

ABSTRACT

CAMPBELL MERTZ, RACHEL E. Enzymatic Bleaching of Whey. (Under the direction of Dr. MaryAnne Drake).

Cheddar cheese produced in the United States is often colored with annatto, an orange carotenoid comprised primarily of norbixin, to achieve the desired color. Some of the annatto remains in the whey and must be bleached to remove color. Increasing regulations on current approved bleaching agents, hydrogen peroxide and benzoyl peroxide, in conjunction with undesirable flavors due to bleaching, are pushing the industry to investigate and implement alternative bleaching agents. Lactoperoxidase (LP), a native enzyme in bovine milk, can be used to effectively bleach whey in conjunction with naturally present thiocyanate (SCN) and low concentrations of added hydrogen peroxide (<50 ppm). The addition of exogenous peroxidase (EP) may also be used to facilitate bleaching. Several studies were conducted to investigate enzyme behavior at various parameters such as bleach point (fluid whey vs. retentate), temperature, pH, and time and their effect on bleaching efficacy. Sensory and volatile compound analyses were conducted to elucidate effects on flavor.

LP activity, in fluid milk, whey, and retentate, did not decrease following 3 d of cold (4C) or frozen (-20C) storage ($p>0.05$). LP activity was higher in retentate than fluid whey as LP was concentrated along with other proteins during ultrafiltration. Among commercial retentates, native LP activity varied widely among both manufacturer and lot. The addition of EP decreased the time requirement for bleaching (>80% norbixin destruction) in both fluid whey and retentate when LP was not highly active. At cold temperatures, LP bleached (>80% norbixin destruction) fluid whey in 12 h whereas the addition of EP decreased

bleaching time from 11 h to 1 h. In commercial retentates, the addition of EP decreased bleaching time by 4 h and 4.5 h at cold (4C) and warm (35C) temperatures, respectively. Enzymatic bleaching, either native LP or with added EP, increased lipid oxidation compounds, aroma intensity and cardboard flavor and decreased sweet aromatic and cooked/milky flavors in fluid wheys and dried whey proteins ($p < 0.05$). Enzymatic bleaching (both LP and EP) occurred faster at 35C than at 4C ($p < 0.05$), although increased off-flavors were observed at 35C as opposed to 4C. Enzymatic bleaching (either LP or EP) destroyed more norbixin than traditional chemical bleaching (hydrogen peroxide (HP) at 250 ppm) ($p < 0.05$). Enzymatic bleaching in fluid whey and retentates is a feasible option for dairy processors to remove color from wheys, however, due to inherent variability in LP activity, the addition of EP may be required for consistent bleaching.

Enzymatic Bleaching of Whey

by
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DEDICATION

To my husband, Billy – I could have never done this without you. I'm glad you talked me out of quitting nearly every week – thank you!

BIOGRAPHY

Rachel Elizabeth Campbell was born on August 17, 1986. Rachel grew up in Chapel Hill, North Carolina and graduated from Chapel Hill High School in 2004. She began pursuing a degree in food science and obtained her BS in Food Science from North Carolina State University in 2008. During college she did 3 internships where she affirmed her love of food science and research. Rachel began her graduate studies in 2008 under Dr. MaryAnne Drake obtaining her MS in 2010 and her PhD in 2013. She has one brother, Dr. Jonathan Campbell. Her parents, Jennifer and Roger Strickland, still reside in her childhood home. After graduation, Rachel, her husband (William Mertz) and their schnoodle (Charlie) will be moving to Memphis, TN.

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The impact of native and non-native enzymes on the flavor of dried dairy ingredients

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ABSTRACT

Dried dairy ingredients are used in a wide array of foods from soups to bars to beverages. The popularity of dried dairy ingredients, including but not limited to sweet whey powder, whey proteins and milk powders, is increasing. Dried dairy ingredient flavor can carry through into the finished product and influence consumer liking, thus it is imperative to produce a consistent product with bland flavor. Many different chemical compounds, both desirable and undesirable, contribute to the overall flavor of dried dairy ingredients, making the flavor very complex. Enzymatic reactions play a major role in flavor. Milk contains several native enzymes such as lactoperoxidase, catalase, xanthine oxidase, proteinases, and lipases, which may impact flavor. In addition, other enzymes are often added to milk or milk products for various functions such as milk clotting (chymosin), bleaching of whey products (fungal peroxidases, catalase to deactivate hydrogen peroxide), flavor (lipases in certain cheeses), or produced during the cheese make process from starter culture or non-starter bacteria. These enzymes and their possible contributions will be discussed in this review. Understanding the sources of flavor is crucial to produce bland, flavor-less ingredients.

KEY WORDS: Dairy ingredients, enzymes, flavor

INTRODUCTION

Brief history of enzymes

The word “enzyme” was first introduced in 1878 by W. Kuhne, and in 1883 E. Duclaux introduced the term “substrate” (Fox and Kelly, 2006a). While enzyme activity was identified previously, it wasn’t until 1926 that B. Sumner crystallized the first enzyme, urease, from jack bean meal at Cornell University, proving definitively that enzymes existed, for which he later received the Nobel prize (1946) (Kosikowski, 1988). Since then, a variety of enzymes have been characterized in a wide array of foods, including milk. The history of indigenous enzymes in bovine milk has been reviewed extensively (Fox and Kelly, 2006a; Fox and Kelly, 2006b).

Nomenclature of enzymes

Enzymes are divided among 6 major classes (Table 1). Each class is then divided into subclasses, each subclass divided into sub-sub classes, and each sub-subclass contains several enzymes. For example, catalase is EC 1.11.1.6. The first number (1) refers to the enzyme class of oxidoreductases, the second number (11) refers to the sub class of enzymes which act on a peroxide as an electron acceptor, the third number (1) refers to the sub-subclass of peroxidases, and the last number (6) identifies this enzyme as a catalase within the sub-subclass (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)).

Enzymes in bovine milk

Indigenous, exogenous, and endogenous enzymes can be found in dairy ingredients. Indigenous enzymes are those that are found naturally in bovine milk. Indigenous enzymes

come from four principal sources in fluid milk: somatic cells, secretory cell cytoplasm, the milk fat globule membrane (MFGM), or blood plasma through “leaky junctions” between mammary cells (Fox and Kelly, 2006a). Over 60 indigenous enzymes have been identified to date in bovine milk; however, milk does not contain the substrates for many of these enzymes (Fox, 2003). Nonetheless, native milk enzymes are of significant importance in the following ways: indices of mastitic infection, preservation of milk quality, indices of thermal history of milk, antimicrobial activity, as a commercial source of enzymes, or deterioration (Fox and Kelly, 2006a). In addition to indigenous enzymes, other enzymes that are not native to milk are often added to milk or milk products for various functions such as milk clotting (chymosin), bleaching of whey products (fungal peroxidases, catalase to deactivate hydrogen peroxide), flavor (lipases in certain cheeses), or produced during the cheese make process from starter culture bacteria (Campbell et al., 2011a; Campbell et al., 2011b). Exogenous enzymes are those that are added to milk during processing (for example, chymosin) while endogenous enzymes are those that are present in a food but not a part of the food such as enzymes produced by microorganisms either by deliberate inoculation (cheese culture) or by unintentional growth (psychrotrophic bacteria) (Table 2). Enzymes can have multiple functions; this review will focus on flavor of milk and milk products, including dried dairy ingredients, due to native and non-native enzymes.

Flavor of dried dairy ingredients

Flavor is considered the single most important factor in determining the success or failure of a food product (Morr and Ha, 1991; Drake, 2007). In general, dairy products display a wholesome and flavorful image making them well accepted with consumers. Dairy

foods can possess two types of off-flavors: those that are already present in the milk and those that develop later as a consequence of actions, such as processing (Bodyfelt et al., 1988). Dried dairy ingredients are expected to have a delicate and bland flavor that is undetectable in finished goods (Drake, 2006). Undesirable flavors that carry through to the finished product limit the utilization of dairy ingredients.

Many different chemical compounds, both desirable and undesirable, contribute to the overall flavor of fluid and dried whey and dried whey ingredients, making the flavor very complex (Carunchia Whetstine et al., 2003, 2005; Drake et al., 2009). Lipid oxidation is responsible for many volatile compounds that contribute to off-flavors (Morr and Ha, 1991; Carunchia Whetstine et al., 2005; Carunchia Whetstine and Drake, 2007; Mortenson et al., 2008; Whitson et al., 2010; Liaw et al., 2011). The reaction of oxygen with unsaturated fatty acids causes a wide variety of flavor-active volatile compounds including methyl esters, ketones, aldehydes, and free fatty acids (Morr and Ha, 1991; Carunchia Whetstine et al., 2003, 2005; Liaw et al., 2011). The role of enzymes as a catalyst for lipid oxidation will be explored in this review.

INDIGENOUS ENZYMES

Lactoperoxidase (EC 1.11.1.7)

A member of the oxidoreductase family, lactoperoxidase (LP) plays an important role in protecting newborn infants against pathogenic microorganisms (Seifu et al., 2005). Lactoperoxidase is a normal component of mammalian milk, and has been found in all mammalian milks (Seifu et al., 2005). Unlike many other enzymes, LP is very heat stable, retaining its activity during the normal pasteurization of bovine milk, only being destroyed

above 78°C (Seifu et al., 2005). The LP molecule contains a heme group in the center (Kussendrager and van Hooijdonk, 2000). Bovine LP consists of a single polypeptide chain containing 612 amino acid residues. Its molecular weight is approximately 78kDa and it has a high isoelectric point of 9.6.

Lactoperoxidase is the second most abundant enzyme found in bovine milk, constituting about 0.5% of the serum proteins in raw milk (de Wit and van Hooijdonk, 1996). Variations in enzyme levels depend on the sexual cycle of the cow, season, feeding regime, and breed (Kussendrager and van Hooijdonk, 2000). The LP system is most traditionally used as an antimicrobial system in developing countries without proper access to pasteurization equipment or cold storage (Seifu et al., 2005). It has recently been applied for bleaching of fluid Cheddar whey (Bottomley et al., 1989; Campbell et al., 2012, 2013).

The LP system consists of three components: lactoperoxidase, hydrogen peroxide (HP) and thiocyanate (SCN). All three of these components are necessary in sufficient amounts in order for the system to be active. The active LP system produces hypothiocyanate (OSCN), the major intermediate oxidation product of SCN, which inhibits bacterial growth and in some cases, is bacteriocidal (Seifu et al., 2005). Other short-lived intermediates that may be formed depending on reaction conditions include cyanogen thiocyanate, cyanosulfurous acid, and cyanosulfuric acid (Seifu et al., 2005). The oxidation of sulfhydryl groups in microbial enzymes and other proteins is considered “key” to the antimicrobial activity of the LP system (Seifu et al., 2005). In addition to OSCN, hydroxyl radicals and superoxide radicals are created possibly causing other reactions in the matrix, such as bleaching or other Fenton-type side reactions causing increased lipid oxidation

(Campbell et al., 2012; Jervis and Drake, 2013). Whether the LP system is used for antimicrobial purposes or for the bleaching of whey, flavor of the milk or whey will be affected by the free radicals created and the oxidation of sulfhydryl groups. Lipid oxidation products have been documented in fluid whey and powdered WPC80 bleached by activating the LP system (Campbell et al., 2012). Lipid oxidation products are major contributors to off-flavors in dried dairy ingredients, often eliciting cardboard/stale flavors (Whitson et al., 2010, 2011). Undesirable flavor associated with sulfur compounds, such as the cabbage flavor produced by dimethyl trisulfide (DMTS) have been previously documented in dried dairy ingredients (Wright et al., 2006, 2009).

While it is evident that the use of the lactoperoxidase system for the bleaching of fluid whey can produce off-flavors that carry through into the final dried whey protein powder (Campbell et al., 2012, 2013) it is unlikely that flavors due to LP activity occur in dried milk ingredients such as milk protein concentrate (MPC) or skim milk powder (SMP). Unlike fluid whey, to activate the lactoperoxidase system in fluid milk, thiocyanate must be added as well as hydrogen peroxide (Boulares et al., 2011). As such, the system cannot activate naturally even if the milk contains enough naturally present HP from aerobic bacteria to activate the LP system. The LP system in milk is used primarily in developing countries without proper processing equipment for pasteurization, thus, this milk would likely not be subjected to membrane filtration. Until recently (2009), CODEX prohibited the use of the lactoperoxidase system in milk and milk products traded internationally. In 2009 this restriction was lifted and “any trade in milk treated by the lactoperoxidase system should only be on the basis of mutual agreement between countries concerned”.

