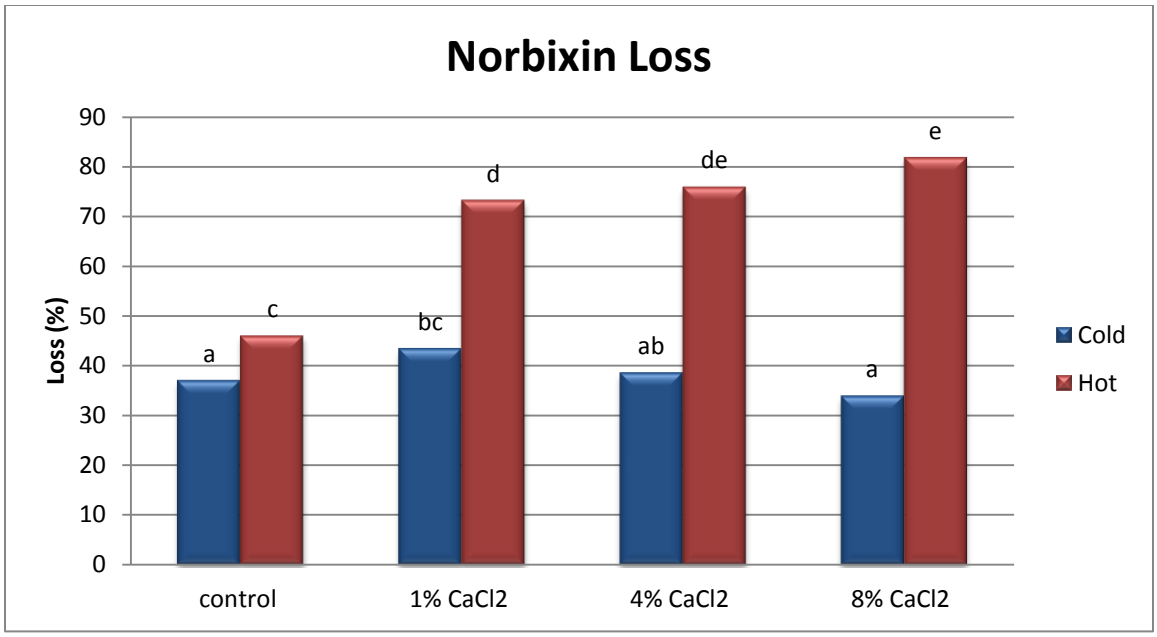

Figure 3.6 Norbixin Loss in Ultrafiltered WPC at Varying CaCl2 Concentrations

Norbixin loss in ultrafiltered WPC at varying $CaCl_2$ concentrations. Cold = 8°C; Hot = 50°C; percent loss as compared to liquid whey

[a-e]Means that do not share a common letter are significantly different ($p<0.05$)

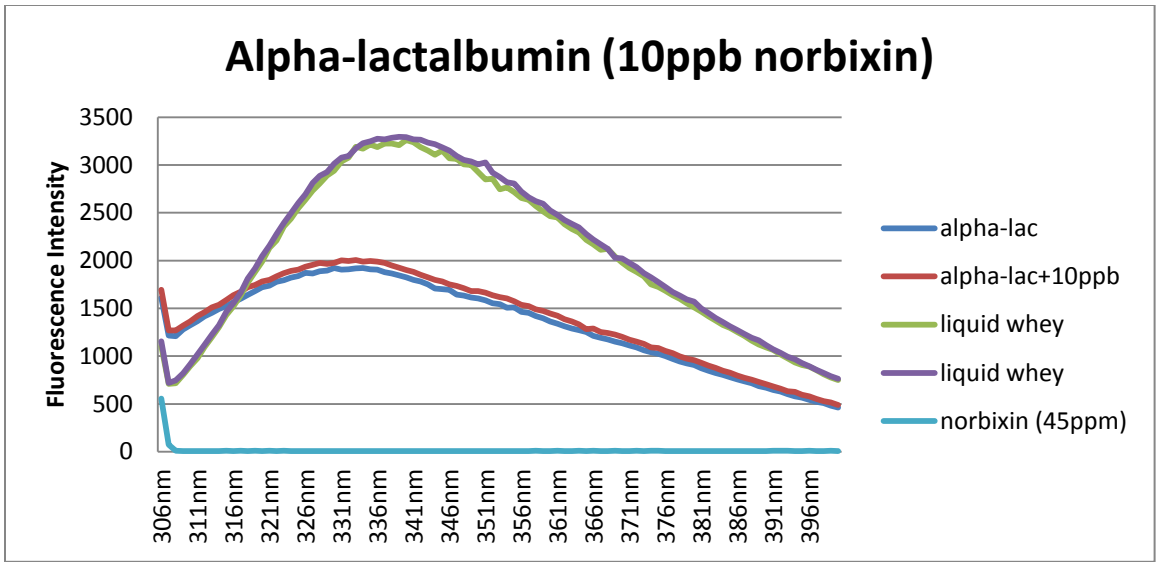

Figure 3.7 Emission Spectra of 10ppb Norbixin in Alpha-Lactalbumin

Emission spectra of alpha-lactalbumin (control), alpha-lactalbumin + norbixin (10ppb), fresh liquid whey, and norbixin standard (45ppm)