Hydrogen Peroxide (HP): Hydrogen peroxide is frequently added to activate the LP system, in either milk or whey depending on the use of the LP system (antimicrobial or bleaching, respectively), if no HP is naturally present (Kussendrager and van Hooijdonk, 2000). Catalase negative organisms (such as lactic acid bacteria) can generate enough HP under aerobic conditions to activate the LP system. Many lactobacilli, lactococci, and streptococci produce sufficient HP under aerobic conditions to activate the LP system (Seifu et al., 2005). If the LP system is not activated by native bacteria producing hydrogen peroxide, exogenous HP must be added to activate the system (Reiter and Harnulv, 1982). Gram negative, catalase positive organisms (such as *pseudomonas*, *coliforms*, *salmonellae*, and *shigellae*) are not only inhibited by the LP system, but may be killed by the activated LP system (Seifu et al., 2005). Gram positive, catalase negative bacteria (such as streptococci and lactococci) are generally inhibited but not killed by the LP system (Seifu et al., 2005). If raw milk is stored at $\leq 15^{\circ}\text{C}$ the LP system can effectively preserve raw milk for 24-26 h (Reiter and Harnulv, 1982). To activate the LP system in raw milk, small amounts (8.5 ppm) of HP are added exogenously (FAO/WHO). Exogenous HP added in small amounts (20 ppm) is effective at activating the LP system for the bleaching of pasteurized, fat-separated fluid whey (Campbell et al., 2012). It is unlikely that the concentration of HP used for activation of the LP system directly causes any flavors in dried dairy ingredients but rather the undesirable flavors are indirectly related to HP concentration and subsequent LP activity.

Thiocyanate (SCN): Thiocyanate is widely distributed in animal tissues and secretions. In fresh bovine milk, 1-15 ppm has been reported (Seifu et al., 2005). SCN concentration in milk varies with breed, species, udder health, and type of feed (Kussendrager and van

Hooijdonk, 2000). There are two major dietary sources of SCN in bovine milk, glucosinolates and cyanogenic glucosides. These can be found in cabbage, kale, brussel sprouts, cauliflower, turnips, rutabaga, cassava, potatoes, maize, millet, sugar cane, peas, and beans (Seifu et al., 2005). There may or may not be enough SCN naturally present to activate the LP system in raw milk (Seifu et al., 2005). As such, Codex recommends that SCN in the raw milk be increased to 15 ppm by adding powdered SCN and mixing thoroughly before the addition of HP to activate the system (Codex, 2005). In fluid whey, SCN appears to be naturally high enough to activate the LP system and bleach sufficiently (Bottomley et al., 1989, Campbell et al., 2012), however, extensive studies have not yet been conducted using various sources of milk.

Catalase (EC 1.11.1.6)

Catalase was among the first enzymes demonstrated in milk (1897), however, it was not formally reported until 1911 (Fox and Kelly, 2006a). Catalase decomposes hydrogen peroxide as follows: $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$. Catalases react with hydrogen peroxide in many ways and none of these reactions are adequately understood. Catalase can be an indigenous, exogenous, or endogenous enzyme as it is found indigenously in milk and can also be produced during cheese manufacture by coryneform bacteria and yeasts; however, it may also be added exogenously in some dairy processes (Kang et al., 2010; Fox and Kelly, 2006a). In raw milk, high levels of catalase are associated with mastitic infection and high levels of somatic cell counts in the milk (Fox and Kelly 2006a). Like many other enzymes, catalase activity varies in milk due to feed, stage of lactation, and mastitis (Fox and Kelly,

2006a). Catalases are usually associated with the MFGM as the specific activity in cream is 12-fold higher than in skimmed milk (Kitchen et al., 1970).

Catalase is relatively heat labile, and is inactivated completely by heating at 65C for 16s (Fox and Kelly, 2006a). It is not a good indicator of cheese made from sub-pasteurized milk as catalase is also produced during cheese ripening (Fox and Kelly, 2006a). Legally, catalase must be added to fluid whey or retentate that has been chemically bleached with hydrogen peroxide to remove residual peroxide (US FDA, 2009). The ability of catalase to break down hydrogen peroxide into water and oxygen helps to prevent the breakdown of hydrogen peroxide into free radicals. Free radicals can go on to cause lipid oxidation thus creating a host of off-flavors commonly found in milk and dairy ingredients. In this sense, catalase may have a positive effect on flavor. However, catalase as a potential source of off-flavors in milk and dairy products has yet to be evaluated in detail.

Xanthine Oxidase (EC 1.17.3.2)

The terminology for the ‘xanthine oxidase’ enzyme throughout the literature is confusing, at best. The enzyme is often more correctly referred to as xanthine oxidoreductase (XOR) (Harrison, 2006), but the term ‘xanthine oxidase’ is used collectively for all forms of the enzyme including not only xanthine oxidase (XO) but also xanthine dehydrogenase (XDH). For the purposes of this paper, xanthine oxidase will solely be used for terminology of the enzyme. Like its name implies, xanthine oxidase is an oxidoreductase and catalyzes oxidation and reduction reactions.

Xanthine oxidase has been found in the milk of all mammals tested, with a particularly high activity in bovine milk (Silanikove and Shapiro, 2007). In milk, XO can be

found in the MFGM (Harrison, 2006) and processing treatments which damage or alter the MFGM will subsequently affect the activity of XO in the milk (Fox, 2003). XO activity is typically measured spectrophotometrically by the rate of uric acid formation monitored at 292 nm but can also be quantified via reverse phase high pressure liquid chromatography (HPLC) (Kelley et al., 2010). XO is a non-specific enzyme and has many different roles, but its most important role is its ability to produce hydrogen peroxide which can then serve as a substrate in the lactoperoxidase system (Fox and Kelly, 2006a; Fox, 2003). Hydrogen peroxide is produced in the presence of oxygen when the reduced FAD (flavin adenine dinucleotide) cofactor reacts with oxygen divalently and forms hydrogen peroxide (Kelley et al., 2010). Unlike other enzymes, treatments such as heating or homogenization cause the release of XO from the MFGM into the aqueous phase of the milk and render the enzyme more active (Dwivedi et al., 1973; Fox, 2003). Until recently it was thought that XO was concentrated in the cream fraction, however, a recent study proved that the highest XO activity in bovine milk was located in the serum phase due to the small contribution of the MFGM to the total mass of the milk (Silanikove and Shapiro, 2007). This enzyme is capable of reducing oxygen to generate reactive oxygen species, superoxide, and hydrogen peroxide (Harrison, 2006). As such, XO may play a role in antimicrobial activity in raw milk. It can also reduce nitrite yielding reactive nitrogen species such as nitric oxide and peroxynitrite (Harrison, 2006). Since XO is a non-specific enzyme, it has a broad range of activities, many applications in food (Dwivedi et al., 1973), and various flavor implications which include:

***As an index of heat treatment:** Literature is conflicting on this issue. Natural variability in the level of XO activity in milk was once said to be too high for XO to be considered a good indicator of heat treatment (Griffiths, 1986). However, later studies suggested that XO could be a good indicator of milk heated in the 80-90C range (Andrews et al., 1987).

***Reduction of nitrate in cheese:** Sodium nitrate is sometimes added to certain cheese varieties to prevent the growth of *Clostridium tyrobutyricum* in order to prevent flavor defects and late gas blowing in Dutch, Swiss, or other cheese varieties (Fox, 2003). XO plays a key role in the bacteriocidal effect of added nitrate as it is necessary to reduce nitrate to nitrite. XO will then go on to reduce nitrite to nitric oxide. In the presence of oxygen, nitric oxide is reduced to superoxide. Superoxide reacts rapidly with nitric oxide to yield peroxynitrite. Both nitric oxide and peroxynitrite exhibit bacteriocidal properties in bovine milk (Harrison, 2006).

***Lipid oxidation:** Due to the non-specificity of the enzyme, XO has long been suspected in the oxidative deterioration of milk flavor (Dwivedi et al., 1973). XO can excite stable triplet oxygen to singlet oxygen, which is a very strong prooxidant (Fox, 2003). It is also possible for XO to produce hydrogen peroxide, which can then in turn react with other compounds to form free radicals and thus increase lipid oxidation (Kelley et al., 2010). Griffiths (1986) found that XO persisted even after heating at 80C for 120s. Lipid oxidation products have been found in a variety of dried dairy ingredients, from WPC to milk powders, and are sources of cardboard flavor (Whitson et al., 2011, Carunchia-Whetstine and Drake, 2007; Croissant et al., 2009; Campbell et al., 2011a; Jarvis et al., 2012). These flavors increase over time and can carry over into the finished product influencing consumer acceptability.

***Production of H₂O₂:** Hydrogen peroxide is produced by XO which serves as a substrate for LP, thus potentially activating the LP system. As discussed previously, the LP system exhibits bacteriostatic and bacteriocidal activity in fluid dairy products and is often used in less developed countries where pasteurization and proper refrigeration are not an option. The LP system in fluid whey can be activated by small amounts of hydrogen peroxide and when the LP system is activated in fluid Cheddar whey, it has great bleaching potential by generation of the oxidative compound hypothiocyanate (Bottomley et al., 1989; Campbell et al., 2012). While enough HP may be produced by XO to activate the LP system, in milk exogenous thiocyanate must also be added so it is unlikely HP produced from XO would unintentionally activate the LP system in milk, thus off-flavors produced due to the LP system would probably not be present in dried milk products. However, in whey, enough thiocyanate is naturally present so it is probable that the HP produced from XO could unintentionally activate the LP system in whey. WPC bleached by the LP system was higher in aldehydes and sulfur compounds compared to traditional bleaching by the dairy industry (Campbell et al., 2012). XO is an indirect source of off flavors and any activation of the LP system in whey directly or indirectly may contribute to lipid oxidation off flavors in dried whey ingredients.

Proteinase (EC 3.4.-.-)

Proteinases in milk can either be inherent in the milk (indigenous) or created by psychrotrophic bacteria during storage (endogenous). Since proteinases span two different categories, they will be discussed within their respective sections. Heat treatments severe enough to eliminate proteolytic activity would be detrimental to milk protein functionality,

thus with the current heat treatments in the dairy industry, proteinases, both indigenous and exogenous, remain active in the final product (Chen et al., 2003). Proteinases can survive all heat treatments applied during the manufacture of milk powder (pasteurization, spray drying, etc.) (Chen et al., 2003). Proteolysis is especially important in aged products such as cheese or in stored products such as dried dairy ingredients. Proteinases break down proteins creating peptides and amino acids. Bitterness in stored milk powders has been attributed to proteolysis (Carunchia Whetstine and Drake, 2007).

Native proteinases: Bovine milk is known to contain several indigenous proteinases, however only two have been studied in detail: the principal milk proteinase plasmin (EC 3.4.21.7) and cathepsin D (EC 3.4.23.5) (Fox and Kelly, 2006a). Many other proteinases exist in milk but are inactive due to the high redox potential of milk and thus are rarely studied (Fox and Kelly, 2006a). The physiological function of plasmin is to dissolve blood clots. Plasmin concentration and activity in bovine milk can vary due to seasonality, stress, diet, lactation, and mastitis and have been well reviewed previously (Bastian and Brown, 1996; Kelly and McSweeney, 2003). The plasmin system in bovine milk is rather complex and consists of several key components including plasminogen activators and inhibitors (Figure 1). In milk, plasmin hydrolyzes peptide bonds (particularly beta-casein) (Fox, 2003). Various processing parameters and storage conditions influence plasmin activity. Thermal treatment associated with the pasteurization of milk can inactivate plasminogen inhibitors and thus increase plasmin activity (Kelly et al., 2006). While optimum plasmin activity occurs at 37C, plasmin can still be active during cold storage of raw or pasteurized milk (Ismail and Nielsen, 2010). Plasmin activity has been linked to increased casein

hydrolysis (Farkye and Fox, 1992). With the degradation of beta-casein, the concentration of gamma-caseins increased concurrently (Farkye and Fox, 1992). Hydrolysis of proteins in milk can lead to bitter taste. Milk that has been processed by ultra-high temperatures (UHT) is often stored at room temperature (22-25C), which is closer to the plasmin optimum and temperature thus more casein hydrolysis may occur. This can ultimately lead to the gelation of UHT milk and poorer protein quality of non-fat dry milk (NFDM) (Ismail and Nielsen, 2010). While plasmin is a very heat stable enzyme and survives even UHT processing as discussed previously, it is not known whether plasmin survives milk powder processing steps such as membrane filtration, evaporation, and spray drying although it is very likely that it does (Chen et al., 2003). In cheese, plasmin contributes to the formation and degradation of water-soluble peptides in cheese, purportedly increasing the sensory quality of cheese (Farkye and Fox, 1992; Bastian et al., 1991). In addition, plasmin contributes to desirable texture changes which occur during cheese ripening (Ismail and Nielson, 2010). Plasmin is active in whey protein products and acid whey products have higher plasmin activity than sweet whey products (Hayes and Nielson, 2000). Plasmin was still present and active in reconstituted commercial whey protein concentrates (both acid and sweet), but its concentration varied considerably (Hayes and Nielsen, 2000). Recent studies of plasmin in whey focused on the effect of high pressure treatment, pH, temperature, or processing (such as filtration) on activity (Crudden and Kelly, 2003; Moatsou et al., 2008; Aaltonen and Ollikainen, 2011; Crudden et al., 2005). It is possible that plasmin activity may have a negative effect on whey proteins and lead to undesirable flavors, however, this has yet to be studied.

Cathepsin D has not been studied nearly as extensively as plasmin and its full significance in fluid milk and milk products is still unknown. The primary biological function of this enzyme is protein degradation in lysosomes. Should this function fail, lipofuscin will accumulate in a variety of tissues and cause neurodegeneration and vision loss (Benes et al., 2008). Cathepsin D is relatively heat sensitive and is inactivated at 70C (Fox, 2003). Like plasmin, cathepsin D is part of a complex system including inactive precursors to the enzyme (Fox and Kelly, 2006a). There is high degree of correlation between somatic cell count and cathepsin D activity in milk, although it is unknown if this is merely due to increased activation or increased production of the enzyme (Hurley et al., 2000). Cathepsin D cleaves alpha-s1-casein, beta-casein, and kappa-casein, although its milk clotting properties are poor (Fox and Kelly 2006a). The peptides created from the cleavage of caseins could possibly create bitter taste in dairy products, however, the role cathepsin D plays on flavor has yet to be directly evaluated. Cathepsin D is relatively heat labile (inactivated at 70C for 10 minutes), however, recent studies suggest that it partially survives HTST pasteurization of milk (Hurley et al., 2000). Thus in fluid milk it is possible capthepsin D plays a direct role in bitter taste although this has yet to be studied. Due to other processing parameters involved in the manufacture of dried dairy ingredients, it is unlikely cathepsin D would still be active in powdered product and play a direct role in flavor as the ingredient ages.

Lipases (EC 3.1.1.-)

Lipids which are present in dairy products can be enzymatically degraded by lipases either via either oxidation or hydrolysis. Lipases can generally be categorized as enzymes

that catalyze the hydrolysis of lipids, which are the major lipid component of milk (Deeth, 2006). Lipoprotein lipase (EC 3.1.1.34) (LPL) accounts for most of the native lipolytic activity in bovine milk and is normally associated with the casein micelle. Lipases may also come from bacterial sources such as *Pseudomonas* during the cold storage of milk. Lipases from these bacteria are notably different from LPL (Table 3). Lipolysis in milk can alter both flavor and functionality of dairy products (Deeth, 2006). In milk there is enough natural LPL to cause raw milk to be unacceptably rancid in less than 10 minutes if the milk fat globule membrane is ruptured and milk fat is exposed to the serum phase of the milk (Deeth, 2006). Realistically, rancidity does not occur at such a rapid pace as LPL is associated with casein micelles and triglycerides are protected by the MFGM. Lipolysis occurs when LPL comes in direct contact with the substrate, triglycerides, and only occurs when the MFGM is physically damaged by various processes including but not limited to agitation, homogenization, or temperature fluctuations (Fox, 2003). Raw milk is not exposed to excessive agitation for this reason. LPL is a relatively unstable and the majority of the enzyme present in milk is inactivated by high temperature short time (HTST) pasteurization.

Lipolysis can be classified as either spontaneous or induced (Deeth, 2006). Specific chemical differences and sources are described below and these result in distinct sensory off flavors: free fatty acid flavors and oxidized flavors. Free fatty acid flavors are due to lipase activity and are caused by short chain free fatty acids with flavors described as vinegar, cheesy, sweaty, and soapy (Drake et al., 2001; Singh et al., 2003). Oxidized flavors are due to auto oxidation of fatty acids and are characterized by cardboard, metallic, and mushroom flavors (Karagul Yuceer et al., 2003).

Spontaneous lipolysis: Some cows produce milk with spontaneous oxidized flavor, often described as fishy or metallic, which develops without the addition of exogenous oxidants or exposure to light (Timmons et al., 2001). Spontaneous oxidation of fluid milk is due to auto-oxidation of polyunsaturated fatty acids (PUFA). Timmons et al. (2001) found that milk with high concentrations of PUFA and copper was the most susceptible to degradation. While the causes are largely unknown, spontaneous or autooxidation of milk may be due to several factors: amount of lipase activity, the integrity of the MFGM, PUFA, and the balance of lipolysis-activating/inhibiting factors (Deeth, 2006). These factors can be due to feed quality, late lactation, and mastitis (Deeth, 2006; Timmons et al., 2001). Havemose et al. (2006) demonstrated that the composition of lipids in milk, especially the concentration of linolenic acid, was very important for auto-oxidation susceptibility and was directly influenced by the type of feed.

Spontaneously oxidized milk can develop rapidly (in the raw milk bulk tank) or more often, develop after pasteurization and several days of storage with flavors intensifying over storage (Timmons et al., 2001). Spontaneous lipolysis occurs primarily on the farm and can be minimized by culling cows with high somatic cell counts, avoidance of freezing on the walls of the bulk tank, avoidance of cooling/warming cycles in the bulk tank, avoidance of excessive agitation (by pumps or agitators) in bulk tank, and proper installation, maintenance, and operation of milking machines (Fox, 2003). Spontaneous auto-oxidation in milk is very complex and is affected by pro and anti oxidants. The influence of antioxidants on the oxidative stability of milk has been studied (Mansson and Akesson, 2000), but the role of antioxidants was determined to not be as important a factor in the

determination of spontaneous lipolysis as fatty acid profile (van Aardt et al., 2005; Havemose et al., 2000). Because spontaneous oxidation is still poorly understood and the resulting flavors are highly undesirable and negatively influence consumer acceptability of milk, it is very important to be able to rapidly evaluate raw milk for spontaneous oxidation susceptibility. Recently, Amamcharla and Metzger (2013) developed a modified ferric reducing antioxidant power assay which measures the total antioxidant capacity of raw milk. This assay was used successfully in both laboratory and commercial facility experiments to identify milk which was susceptible to oxidation (Amamcharla and Metzger, 2013).

Induced lipolysis: Induced lipolysis occurs when the MFGM is disrupted by physical forces exposing the lipid substrate to the lipase. The harsher the treatment or the longer time the treatment is applied, the greater the exposed surface area of the lipid and consequently, the greater the rancidity. Damage during milk processing (agitation, homogenization, temperature changes) can result in lipolysis, however, “most, if not all” LPL is deactivated during pasteurization so rancidity is usually only seen in butter, cheese, or milk powders after a period of storage (Hickey et al., 2007; Deeth, 2006; Farkye and Fox, 1992). Lipolysis, the hydrolysis of lipids, leads to the formation of free fatty acids (FFA). Short chain free fatty acids are of particular importance in the flavor of not only cheese, but other dairy products as well. Acetic acid (vinegar), butanoic acid (cheesy), pentanoic acid (sweaty), hexanoic acid (sweaty), heptanoic acid (sweaty), octanoic acid (sweaty/waxy) and nonanoic acid (sweaty) have previously been identified in both SMP and WMP as odor active compounds (Carunchia-Whetstine and Drake, 2007). During storage of these powders, FFA and consequently undesirable flavors increased (Carunchia-Whetstine and

Drake, 2007) suggesting that lipolysis is due primarily to post process contamination due to bacterial lipase (Collins et al., 2003). In order to create a product with acceptable flavor over time, not only must high quality milk be used, but also extra care must be taken during processing to minimize bacterial lipases. For example, extra care should be taken to avoid product exposure to light. Should the product be exposed to light for extended periods of time either during storage or processing, odor active compounds associated with light-induced oxidation may be present including but not limited to pentanal, dimethyl disulfide, hexanal, heptanal and nonanal (van Aardt et al., 2005).

ENDOGENOUS ENZYMES

Bacterial Proteinases

Two main types of spoilage bacteria can be found in milk and milk products: *Pseudomonas* and *Bacillus* species. *Pseudomonas* species are psychrotrophs and found commonly in raw milk due to contamination from the interior of the udder, cows' teats, or milking and storage equipment (Chen et al., 2003). In pasteurized milk, psychrotrophs are the main cause of spoilage (Chen et al., 2003). Hayes et al. (2002) evaluated sensory aroma characteristics of milk spoilage by *Pseudomonas* species and generated 6 terms to describe spoiled milk: rotten hamburger, barn/fecal, shrimpy, medicinal, fruity, and baby vomit/cheesy. Proteinases from psychrotrophs, like many other enzymes, preferentially attack casein over whey proteins (Chen et al., 2003). *Pseudomonas* species have long been known to cause flavor defects in dairy products with the earliest study on this subject being published in 1902 (Morgan, 1976). In particular, *Pseudomonas fragi* is responsible for the development of "fruity" flavors in processed dairy products. Lipases from this organism are

able to hydrolyze milk fat and esterify butyric and caproic acids with ethanol (Morgan, 1976). Fruity flavor has been documented in skim and whole milk powders (Carunchia Whetstine and Drake, 2007). While “fruity” flavor in fluid milk and dried dairy ingredients is not desirable, this flavor at low intensities may be desirable in Cheddar cheese (Carunchia Whetstine and Drake, 2007). In Cheddar cheese, the production of ethanol by the lactic starter culture coupled with the presence of the esterase enzyme (similar to that found in *Pseudomonas*) commonly in some lactic acid bacteria is responsible for fruity volatiles in Cheddar cheese (Morgan, 1976). Esterases are different from lipases in that esterases prefer soluble rather than emulsified ester substrates (Fox and Kelly, 2006a), thus esterases are more likely to be found in whey while lipases are more likely to be found in fluid milk.

With the improved control of post-pasteurization contamination of non-heat resistant psychrotrophs, the research focus in fluid milk has shifted to psychrotrophic spore formers and their potential impact on milk quality (Meer et al., 1999; Scheldeman et al., 2006). *Bacillus* species are environmentally ubiquitous and can be present in the raw milk or introduced during production, handling, and processing (Huck et al., 2008). In addition, *Bacillus* species have the capability to sporulate during adverse conditions, such as during high temperature short time processing (HTST) or during milk powder processing steps such as membrane filtration, evaporation, or spray drying. In a study conducted by Ranieri and Boor (2009), samples from pasteurized fluid milk across 18 dairy plants in 5 geographical regions of the United States revealed that more than 84% of gram-positive spore-forming isolates at 1 day, 7 days, and 10 days of refrigerated storage were of the *Bacillus* genus. Spores are very resistant to heat and chemical cleaning agents and may become a persistent

problem on factory equipment leading to further contamination during the powder making process (Chen et al., 2003). International concerns over spore counts in dried dairy ingredients are high (Yuan et al., 2012). Thermophilic spores are often used often used as hygiene indicators in processed products. Spores are considered spoilage organisms and if they germinate can display proteolytic and lipolytic activity and cause undesirable flavors (Reginensi et al., 2011; Burgess et al., 2010). *Bacillus* species are capable of producing multiple types of proteinases thereby having more diverse proteolytic activity than proteinases from *Pseudomonas* species (Chen et al., 2003). Enzymatic activity results in the development of objectionable flavors and quality defects in dairy products (Meer et al., 1999). In addition, spores are capable of forming a biofilm on dairy equipment leading to fouling and loss of production time (Burgess et al., 2010). Proteinases from both *Bacillus* species and *Pseudomonas* species have been documented to be heat stable, not only surviving processing but also remaining active in milk powders over long periods of storage (Chen et al., 2003). The direct effects of bacterial proteinases, particularly those due to *Bacillus* and the role of spore counts on sensory quality of dried milk ingredients has not been determined. Because dried dairy products are generally stored below 37C, it is unlikely that spoilage due to spore germination and vegetative growth would be problematic in dried ingredients (Burgess et al., 2010).