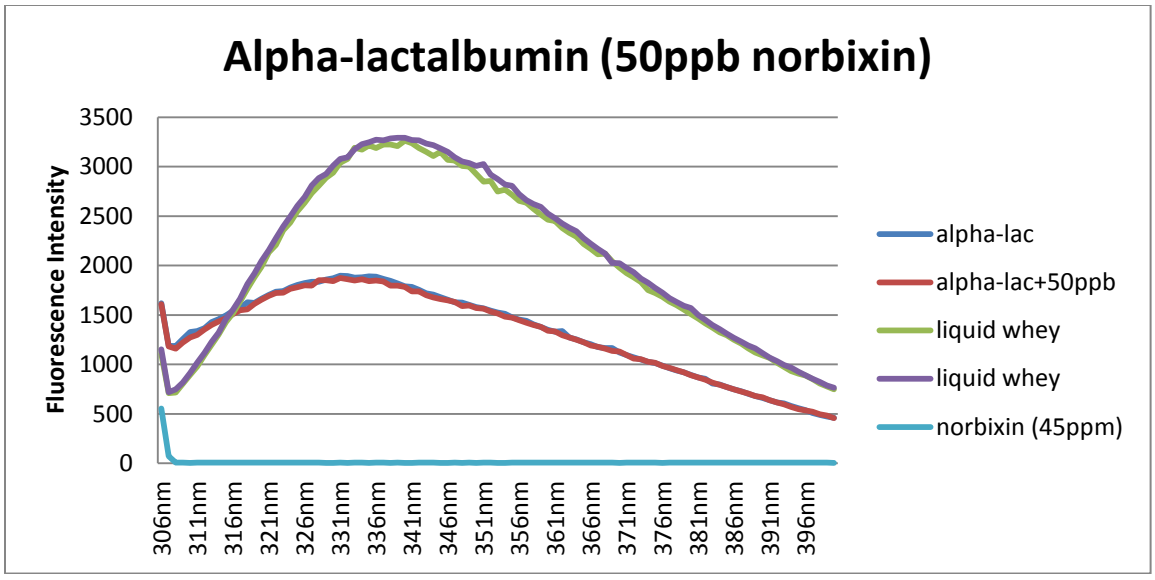

Figure 3.8 Emission Spectra of 50ppb Norbixin in Alpha-Lactalbumin

Emission spectra of alpha-lactalbumin (control), alpha-lactalbumin + norbixin (50ppb), fresh liquid whey, and norbixin standard (45ppm)