Starter Culture

Cheese flavor cannot be produced without starter bacteria and starter culture has been attributed to off-flavors in dried dairy ingredients including WPC80 and whey powder (Carunchia Whetstine et al., 2003; Tomaino et al., 2004; Liaw et al., 2010, 2011; Campbell

et al, 2011a). Cheeses made from different starters have different flavors and thus wheys from different cheeses have different flavors (Carunchia Whetstine et al.,2003; Gallardo-Escamilla et al., 2005; Drake et al., 2009; Campbell et al. 2011b). Flavor of fluid whey from thermophilic starter differed from the flavor of fluid whey from mesophilic starter cultures (Liaw et al., 2011; Campbell et al., 2011b) and the flavor of whey from acid-set curd differed even further (Gallardo-Escamilla et al., 2005).

In the United States, the two main sources of liquid whey are Mozzarella and Cheddar. Liaw and others (2011) reported that Mozzarella and Cheddar liquid whey were distinct in flavor and volatile compounds initially but that Cheddar whey was also more prone to lipid oxidation than Mozzarella whey. Whitson and others (2011) reported similar findings with liquid Mozzarella and Cheddar whey retentates. Mesophilic *Lactococcus lactis* starter cultures, used to produce Cheddar cheese, influence flavor and oxidative stability of liquid whey (Carunchia Whetstine and others 2003; Tomaino and others 2004). Campbell and others (2011a) recently demonstrated that lipid oxidation products were higher in concentration in dried whey protein concentrate (WPC) manufactured from mesophilic starter culture (Cheddar) whey compared to WPC from rennet-set whey. Lactic acid bacteria can produce hydrogen peroxide, which can then in turn activate the lactoperoxidase system as previously discussed. The lactoperoxidase system produces free radicals which then in turn create lipid oxidation products. Cheddar whey contains more lipid oxidation products than Mozzarella whey suggesting that mesophilic starter cultures produce more hydrogen peroxide and are more likely to activate the lactoperoxidase system than thermophilic starter cultures. In addition, the activity of the LP system is highly

influenced by the heating temperatures that the curd and whey are subjected to and the pH at which the whey is drained, thus, more research needs to be conducted to pinpoint the cause of higher oxidation in Cheddar vs. Mozzarella wheys.

Lactic acid bacteria convert lactose to lactic acid, along with the production of diacetyl and acetaldehyde which contribute to flavor differences (Smit et al., 2005; Urbach et al., 1995). While starter lactic acid bacteria are well known to cause flavor defects in dried dairy ingredients, non-starter lactic acid bacteria (NSLAB) can also contribute to flavor. Naturally, NSLAB are very diverse between strains and since not all enzymes are present in a single strain, often multiple strains are often used to create one cheese with desirable flavor (Smit et al., 2005). NSLAB can grow to large numbers (approx. 10^8 cfu/g) in cheese after storage and thus have become a widely studied topic (Cogan et al., 2007). Since NSLAB are known to effect cheese flavor, potentially they also contribute to flavor in dried dairy ingredients, although this topic has yet to be directly studied.

EXOGENOUS ENZYMES

Rennet (Chymosin)

Rennet is used to coagulate milk for the production of cheese. Most of the rennet added to the milk is lost in the whey (Bansal et al., 2007). While residual rennet is minimal in cheese (5-15% of the original enzyme activity), it can have major flavor implications, primarily contributing to proteolysis during cheese ripening (Singh et al., 2003; Bansal et al., 2007). Bitter taste, from the production of bitter peptides, has also been attributed to residual chymosin in the cheese (Singh et al., 2005). Although chymosin carries over into fluid whey, it does not survive in dried whey protein as fluid whey typically undergoes a

pasteurization step either prior to or directly after fat separation. Pasteurization of the fluid whey inactivates the residual milk clotting enzymes (Thunell et al., 1979). Should pasteurization be inadequate or not performed in a timely fashion, the chymosin may cause flavor defects due to proteolysis.

Fungal Peroxidase

In the United States, much of the whey comes from Cheddar cheese colored with annatto, a natural yellow-orange carotenoid, in order to impart desired cheese color (Kang et al., 2010). While most of the annatto stays in the Cheddar cheese, some of the color goes into the whey (Kang et al., 2010). In order to produce the desired dried whey ingredients free of color, the whey must be bleached. Chemical bleaches, such as benzoyl peroxide or hydrogen peroxide, are most commonly used. However, recently, enzymatic bleaching, either with the native lactoperoxidase or with exogenously added fungal peroxidase has gained momentum. Currently there is one commercially marketed fungal peroxidase, Maxibright™ (DSM, The Netherlands), for use in dairy products. Maxibright™ works on a similar mechanism as lactoperoxidase in that it needs small amounts of hydrogen peroxide to activate the system. Once activated, Maxibright™ will bleach fluid whey faster than lactoperoxidase alone at cold temperatures (Campbell et al., submitted). The addition of fungal peroxidase may also provide extra insurance against the variable levels of lactoperoxidase in milk (Campbell et al. submitted). Like other chemical and enzymatic bleaching steps, other non-specific oxidative reactions may occur and influence the flavor of the subsequent dried ingredients.

CONCLUSION

Flavor directly influences liking of a product and thus purchase intent. The flavor of dried dairy ingredients can carry through into the finished product and influence consumer perception. Dried dairy ingredients are susceptible to a host of off-flavors from various processing parameters. Enzymes, both those naturally present and those added, can have great impact on flavor and thus steps must be taken to ensure minimize undesirable flavors caused by enzymatic reactions. Understanding enzymes, their reactions and subsequent off-flavors are key to designing a process which minimizes the negative effects of enzymes. Future work is needed to close the knowledge gap in previously discussed areas so that manufacturers can produce bland, desirable, consistent dried dairy ingredients for use in food processing.

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TABLES AND FIGURES

Table 1: Major classes of enzymes (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB))

Enzyme Class (EC) Number	Class Name	Function
1	Oxidoreductases	Catalyze oxidation or reduction reactions
2	Transferases	Catalyze the transfer of specific chemical moieties
3	Hydrolases	Hydrolyze substrates with concomitant uptake of water molecules
4	Lyases	Remove or add specific chemical moieties to their substrates
5	Isomerases	Catalyze isomerization
6	Ligases	Catalyze the synthesis or bonding together of substrate units

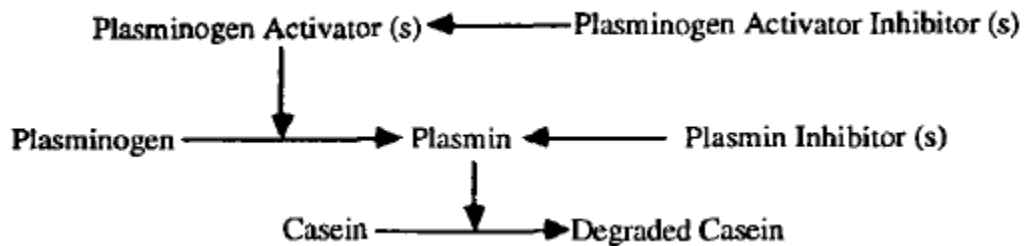
Table 2: Indigenous, exogenous, and endogenous enzymes found in dried dairy ingredients and their potential to influence final ingredient flavor

Enzyme	Indigenous, Exogenous, or Endogenous	Yes	No	Maybe
Lactoperoxidase	Indigenous	X		
Catalase	Indigenous/Exogenous/Endogenous			X
Xanthine Oxidase	Indigenous	X		
Proteinase	Indigenous/Endogenous	X		
Lipase	Indigenous/Exogenous	X		
Starter Culture and Non Starter Bacteria	Endogenous	X		
Chymosin	Exogenous			X
Fungal Peroxidase	Exogenous	X		

Table 3: Comparison of native and non-native lipases in fluid milk (adapted from Deeth, 2006)

Milk Lipoprotein Lipase (LPL)	Lipases from bacteria
Heat sensitive; destroyed by high temperature short time (HTST) pasteurization	Not very heat sensitive; stable to HTST and even ultra high temperature (UHT) treatment
Milk fat globule membrane (MFGM) acts as a physical barrier to lipid substrate	MFGM present no barrier
Associated with fresh milk and cream	Associated with stored products: UHT milk, cheese, butter, milk powders
High levels present in raw milk	Trace levels present in raw milk

Figure 1: Plasmin System in Fluid Milk (Bastian and Brown, 1996)



The use of lactoperoxidase for the bleaching of fluid whey

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INTERPRETIVE SUMMARY

The United States is a major consumer of yellow Cheddar cheese, in which the natural colorant, annatto, is added. It is necessary to bleach colored whey in order to achieve the desired lack of color in dried whey ingredients. This study found that milk lactoperoxidase activity remains at a viable amount for bleaching throughout whey processing. Bleaching using the lactoperoxidase system resulted in higher norbixin destruction compared to traditional chemical bleaching (hydrogen peroxide).

ABSTRACT

Lactoperoxidase (LP) is the second most abundant enzyme in bovine milk and has been used in conjunction with hydrogen peroxide (HP) and thiocyanate (SCN) to work as an antimicrobial in raw milk where pasteurization is not feasible. Thiocyanate is naturally present and the lactoperoxidase system purportedly can be used to bleach dairy products, such as whey, with the addition of very little hydrogen peroxide to the system. There were three objectives in this study: 1) to quantify the amount of HP necessary for bleaching of fluid whey using the LP system, 2) to monitor LP activity throughout cheesemaking and whey processing, and 3) to compare the flavor of WPC 80 bleached by the LP system to that bleached by traditional HP bleaching. Cheddar cheese whey with annatto (15 mL annatto /454 kg milk, annatto with 3% w/v norbixin content) was manufactured using a standard Cheddar cheese make procedure. Various levels of HP (5-100 ppm) were added to fluid whey to determine the optimum concentration of HP for LP activity which was measured using an established colorimetric method. In subsequent experiments, fat-separated whey was bleached for 1 h with 250 ppm HP (traditional) or 20 ppm HP (LP system). WPC80 was manufactured from whey bleached with 250 ppm HP or 20 ppm HP. All samples were subjected to color analysis (Hunter Lab and norbixin extraction) and proximate analysis (fat, protein, moisture). Sensory and instrumental volatile analyses were conducted on WPC 80. Optimal LP bleaching in fluid whey occurred with the addition of 20 ppm HP. Bleaching of fluid whey at either 35 or 50°C for 1 h with LP resulted in >99% norbixin destruction compared to 32 or 47% destruction from bleaching with 250 ppm HP, at 35 or 50°C for 1 h respectively. Higher aroma intensity and increased lipid oxidation compounds were

documented in WPC80 from bleached whey compared to WPC80 from unbleached whey. Monitoring of LP activity throughout cheese and whey manufacture showed that LP activity sharply decreased after 30 min of bleaching (17.01 ± 1.4 U/mL to <1 U/mL), suggesting that sufficient bleaching takes place in a very short amount of time. LP averaged 13.01 ± 0.7 U/mL in unpasteurized, fat-separated liquid whey and 138.6 ± 11.9 U/mL in concentrated retentate (11% solids). LP may be a viable alternative for chemical whey bleaching.