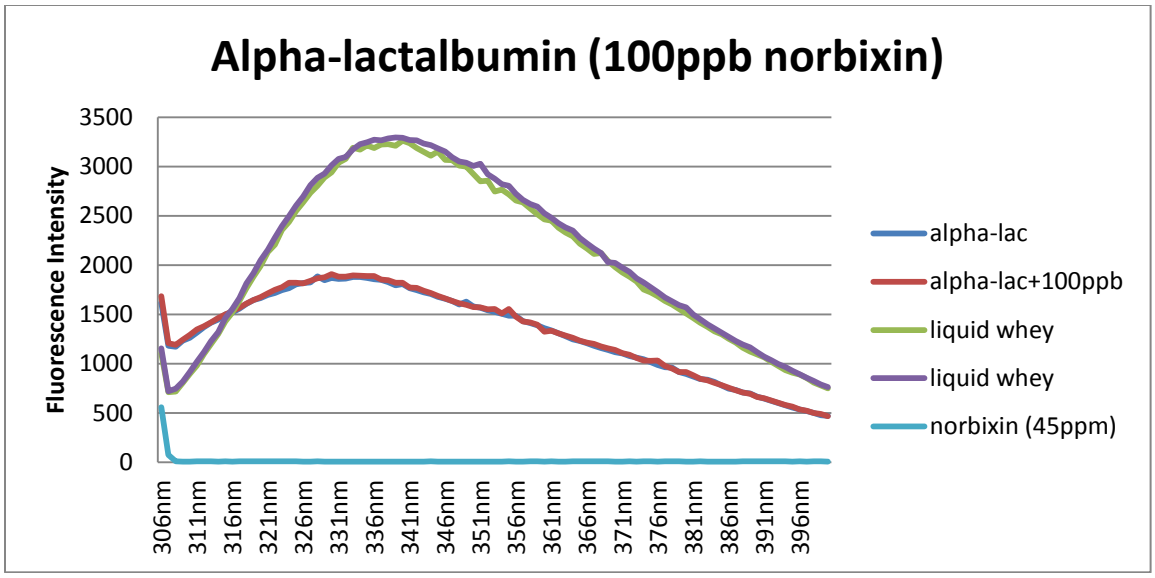

Figure 3.9 Emission Spectra of 100ppb Norbixin in Alpha-Lactalbumin

Emission spectra of alpha-lactalbumin (control), alpha-lactalbumin + norbixin (100ppb), fresh liquid whey, and norbixin standard (45ppm)

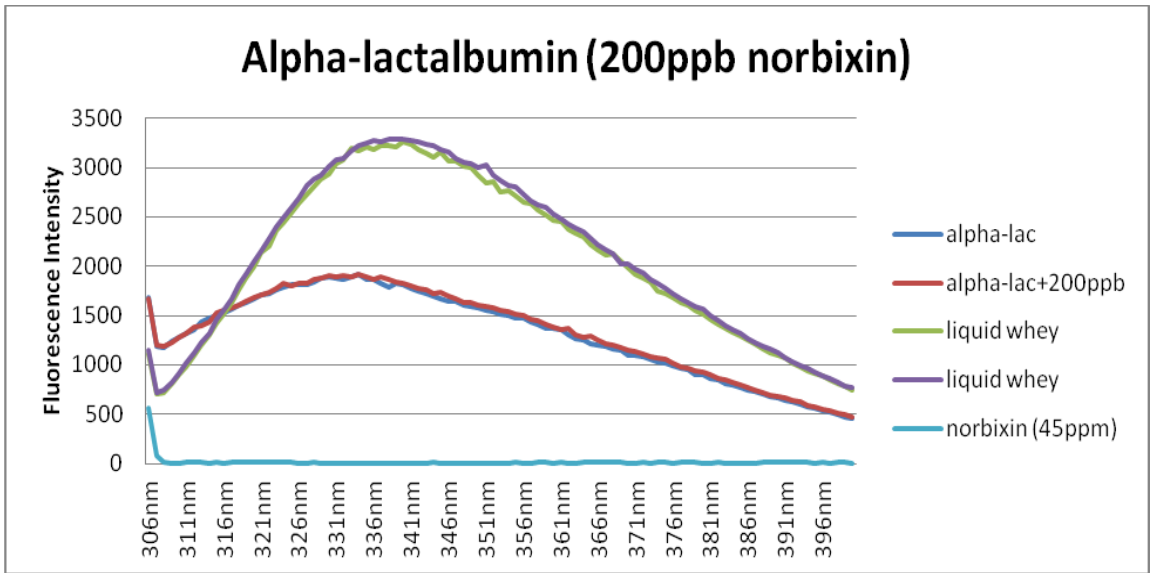

Figure 3.10 Emission Spectra of 200ppb Norbixin in Alpha-Lactalbumin

Emission spectra of alpha-lactalbumin (control), alpha-lactalbumin + norbixin (200ppb), fresh liquid whey, and norbixin standard (45ppm)

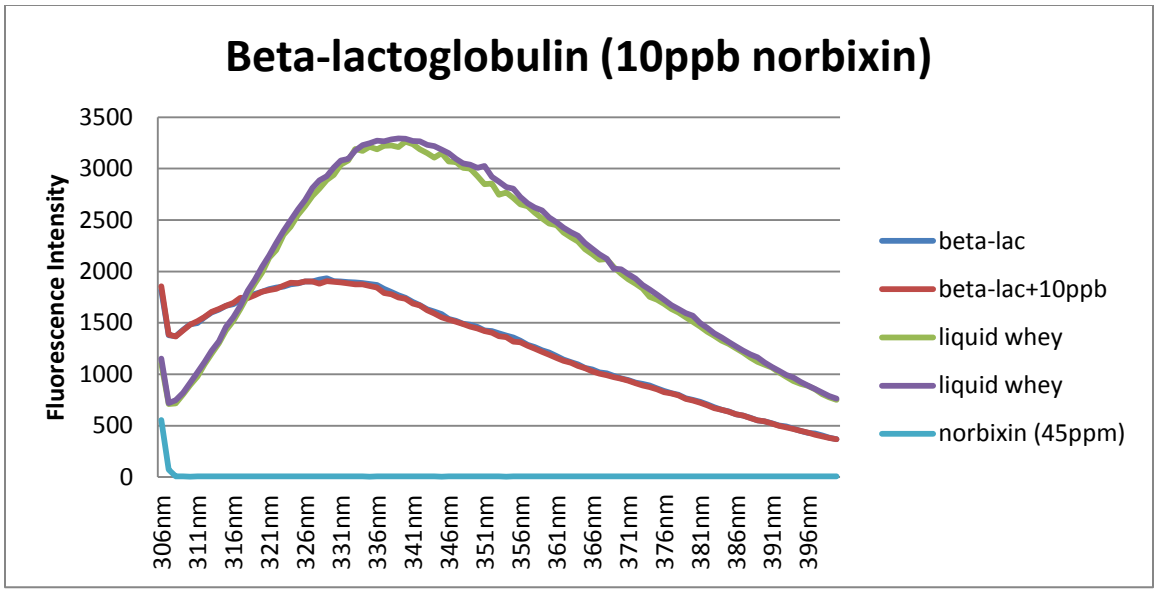

Figure 3.11 Emission Spectra of 10ppb Norbixin in Beta-Lactoglobulin

Emission spectra of beta-lactoglobulin (control), beta-lactoglobulin + norbixin (10ppb), fresh liquid whey, and norbixin standard (45ppm)

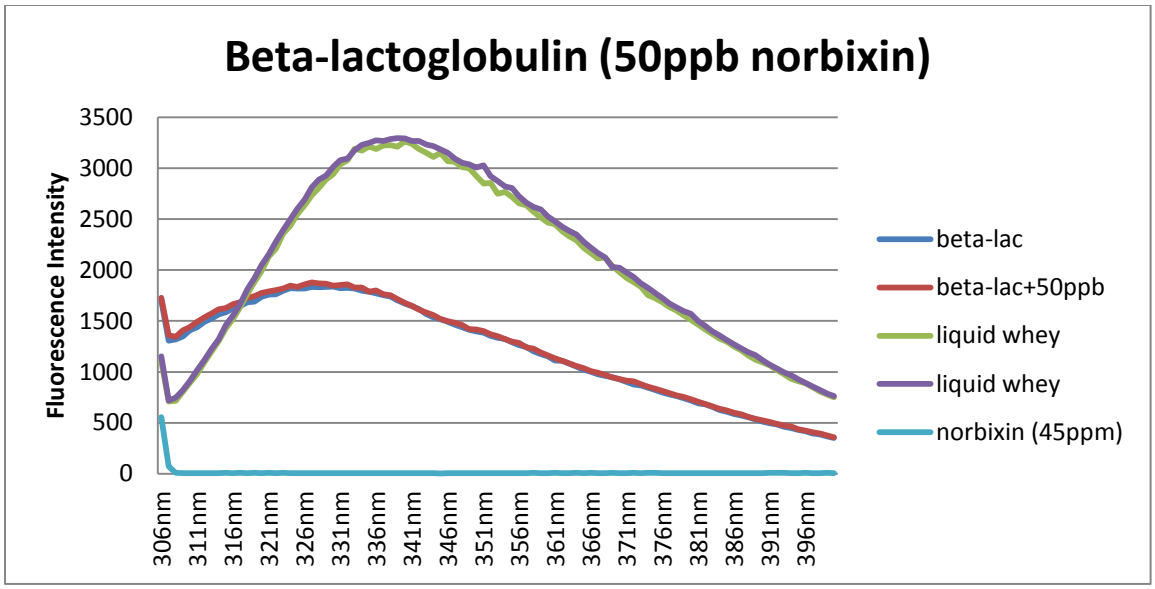

Figure 3.12 Emission Spectra of 50ppb Norbixin in Beta-Lactoglobulin

Emission spectra of beta-lactoglobulin (control), beta-lactoglobulin + norbixin (50ppb), fresh liquid whey, and norbixin standard (45ppm)