Key Words: whey, flavor, bleaching, lactoperoxidase

INTRODUCTION

Lactoperoxidase (LP) is an oxidoreductase enzyme belonging to the peroxidase family and is found in a wide range of mammalian milks, including humans (Seifu et al., 2005). This enzyme is heat stable and is inactivated after 15s at 78°C (de Wit and van Hooijdonk, 1996). Historically, the LP system has been used to inhibit microbial growth in bovine milk. The LP system consists of three components: LP, thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2). The system is not active unless all three components are present in sufficient amounts (Seifu et al., 2005). The major intermediate oxidation product of the LP-catalysed oxidation of SCN^- is the hypothiocyanate ion (OSCN^-), which is bactericidal (Seifu et al., 2005). Hydrogen peroxide is sometimes added to activate the system if no HP is naturally present. Catalase negative organisms (such as lactic acid bacteria) can generate HP under aerobic conditions and thus can also activate the LP system. Many lactobacilli, lactococci, and streptococci produce sufficient HP under aerobic conditions to activate the LP system (Seifu et al., 2005). Exogenous HP must be added to activate the system (Reiter and Harnulv, 1982) if it is not supplied by catalase negative organisms. Gram negative, catalase positive organisms (such as *pseudomonas*, *coliforms*, *salmonellae*, and *shigellae*) are not only inhibited by the LP system, but may be killed provided that HP is supplied exogeneously (Seifu et al., 2005). Gram positive, catalase negative bacteria (such as streptococci and lactococci) are generally inhibited but not killed by the LP system (Seifu et al., 2005). If raw milk is stored at $\leq 15^\circ\text{C}$ the LP system can effectively preserve raw milk for 24-26 h (Reiter and Harnulv, 1982).

Measurement of LP can be done using a variety of methods, and as such, LP activity values vary widely in the literature. In 1994, Pruitt and Kamau established a method to quantify LP activity. This assay uses 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) as a chromophore and the measurement is carried out at 412 nm. This method has been widely accepted (Pruitt and Kamau, 1994). Bovine milk contains 1.2 to 19.4 units/mL of LP (Seifu et al., 2005), although levels in liquid whey are reported to be 30 mg/l or about 0.5% (w/w) of whey proteins (de Wit and van Hooijdonk, 1996). Levels of LP can vary depending on the lactation cycle of the cow, season, feeding regime, and breed (Kussendrager and van Hooijdonk, 2000). Like LP, thiocyanate can vary widely due to feeding regime (Seifu et al., 2005). The third component of the LP system, hydrogen peroxide, is not normally detected in raw milk and is typically added exogenously. HP can be generated endogenously by bacteria, although amounts sufficient to activate the LP system may not be generated (Seifu et al., 2005). Depending on the milk, any one of the three components which make up the LP system could become the limiting factor.

In addition to inhibiting microbial growth, hypothiocyanate (produced when LP reacts with SCN), also has bleaching capabilities. The strong oxidizing capacity allows hypothiocyanate to react with carotenoids leading to destruction of conjugation and subsequent color loss of norbixin. Very few papers have investigated the use of the LP system for decolorization of whey, and no scholarly journal articles to our knowledge have been published. Bottomley et al. (1989) published a patent describing the decolorizing of whey and products derived from whey using the LP system. Though some process details were provided, quantitative norbixin destruction was not addressed, LP activity was not

monitored throughout processing, and flavor of finished product was not addressed. Subsequently, all of these items were a focus of the current study. The United States is a major consumer of yellow Cheddar cheese, in which the natural colorant, annatto, comprised of the carotenoids bixin and norbixin, is added. Some of the color remains in the liquid whey following curd separation and it is necessary to bleach the whey in order to achieve a desired lack of color in dried whey ingredients (Kang et al., 2010). The objectives of this study were to quantify the amount of HP necessary for optimal bleaching using the LP system in fluid whey, to monitor LP activity throughout cheesemaking and whey processing, and to compare the flavor of WPC80 bleached by the LP system to that bleached by high levels of hydrogen peroxide (traditional chemical bleaching). The overall goal of this study was to determine if whey bleaching via the LP system would be a viable alternative to chemical HP bleaching for the dairy industry.

METHODS

Experimental Design Overview

There were two experimental components: liquid whey trials and the production of WPC 80. Optimum HP levels to activate the LP system were first determined. Liquid whey trials were then conducted as a 2 by 3 factorial design with temperature (35 or 50°C) and bleach treatment (control (C), 20 ppm HP (LP), or 250 ppm HP (HP)). The samples with the highest bleaching efficacy were then selected for WPC80 manufacture. All samples within each trial were made from the same lot of milk. LP and SCN were monitored throughout the entire process of cheese and whey manufacture. All experiments were conducted in triplicate.

Production of Liquid Whey

Cheddar whey was manufactured from vat pasteurized whole bovine milk (195 kg) as described by Campbell et al. (2011). The whey was drained from the curds at pH 6.35 and a sieve was used to remove any remaining particles. The whey was immediately processed with a hot bowl cream separator (Model SI600E, Agri-Lac, Miami, FL) to reduce the fat content.

Activation of LP System

The optimum level of HP to activate the LP system was determined by adding 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 ppm HP to unpasteurized fat-separated liquid Cheddar whey. Bleaching was then carried out as described below. The concentration of HP which resulted in the most bleaching, 20 ppm, (according to % destruction via b^* reflectance values) was selected for further trials.

LP and HP Bleaching

Small aliquots (50 mL) of liquid whey were placed in amber glass jars to prevent light degradation. The jars were placed in a water bath at either 35°C or 50°C and allowed to equilibrate. To activate the LP system, 20 ppm of hydrogen peroxide (35% w/v, Nelson Jameson, Inc., Marshfield, WI) was added and allowed to bleach in the water bath at either 35°C or 50°C for 1 h with gentle agitation. For HP chemical bleaching, 250 ppm HP was added to liquid whey and allowed to bleach for 1 h with gentle agitation. The latter concentration of HP was selected since it represents the mid-range of the legally allowed amount of HP for traditional chemical bleaching of whey and also represents a concentration that might be applied by industry (Kang et al., 2010; Listiyani et al., 2011). Peroxide test

strips (EMD Chemicals, VWR International, West Chester, PA) were used to determine if any HP was leftover after the bleaching treatment. If so, catalase (20 mg/kg, FoodPro CAT, Danisco, New Century, NJ) was added at a rate of 20 ppm to deactivate HP. The samples were then pasteurized (63°C for 30 min) followed by immediate cooling on ice.

Measurements, including amount of LP and SCN⁻, L*a*b* values, and volatile compound analyses were performed immediately. Samples were frozen at -80°C for norbixin, mineral, and proximate analysis at a later date (within 90 d).

Production of WPC 80

Treatments were selected based on current industrial practices and from the previous liquid whey trials (LP (20 ppm HP) at 35°C, HP 250 ppm at 50°C, and Control 50°C) for manufacture of WPC 80. Colored fat separated cheese whey was freshly manufactured as previously described. The separated whey was transferred into a 102 liter stainless vat (FermentorTM, Blichmann EngineeringTM, Lafayette, IN) equipped with a coil heater (1/2" outer diameter, PAC Stainless LTD, Seattle, WA). One of three treatments: LP (35°C, 20 mg/kg HP (35% w/v, Nelson Jameson, Inc., Marshfield, WI)), HP (50°C, 250 mg/kg), or control (50°C, no bleach added) were administered. The whey was allowed to bleach, or held at 50°C (control), for 1 h while recirculating using a peristaltic pump (Model 77410-10, Millipore Inc., Billerica, MA). If any HP remained after treatment, it was deactivated using 20 ppm of catalase (20 mg/kg, FoodPro CAT, Danisco, New Century, NJ). Wheys were then heated to 63°C for 30 min to pasteurize. Following pasteurization, the whey was cooled to the appropriate temperature (either 35°C or 50°C) before ultrafiltration commenced. The ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica,

MA) was equipped with 5 polyethersulfone cartridge membrane filters (Model P2B010V05, 10 kDa nominal separation cutoffs, 0.5 m² surface area, Millipore Inc., Billerica, MA). Each sample was run through a peristaltic pump (Model 77410-10) and the UF assembly using silicone tubing (Model 96440-73) that was connected to the vat. Pumps, pumpheads, and tubing were all obtained from Cole-Palmer (Vernon Hills, IL). This process continued until the retentate reached 80% protein (w/v) content confirmed by a SprintTM rapid protein analyzer (CEM, Matthews, NC). Retentates were then collected and spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark). Inlet temperature was 150°C and the outlet temperature was at 80°C. Total spray drying time was about 1 h. The powder was collected and stored in mylar bags at -80°C following production. All treatments were manufactured from the same lot of milk and the experiment was carried out in triplicate.

Compositional Analysis

Total solids of liquid whey and WPC80 were determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). In powdered WPC80, fat was quantified by soxhlet ether extraction (AOAC, 2000; method number 947.05). In liquid wheys, fat was quantified using the Pennsylvania modified Babcock method (AOAC, 2000; method number 989.04). Protein was determined using the Kjeldahl method in powdered wheys (AOAC, 2000; method number 991.20; 33.2.11) or using the SprintTM Rapid Protein Analyzer (CEM, Matthews, NC) if the whey was liquid. Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) was determined by the NCSU 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.

Hunter L* a* b* values

WPC 80 samples were measured in both powder form (10 g) and liquid form (10 mL of 10% w/v solution). Ten mL of the sample (rehydrated at 10% w/v solids if necessary) was placed into the bottom of a 60mm x 15mm polystyrene petri dish (Beckton Dickinson, Franklin Lakes, NJ). Color of samples was measured using a Minolta Chroma meter (CR-410, Ramsey, NJ). Each sample was measured in duplicate and duplicate measurements were taken. Before measurements being taken, a factory-supplied calibration plate was used to calibrate the instrument. The Hunter CIE Lab color scale was used. Reflectance values were taken with a white calibration plate as the background.

Norbixin Extraction and Quantification

Norbixin is the primary carotenoid in water soluble annatto extracts and was extracted and measured to determine percent annatto destruction and bleaching efficacy (Kang et al., 2010). Norbixin was extracted using the methods described by Campbell et al. (2011) and quantified by HPLC. Briefly, 0.6 g of sample was weighed into a 50 mL centrifuge tube (Nalgene, Rochester, NY). To this, 6 mL water was added and the sample was vortexed. Three mL ethanol was added and the solution was then vortexed again. Three mL chloroform (VWR International, West Chester, PA) and 1 mL glacial acetic acid (1% w/v; JT Baker, Phillipsburg, NJ) was added to the previous solution. The sample was vortexed and centrifuged at $16500 \times g$ for 10 min at 4°C (Model RC5B, Thermo Scientific, Waltham, MA). The bottom chloroform layer 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).

To further filter and purify the extracted norbixin, solid phase microextraction was utilized. The column selected was the Strata-NH₂ SPE column (500 mg/3 mL, Phenomenex, Torrance, CA). To condition the column, 4 mL of *n*-hexane (VWR International, West Chester, PA) was run through the column. An aliquot of the extract collected previously (1 mL) was transferred onto the conditioned SPE column. The column was rinsed with 2.5 mL *n*-hexane:diethyl ether (1:1 (v/v)) and 1 mL acetone (VWR International, West Chester, PA). The norbixin was eluted with 2 mL methanol:glacial acetic acid (7:3 (v/v)). Final volume was measured and samples placed in vials for quantification by HPLC (Waters 1525 Binary Pump) and quantification (Waters, Milford, MA). Isocratic mobile phase (70% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/30% water (EMD Chemicals, VWR International, West Chester, PA) with 0.1% (w/v) formic acid (EMD Chemicals, VWR International, West Chester, PA)) was used at a flow rate of 1 mL/min pumped through a binary pump (Waters 1525, Waters, Milford, MA). Fifteen microliters of the sample was injected (Waters 2707 Autosampler) onto the column (Phenomenex Kinetex 2.6µm particle size, 10 cm length, 4.6mm inner diameter, 100Å pore size) which was heated to 40°C. The injector temperature was set to 4°C. Sample was sent through a photodiode array detector (Waters 2998). A standard curve was created by rehydrating norbixin powder (45% (w/v), Chr. Hansen, Milwaukee, WI) in 2.5% (w/v) potassium hydroxide (BDH, VWR International, West Chester, PA) and then diluting in methanol:glacial acetic acid [7:3 (vol/vol)]. The maxima used for calculation was 460 nm. Norbixin concentration was

calculated by total solids and correction for dilution during the extraction and SPE processes.