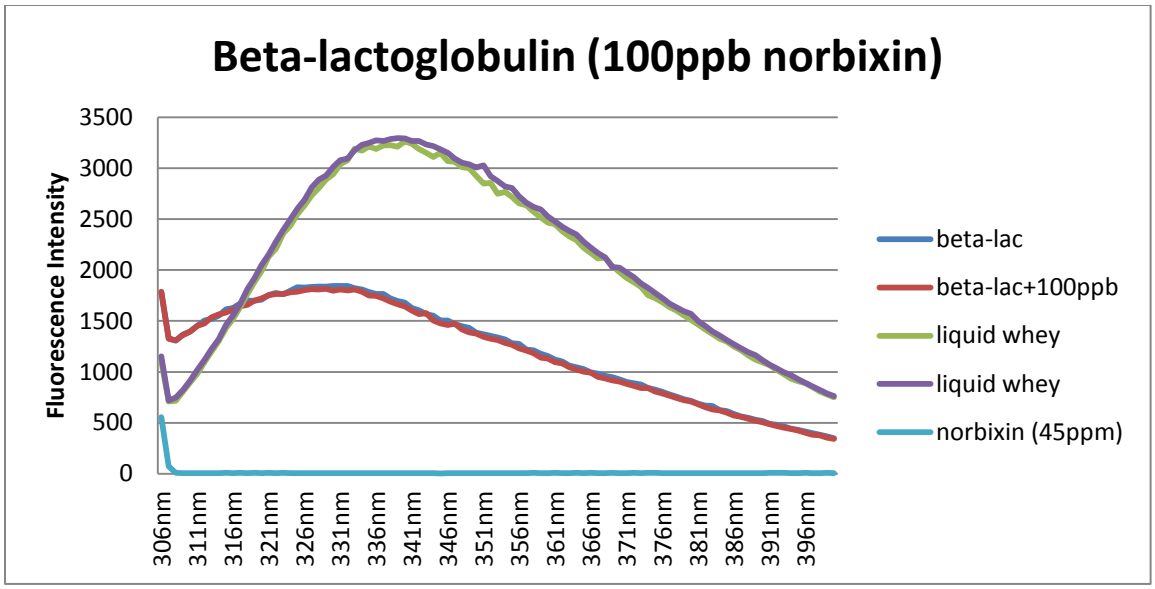

Figure 3.13 Emission Spectra of 100ppb Norbixin in Beta-Lactoglobulin

Emission spectra of beta-lactoglobulin (control), beta-lactoglobulin + norbixin (100ppb), fresh liquid whey, and norbixin standard (45ppm)

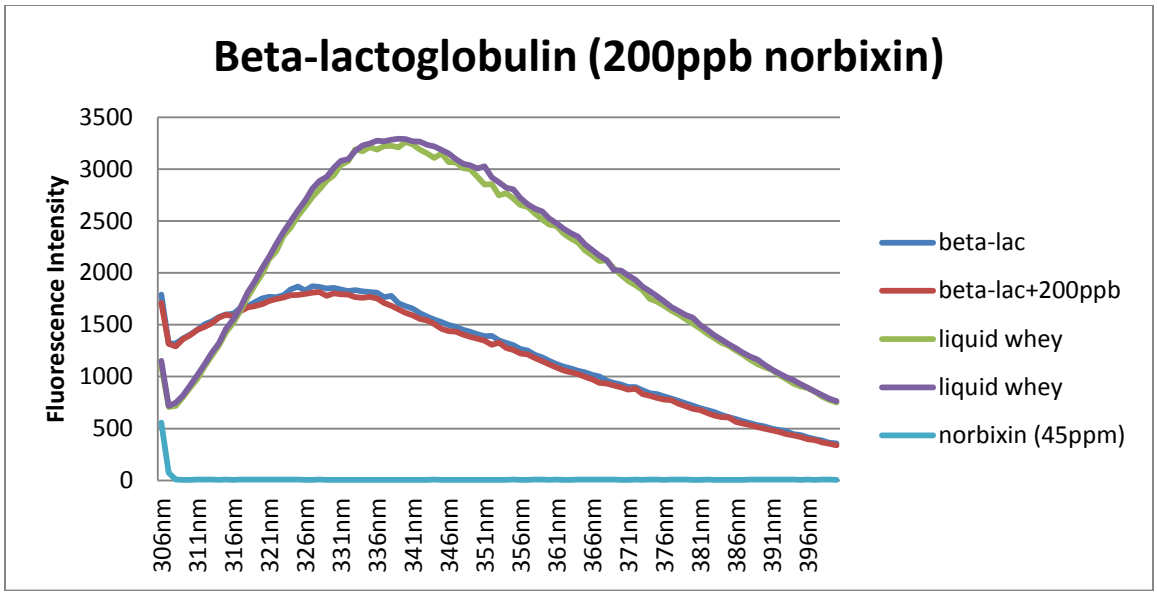

Figure 3.14 Emission Spectra of 200ppb Norbixin in Beta-Lactoglobulin

Emission spectra of beta-lactoglobulin (control), beta-lactoglobulin + norbixin (200ppb), fresh liquid whey, and norbixin standard (45ppm)

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