Lactoperoxidase (LP) Measurement

LP measurements and calculations were performed according to the International Dairy Federation method (Pruitt and Kamau, 1994). Two mL of ABTS stock solution (1mM ABTS (TCI, Tokyo, Japan) in 0.1mM phosphate buffer pH 6.0 (EMS, Hatfield, PA)) was placed in a disposable plastic cuvette (VWR International, West Chester, PA). To start the reaction, 0.10 mL of sample and 1.0 mL hydrogen peroxide solution (0.3 mM; Nelson Jameson, Inc., Marshfield, WI) was added simultaneously and mixed thoroughly. The absorbance was monitored at 412 nm every 5 seconds until the reaction reached a plateau (2-5 min). Calculations were determined using the least squares regression of the absorbance data and Beer's law (Pruitt and Kamau, 1994).

Thiocyanate (SCN) Measurement

Thiocyanate measurements were performed according to the International Dairy Federation method (IDF, 1988). Four mL of sample was mixed with 2.0 mL (w/v) of 20% trichloroacetic acid (TCA) solution (BDH, VWR International, West Chester, PA). The mixture was blended well and allowed to sit for at least 30 min. The mixture was then centrifuged at $16,500 \times g$ for 10 min (Model RC5B, Thermo Scientific, Waltham, MA). After centrifugation, 1.5 mL of the supernatant was mixed with 1.5 mL of ferric nitrate solution (16g Fe (NO₃)₃ (EMD Chemicals, VWR International, West Chester, PA) dissolved in 50 mL of 2M nitric acid (HNO₃) (BDH, VWR International, West Chester, PA) and then diluted with DI water to 100 mL). The measurement was performed at 460 nm within 10

min of ferric nitrate solution addition. The concentration of SCN (Sigma Aldrich, Milwaukee, WI) was determined by the use of external standard curves.

Descriptive Sensory Analysis

Sensory analysis was conducted on rehydrated WPC80 (10% w/v) using a trained descriptive sensory panel and an established dairy flavor language (Drake et al., 2003, 2009). Panelists (n =8) each had more than 150 h of previous experience with the sensory analysis of fluid and dried whey products using the Spectrum™ descriptive analysis method (Meilgaard et al., 1999). All sensory testing was conducted in accordance with the NCSU Institutional Review Board for Human Subjects guidelines.

Reconstituted WPC80 (10% solids w/v) was evaluated by placing 30 mL in three-digit-coded 60 mL lidded cups (Solo Cup Company, Champaign, IL). Preparations were conducted with overhead lights off to avoid exposure to light. WPC80 were evaluated within 7 days of production. Samples were evaluated by each panelist in duplicate. Sensory data were collected on paper ballots or using Compusense™ five, release 4.8 (Compusense, Guelph, Canada).

Gas Chromatography Mass Spectrometry (GCMS)

Volatile compounds in fluid whey and WPC 80 powder were extracted by solid phase microextraction (SPME) using selective ion monitoring (SIM). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using a modified method of Liaw et al. (2010). Liquid samples were tested the day of manufacture and spray dried powders were reconstituted at 10 % solids (w/v) and evaluated within 7 days. All samples contained 10 % (w/v) sodium chloride (Fischer Scientific), and 10 ul

internal standard solution (2-methyl-3-heptanone in methanol at 81 ppm (Sigma Aldrich, Milwaukee, WI)) in 20 ml 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 MSD (Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1 cm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA) at 31 mm with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm.

The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 250°C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250°C. An Zb-5ms column (Zb-5ms 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) was used for all analyses at a constant flow rate of 1 ml/min. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°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 compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound. Retention indices were calculated using an alkane series (Sigma Aldrich, Milwaukee, WI) (Van den Dool and Kratz, 1963).

Statistical Analysis

All data was analyzed by a one-way analysis of variance (ANOVA) using a general linear model (PROC GLM) in SAS (SAS Statistical Analysis Software, version 9.2, SAS Institute, Cary, NC). Replication was designated as a random effect. Principal component analysis was conducted and biplots were generated using Microsoft Excel statistical software (Excel Stat, New York, New York).

RESULTS

Liquid Whey

The most effective bleaching with LP in unpasteurized, fat-separated liquid whey occurred with an addition of 20 ppm HP (Figure 1) and this concentration was chosen for subsequent sets of experiments. Liquid whey was treated at either 35°C or 50°C and received one of three treatments: HP 250 ppm, LP (HP 20 ppm), or nothing (Control). Samples did not differ in composition (solids 6.49 ± 0.08 , protein 0.82 ± 0.05 , and fat 0.03 ± 0.01). Samples bleached by the LP system showed the greatest bleaching efficacy, >99% destruction of norbixin. Bleaching by LP did not vary with temperature for the bleaching time evaluated, however, bleaching by HP was more effective at 50°C than at 35°C with 46.9% and 31.8% norbixin destruction, respectively ($p < 0.05$). Reflectance values were consistent with norbixin extraction values in that LP bleached better than HP ($p < 0.05$).

Both LP and SCN were monitored throughout the cheesemaking process through whey pasteurization, which immediately followed bleaching to ensure that neither of these changed over time or limited the system. SCN did not vary with bleaching type (LP, HP, or control) nor did it decrease over time ($p > 0.05$). Using an external standard curve for

quantification, the SCN level was 3.8 ± 2.2 ppm in fluid whey, which is consistent with previously reported values of 3.2 to 4.6 ppm (Seifu et al., 2005). LP was monitored throughout cheesemaking and was still active in samples receiving no bleach treatment after whey pasteurization (17.01 ± 1.4 U/mL). LP activity levels in unpasteurized fluid whey were not affected by fat separation ($p > 0.05$) but slightly increased following whey pasteurization with values averaging 13.01 ± 0.7 U/mL prior to pasteurization and 17.01 ± 1.4 U/mL after pasteurization ($p < 0.05$). Previous studies have shown a decrease in LP after pasteurization in fluid milk (Seifu et al., 2005), although in fluid whey we observed an increase in LP. All replicates followed the same trend. It is unknown as to why this is occurring and needs further investigation. A possible explanation lies in the pH of the system. The pH optimum for LP activity is between 5-6. The pH will continue to slowly decline due to the activity of the starter until proper temperature is reached to kill these organisms. As the pH declines closer to the optimal pH, the activity of LP increases (Kussendrager and van Hooijdonk, 2000). In samples which received bleach treatment (either HP 20 ppm (LP) or HP 250 ppm), no LP activity was detected after 30 min. This suggests that bleaching by the LP system was a fast process, occurring within 30 min.

Volatile compound differences indicated that treatment had more of an effect than temperature with very few interactions. Octanal and 2,6 nonadienal were impacted by temperature while 2-pentylfuran, 1-octen-3-one, octanal, 2,4 nonadienal, 2,5 nonadienal, hexanal and diacetyl were impacted by treatment ($p < 0.05$) (Table 1). Only two interaction effects (temperature*treatment) were found: nonanal and heptanal ($p < 0.05$) (Table 1). Methional, DMTS, and 1-hexen-3-one were not different between treatments or temperature

($p > 0.05$) (Table 1). These volatile compounds have been previously documented to be associated with off flavors in both liquid and dried whey protein (Listinanyi et al., 2011; Whitson et al., 2010, 2011; Croissant et al., 2009).

Whey Protein Concentrate (80%)

WPC80 did not differ in composition (ash $3.5 \pm 0.1\%$, protein $77.1 \pm 0.7\%$, fat $4.7 \pm 0.1\%$). Mineral composition was also not different (results not shown). WPC80 from LP bleached whey had lower norbixin than WPC80 from HP bleached whey (99.4 vs. 48.3% destruction respectively ($p < 0.05$)), consistent with fluid whey trials. Reflectance values, in rehydrated WPC80 (Table 2), was consistent with norbixin extraction results. Powdered WPC80 reflectance values were not in agreement with norbixin extraction results (Table 2). This is not unusual as b^* measurements are a value of all yellow pigments, not just norbixin, which can not only survive the whey concentrate process (such as beta-carotene found naturally in milk) and also compounds, such as Maillard browning products, which can also contribute to color.

WPC80 from bleached wheys (either HP or LP) were higher in aroma intensity and cardboard flavor and lower in sweet aromatic and cooked/milky flavors compared to the control WPC80 ($p < 0.05$) (Table 3). Bleached whey proteins varied from each other in flavor in that LP bleached WPC80 contained a very noticeable cabbage flavor while WPC80 from HP bleached whey displayed a fatty flavor ($p < 0.05$). Volatile results were consistent with sensory results. WPC80 from either LP or HP bleached wheys were higher in hexanal, decanal, 1-hexen-3-one, 2,3 octadien-one, benzacetaldehyde, heptanal, octanal, and 2-pentylfuran than the control unbleached WPC80 ($p < 0.05$) (Tables 4). WPC80 did not differ

in pentanal, DMS, DMTS, sotolon, or diacetyl ($p>0.05$) (Table 4). WPC80 from whey bleached with LP was significantly higher in heptanal, octanal, DMDS, and 2-pentylfuran than those bleached with HP ($p<0.05$) (Table 4).

DISCUSSION

Liquid Whey

Lactoperoxidase is one of the most heat stable enzymes, even retaining activity during normal pasteurization of milk (Seifu et al., 2005). Monitoring LP throughout cheesemake (raw milk, pasteurized milk, whey, pasteurized whey) showed minimal loss throughout processing with LP still active in pasteurized whey. LP constitutes about 1% of whey proteins in raw bovine milk with reported activity ranging widely from 1.2 to 19.4 U/mL, however, most recent literature in bovine milk points to the average being between 1.5 and 2.7 U/mL (Seifu et al., 2005). Variations of enzyme level in bovine milk can be attributed to sexual cycle of the cow, season, feeding regime, and breed (Kussendrager and van Hooijdonk, 2000). Thiocyanate (SCN) levels were not different between samples or during processing ($p>0.05$). Thiocyanate levels can vary depending on the feeding regime of the animal (Reiter and Harnulv, 1984).

The bleaching of whey by LP has not been well documented. Bottomley et al. (1989) found decolorization of whey to be between 50-85% depending on amount of SCN, HP, and LP. The level of bleaching achieved in this study was >99% suggesting that the LP system was operating under optimal conditions. Previously, LP activity had been reported to be optimal between 11 and 15ppm (Bottomley et al., 1989) which is in general agreement

with the curve generated in this paper as LP is known to vary slightly which was previously discussed (Figure 1).

Previous research has not reported the volatile compounds produced specifically from the bleaching of whey using the LP system. Bleaching whey (by either hydrogen peroxide or benzyol peroxide) increased lipid oxidation compounds (Croissant et al., 2009; Listiyani et al., 2011, 2012). Bleaching whey via the LP system yielded levels of hexanal, heptanal, ocatanal, nonanal, 2,4 nonadienal, and 2,6 nonadienal higher than unbleached wheys ($p < 0.05$) and similar to or higher than concentrations found in wheys with chemical bleaching (Table 1) suggesting that LP catalyzes lipid oxidation. The LP system generates hypothiocyanate and ferrous iron not only allowing for bleaching efficacy from hypothiocyanate but also from Fenton-type reactions that may further participate in bleaching efficacy and possible side reactions such as lipid oxidation. Lipid oxidation compounds have been directly linked to the cardboard flavor commonly found in wheys (Whitson et al., 2010). Specifically, hexanal is a great indicator of lipid oxidation, however it is not directly linked to cardboard flavor (Whitson et al., 2010). The flavors in fluid whey carries over into the finished WPC spray dried products (Croissant et al., 2009). These flavors can in turn influence the acceptability of the final product.

Whey Protein Concentrate (80%)

In agreement with the first part of the study, WPC80 from whey bleached using the LP system contained <1% residual norbixin, more norbixin destruction than those samples from whey bleached by HP. Reflectance values were consistent with norbixin destruction

values in that LP bleached better than HP alone ($p < 0.05$). Whey products devoid of any color are highly desirable (Kang et al., 2010).

Bleaching and bleach type have been previously shown to negatively impact the flavor of whey and spray dried whey proteins (Croissant et al., 2009; Listiyani et al., 2011, 2012). Lipid oxidation products are the primary off flavors found in dried whey proteins (Whitson et al., 2010; Whitson et al., 2011) and contribute to undesirable off-flavors. Consistent with previous studies, bleached whey and WPC80 from bleached whey had higher concentrations of lipid oxidation products (such as aldehydes), cardboard flavor, and higher aroma intensities compared to unbleached whey and WPC80 from unbleached whey ($p < 0.05$). Source of bleaching also influenced flavor. WPC80 from HP bleached whey displayed a fatty flavor while WPC80 from LP bleached whey contained cabbage flavor. In addition, WPC80 bleached with LP were higher in heptanal, octanal, DMDS and 2-pentylfuran than those bleached with HP ($p < 0.05$). Bleaching with LP resulted in a whiter product (more norbixin destruction) than chemical bleach (HP), but also resulted in increased off-flavors. The high amount of norbixin destruction ($> 99\%$) using LP suggests that conditions might be optimized to decrease the amount of norbixin destruction while minimizing flavor contributions or to use LP in combination with another bleaching method.

CONCLUSION

The lactoperoxidase system has long been used in the dairy industry as an antimicrobial in raw milk but shows other potential applications, such as the bleaching of fluid whey. The LP system was activated by the addition of 10-40 ppm HP with an optimal concentration of 20 ppm. Compared to HP bleaching, enzymatic bleaching was more

effective in norbixin destruction, bleaching >99%, in both fluid whey and WPC 80. Lipid oxidation products were higher in bleached wheys (either HP or LP) than unbleached wheys and LP bleached wheys contained more sulfur products. These results suggest that sufficient bleaching may be obtained in less time thereby optimizing bleaching efficacy while reducing volatile loads and optimizing flavor. These results demonstrate that LP is active during milk and fluid whey processing and that the LP system can be used to effectively bleach fluid whey with a small addition (20 ppm) HP. Future experiments should focus on the optimization of enzymatic bleaching in both fluid whey and liquid retentate.

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TABLES AND FIGURES

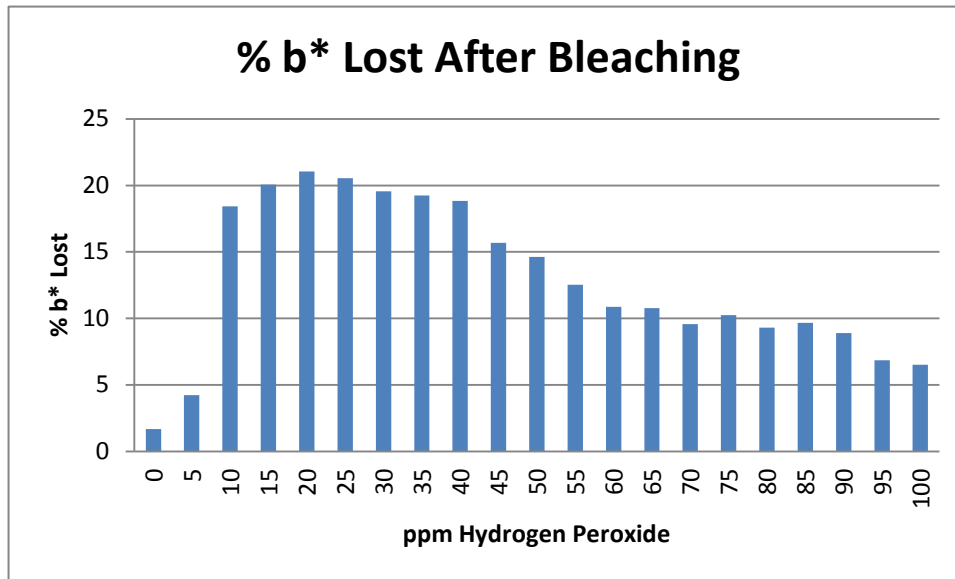


Figure 1: Bleaching efficacy of lactoperoxidase across various levels of hydrogen peroxide in unpasteurized, fat-separated fluid Cheddar whey at 35°C for 1 h.

Table 1: Relative abundance (ppb) of selected volatile compounds in unpasteurized, fat-separated fluid Cheddar whey after bleaching for 1 h

	Control 35C	Control 50C	HP 35C	HP 50C	LP 35C	LP 50C
Hexanal	0.146b	0.099b	0.894b	1.08b	4.00a	3.17a
Heptanal	0.110c	0.088c	0.366c	0.476bc	2.20a	1.21b
Octanal	0.152c	0.078c	0.221bc	0.211bc	1.21a	0.658b
Nonanal	0.415bc	0.259c	0.384bc	0.358bc	1.29a	0.759b
2,4 nonadienal	0.020b	0.020b	0.034b	0.027b	0.137a	0.107a
2,6 nonadienal	0.043ab	0.023b	0.027b	0.022b	0.054a	0.039ab
Methional	0.022a	0.018a	0.016a	0.019a	0.016a	0.015a
DMTS	0.979a	0.960a	0.966a	0.845a	0.973a	0.929a
2-pentylfuran	0.431b	0.451b	1.05b	1.37b	13.3a	11.2a
1-octen-3-one	0.084b	0.084b	0.198b	0.203b	0.647a	0.450a
1-hexen-3-one	0.015a	0.164a	0.034a	0.033a	0.095a	0.088a
Diacetyl	0.184a	0.169ab	0.116bc	0.094c	0.156ab	0.161ab

a–c Means in a row not sharing a common superscript are different ($P < 0.05$).

Control samples received no bleach treatment, HP samples received 250ppm addition of hydrogen peroxide, and LP samples received 20ppm addition of hydrogen peroxide thus activating the lactoperoxidase (LP) system. Samples were bleached for 1h at either 35 or 50C.

Table 2: L*a*b* values for dried and rehydrated WPC80 (10% w/v)

Powder	L*	a*	b*	Rehydrated	L*	a*	b*
Control	87.4b	-0.7a	22.0a	Control	64.6b	-0.3a	33.2a
HP	92.0a	-0.8a	16.8b	HP	69.7a	-2.3c	27.3b
LP	91.5a	-0.6a	17.8c	LP	70.0a	-1.7b	18.7c

a–c Means in a column not sharing a common superscript are different ($P < 0.05$).

Table 3: Descriptive sensory profiles of WPC80

Category	Aroma Intensity	Sweet Aromatic	Cardboard	Cabbage	Cooked/Milky	Fatty	Astringency
Control	2.1b	1.9a	1.1b	ND	2.2a	ND	2.0a
HP	2.8a	0.5b	2.3a	ND	1.5b	1.2	1.8a
LP	2.6a	0.6b	2.2a	2.1a	1.5b	ND	1.8a

a–b Means in a column not sharing a common superscript are different ($P < 0.05$). Attribute intensities were scored on a 0 to 15 point universal intensity scale (Meilgaard et al., 1999). Most dried ingredient flavors fall between 0 and 4 (Croissant et al., 2009; Listiyani et al., 2011).

Control samples received no bleach treatment, HP samples received 250ppm addition of hydrogen peroxide, and LP samples received 20ppm addition of hydrogen peroxide thus activating the lactoperoxidase (LP) system.

Table 4: Relative abundance (ppb) of selected volatile compounds in WPC80

	Control	HP	LP
Pentanal	3.00a	2.84a	3.32a
Hexanal	3.08b	8.06a	6.00a
Heptanal	0.916c	2.77b	4.27a
Octanal	0.481c	1.89b	3.63a
Nonanal	2.87b	4.00ab	5.44a
Decanal	0.070b	0.124a	0.131a
DMS	0.387a	0.371a	0.424a
DMDS	0.131b	0.313b	3.12a
DMTS	0.483a	0.519a	0.515a
1-hexen-3-one	0.143b	0.432a	0.460a
2,3 octadien-one	2.00b	5.09a	5.49a
Benzacetaldehyde	0.470b	1.09a	1.35a
Sotolon	0.051a	0.039a	0.040a
2-pentyl furan	4.80c	15.9b	49.1a
Diacetyl	0.048a	0.027a	0.040a

a–c Means in a row not sharing a common superscript are different ($P < 0.05$).

Control samples received no bleach treatment, HP samples received 250ppm addition of hydrogen peroxide, and LP samples received 20ppm addition of hydrogen peroxide thus activating the lactoperoxidase (LP) system.

Cold Enzymatic Bleaching of Fluid Whey

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ABSTRACT

Chemical bleaching of fluid whey and retentate with hydrogen peroxide (HP) alone requires high concentrations (100 – 500 mg/kg HP) and recent studies have demonstrated that off flavors are generated during chemical bleaching that carry-through to spray dried whey proteins. Bleaching of fluid whey and retentate with enzymes such as naturally present lactoperoxidase (LP) or an exogenous commercial peroxidase (EP) at cold temperatures (4C) may be a viable alternative to traditional chemical bleaching of whey. The objective of this study was to determine the optimum level of HP for enzymatic bleaching (both LP and EP) at 4C and compare bleaching efficacy and sensory characteristics to HP chemical bleaching at 4C. Selected treatments were subsequently applied for WPC80 manufacture. Fluid Cheddar whey and retentate (80% protein) were manufactured in triplicate from pasteurized whole milk. The optimum concentration of HP (0 to 250 mg/kg) to activate enzymatic bleaching at 4C was determined by quantifying the loss of norbixin using previously established HPLC (high performance liquid chromatography) measurements. In subsequent experiments, bleaching efficacy, descriptive sensory analysis, and volatile compounds were monitored at selected time points. A control with no bleaching was also evaluated. Enzymatic bleaching of fluid whey and retentate at 4C resulted in faster bleaching and higher bleaching efficacy (color loss) than bleaching with HP alone at 250 mg/kg ($p < 0.05$). Due to concentrated levels of naturally present lactoperoxidase, retentate bleached to completion (>80% norbixin destruction in 30 min) faster than fluid whey ($p < 0.05$) at 4C (>80% norbixin destruction in 12 h). In fluid whey, the addition of EP decreased bleaching time ($p < 0.05$). Spray dried WPC80 from bleached

wheys, regardless of bleach treatment, were characterized by a lack of sweet aromatic and buttery flavors, and the presence of cardboard flavor concurrent with higher relative abundance of 1-octen-3-ol and 1-octen-3-one ($p < 0.05$). Among enzymatically bleached WPC80, lactoperoxidase bleached WPC80 contained higher relative abundance of 2,3 octadienone, 2-pentyl furan, and hexanal than those bleached with added EP ($p < 0.05$). Bleach times, bleaching efficacy, and flavor results suggest that enzymatic bleaching may be a viable and desirable alternative to HP bleaching of fluid whey or retentate.

KEY WORDS: Whey, lactoperoxidase, flavor, bleach

INTRODUCTION

Whey is a by-product of cheese manufacture and is often further processed into value added products, such as whey protein concentrate 34% or 80% or whey protein isolate (>90% protein). Typical whey processing steps include fat separation, pasteurization, bleaching, ultrafiltration, diafiltration, and spray drying. The flavor of fluid whey carries through into the final spray dried products (Croissant et al., 2009), and consumers and product manufacturers demand that dried whey ingredients be colorless with a bland flavor (Kang et al., 2010).

The manufacture of Cheddar cheese has continued to increase, and Cheddar whey is one of the main sources of cheese whey. Norbixin, a natural orange colored carotenoid, is added to Cheddar cheese milk to impart the desired orange color and a portion of the norbixin is retained in the fluid whey (Kang et al., 2010), and must be bleached. Off-flavors in dried whey proteins associated with bleaching, either with benzoyl peroxide (BP) or hydrogen peroxide (HP), have been well documented in the literature (Croissant et al., 2009; Listiyani et al., 2011, 2012; Jervis et al., 2012). Due to the increased demand for bland, colorless whey ingredients and international concerns with the use of BP and increasing concerns with HP, chemical bleaching alternatives are desirable (Kang et al., 2012; Campbell et al., 2012). Campbell et al. (2012) recently demonstrated that as little as 10 ppm HP was sufficient for greater than 80% norbixin destruction by lactoperoxidase in fluid whey at 35C. Enzymatic bleaching, either utilizing the native lactoperoxidase system (LP) or by adding an exogenous peroxidase (EP) has yet to be fully explored.

Lactoperoxidase (LP), a native enzyme found in milk, is often used to increase storage stability and reduce the loss of fresh milk quality due to microbial spoilage. Lactoperoxidase is a member of the peroxidase family and when its activators, thiocyanate and hydrogen peroxide, are present, hypothiocyanate, a potent antimicrobial, is produced (Reiter and Harnulv, 1982). In addition to milk preservation, the lactoperoxidase system can be used to bleach whey (Bottomley et al., 1989; Campbell et al., 2012). The strong oxidizing capacity of hypothiocyanate results in the destruction of carotenoid conjugation and subsequent color loss of norbixin in cheese whey. Using the LP system to bleach whey can be highly variable as levels of LP can vary depending on the lactation cycle of the cow, season, feeding regime, and breed (Kussendrager and van Hooijdonk, 2000). Like LP, thiocyanate concentration in milk and whey can vary widely due to feeding regime (Seifu et al., 2005). The third component of the LP system, hydrogen peroxide, is not normally detected in raw milk and is typically added exogenously. HP can be generated endogenously by bacteria, although amounts sufficient to activate the LP system may not be generated (Seifu et al., 2005). Depending on the milk, any one of the three components which make up the LP system could limit LP activity.

In order to facilitate enzymatic whey bleaching, a commercial exogenous peroxidase (EP) is available and can be added to fluid whey product in small quantities to help achieve desired and consistent bleaching efficacy. This enzyme, Maxibright™ (MB), is derived from a mushroom, *Marasmius scorodoni* (Zorn et al., 2003). Very little is known about the enzyme mechanism compared to that of lactoperoxidase, however, it is known that both of these enzymes require similar amounts of hydrogen peroxide to activate their respective

systems (Bottomley et al., 1989; Zorn et al., 2003). Since the original patent was filed in 2006, several studies have addressed the bleaching capacity of MB on beta-carotene in model systems (Scheibner et al., 2008; Puhse et al., 2009; Zelena et al., 2009), however, the bleaching efficacy and subsequent effects on flavor of MB in conjunction with the natural LP system in fluid whey has yet to be investigated. Studies have demonstrated that chemical bleaching at colder temperatures (<10C) results in less lipid oxidation (Listiyani et al., 2011). Additionally, colder temperatures enhance membrane stability, microbial quality, and protein integrity. As such, cold bleaching is an attractive process. The objective of this study was to optimize enzymatic bleaching of whey and retentates with both LP and EP at 4C and to evaluate their subsequent effects on flavor of WPC80.

METHODS

Experimental Design Overview

There were two experimental components: liquid whey and retentate trials and the manufacture of WPC 80. Optimum HP levels to activate the LP and EP systems were first determined. Liquid whey and retentate trials were then conducted to determine optimum bleach times at 4C. The liquid whey treatments with the most bleaching and the fastest bleaching times were then selected for WPC80 manufacture. All treatments within each trial were made from the same lot of milk. All experiments were conducted in triplicate.

Production of Liquid Whey

Cheddar whey was manufactured from HTST (high temperature short time; 15s at 72C) pasteurized whole milk (720 kg/h; model T4 RGS-16/2, SPX Flow Technology, Greensboro, NC). The milk was then cooled to 31°C and transferred to a cheese vat (Kusel

Equipment, Watertown, WI). Colored Cheddar whey manufacture proceeded as described by Campbell et al. (2011). The whey was drained from the curds at pH 6.3 and a sieve was used to remove any remaining particles. The whey was immediately processed with a hot bowl cream separator (Model SI600E, Agri-Lac, Miami, FL) to reduce the fat content. Fat separated, fluid whey was then HTST pasteurized as described previously. Whey was cooled to 4°C prior to bleaching experiments.

Production of Retentate

Fat separated, pasteurized fluid whey was transferred into a 102 liter stainless vat (FermentorTM, Blichmann EngineeringTM, Lafayette, IN) equipped with a coil heater (1/2" outer diameter, PAC Stainless LTD, Seattle, WA) and heated to 50°C while recirculating using a peristaltic pump (Model 77410-10, Millipore Inc., Billerica, MA). Once temperature was reached, ultrafiltration commenced. The ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) was equipped with 10 polyethersulfone cartridge membrane filters (Model P2B010V05, 10 kDa nominal separation cutoffs, 0.5 m² surface area, Millipore Inc., Billerica, MA). Each sample was run through a peristaltic pump (Model 77410-10) and the UF assembly using silicone tubing (Model 96440-73) that was connected to the vat. Pumps, pumpheads, and tubing were all obtained from Cole-Palmer (Vernon Hills, IL). Ultrafiltration and diafiltration continued until the retentate reached 80% protein dry basis (w/w) content confirmed by a SprintTM rapid protein analyzer (CEM, Matthews, NC). Retentates were then collected and cooled to 4°C prior to bleaching experiments.

Activation of LP or EP System

The optimum level of HP to activate the LP or EP system was determined by adding 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 250 mg/kg HP to pasteurized fat-separated liquid Cheddar whey or retentate (80% protein dry basis, 10% solids). Bleaching was then carried out as described below. The concentration of HP which resulted in the most bleaching, 10 mg/kg in fluid whey and 15 mg/kg in retentate, (according to norbixin destruction determined via HPLC) was selected for further trials.

Optimum Bleaching Time

Liquid whey or retentate (10% solids w/v, 80% protein dry basis w/w) was placed in amber glass jars in an ice bath and allowed to equilibrate to 4°C. To activate the LP system, 10 mg/kg of hydrogen peroxide (liquid whey) or 15 mg/kg of hydrogen peroxide (retentate) (35% w/v, Nelson Jameson, Inc., Marshfield, WI) was added and allowed to bleach in an ice bath with gentle agitation. To activate the EP system, 2 DBLU MB (**Dairy BLEaching Unit**; DSM, Delft, Netherlands) was added to the liquid whey or retentate and agitated gently. To that, 10 or 15 mg/kg HP was added and allowed to bleach in the ice bath with gentle agitation. Aliquots of samples were removed at appropriate time points (Fluid whey: 0h, 0.5h, 1h, 2h, 4h, 6h, 24h; Retentate: 0 min, 3 min, 5 min, 7 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, 1h, 2h, 4h, 6h, 24h). Peroxide test strips (EMD Chemicals, VWR International, West Chester, PA) were used to determine if any HP remained after the bleaching treatment. To consume the remaining HP in order to stop peroxidase activity, catalase (FoodPro CAT, Danisco, New Century, NJ) was added at a rate of 20 mg/kg.

Measurements, including norbixin, volatile compound analyses, and descriptive analysis were performed immediately.

Production of WPC 80

Industrially, due to manufacturing constraints, bleaching most frequently occurs at the fluid whey level. We also observed differences in the amount of time required for LP and EP bleaching in fluid whey. For these reasons, liquid whey was selected as the bleaching point for WPC80 trials. Treatments were selected based on current industrial practices and from the previous liquid whey trials to achieve maximum bleaching at 4C (**LP** 10 mg/kg HP for 12h, **EP** 2 DBLU MB and 10 mg/kg HP 1h, **HP** 250 mg/kg 12h, **Control** (no bleaching) 12h 4°C) for manufacture of WPC 80. Colored fat separated cheese whey was freshly manufactured as previously described. The pasteurized separated whey was placed in sanitized 38L milk cans at 4°C. Two of three treatments were administered immediately: LP (10 mg/kg HP (35% w/v, Nelson Jameson, Inc., Marshfield, WI)) or HP (250 mg/kg) and allowed to bleach overnight. The following morning the last treatment (EP) was administered (2 DBLU MB, 10 mg/kg HP) and allowed to bleach for 1 h. If any HP remained after treatment, catalase was added at a rate of 20 mg/kg (FoodPro CAT, Danisco, New Century, NJ) to consume the remaining HP and stop peroxidase activity.

Bleached wheys were transferred into a 102 liter stainless vat (FermentorTM, Blichmann EngineeringTM, Lafayette, IN) equipped with a coil heater (1/2" outer diameter, PAC Stainless LTD, Seattle, WA) and heated to 50°C while recirculating using a peristaltic pump (Model 77410-10, Millipore Inc., Billerica, MA). Once temperature was reached, ultrafiltration commenced. The ultrafiltration (UF) system (Model Pellicon 2, Millipore

Inc., Billerica, MA) was equipped with 5 polyethersulfone cartridge membrane filters (Model P2B010V05, 10 kDa nominal separation cutoffs, 0.5 m² surface area, Millipore Inc., Billerica, MA). Each sample was run through a peristaltic pump (Model 77410-10) and the UF assembly using silicone tubing (Model 96440-73) that was connected to the vat. Pumps, pumphheads, and tubing were all obtained from Cole-Palmer (Vernon Hills, IL).

Ultrafiltration and diafiltration continued until the retentate reached 80% protein dry basis (w/w) content confirmed by a SprintTM rapid protein analyzer (CEM, Matthews, NC).

Retentates were then collected and spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark). Inlet temperature was 200°C and the outlet temperature was at 90°C. Total spray drying time was about 1 h. The powder was collected and stored in mylar bags at -80°C following production. All treatments were manufactured from the same lot of milk and the experiment was conducted in triplicate.

Compositional Analysis

Total solids of defatted liquid whey and WPC80 were determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). In powdered WPC80, fat was quantified by Mojonnier (AOAC, 2000; method number 932.06 and 989.05). In defatted liquid wheys, fat was quantified using the CEM Smart Trac rapid fat analyzer (CEM, Matthews, NC).

Protein was determined using the Kjeldahl method in powdered whey proteins (AOAC, 2000; method number 991.20; 33.2.11) or using the SprintTM Rapid Protein Analyzer (CEM, Matthews, NC) if the whey was liquid. Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) was determined by the NCSU 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.

Hunter L* a* b* values

WPC 80s were measured in both powder form (10 g) and liquid form (10 mL of 10% w/v solution). Ten mL of the sample (rehydrated at 10% w/v solids if necessary) was placed into the bottom of a 60mm x 15mm polystyrene petri dish (Beckton Dickinson, Franklin Lakes, NJ). Color of WPC80 was measured using a Minolta Chroma meter (CR-410, Ramsey, NJ). Each sample was measured in duplicate. Before measurements were taken, a factory-supplied calibration plate was used to calibrate the instrument. The Hunter CIE Lab color scale was used. Reflectance values were taken with a white calibration plate as the background.

Norbixin Extraction and Quantification

Norbixin is the primary carotenoid in water soluble annatto extracts and was extracted and measured to determine percent annatto destruction and bleaching efficacy (Kang et al., 2010). Norbixin was extracted and quantified using HPLC. To extract defatted fluid wheys, 200ul was placed into a 2 mL microcentrifuge tube (VWR International, West Chester, PA). To this, 800ul of dilution solution (80% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/20% water (EMD Chemicals, VWR International, West Chester, PA with 0.1% (w/v) formic acid (EMD Chemicals, VWR International, West Chester, PA)) was added. The solution was vortexed and centrifuged (14,000 x g, Microfuge® 18 Centrifuge, Beckman Coulter, Brea, CA). The supernatant was removed and placed into vials for quantification by HPLC. To extract powders, samples

were first reconstituted to 10% solids using DI water. Then, 100ul of sample was placed into a 2 mL microcentrifuge tube. To this, 900ul dilution solution was added and the sample was vortexed and centrifuged as previously described. The supernatant was removed and placed into vials for quantification by HPLC. 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).

Quantification was conducted using HPLC (Waters 1525 Binary Pump, Waters, Milford, MA). Isocratic mobile phase (70% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/30% water (EMD Chemicals, VWR International, West Chester, PA) with 0.1% (w/v) formic acid (EMD Chemicals, VWR International, West Chester, PA)) was used at a flow rate of 1mL/min pumped through a binary pump (Waters 1525, Waters, Milford, MA). Fifty microliters of the sample was injected (Waters 2707 Autosampler) onto the column (Phenomenex Kinetex 2.6µm particle size, 10 cm length, 4.6mm inner diameter, 100A pore size) which was heated to 40°C. The injector temperature was set to 4°C. Sample was sent through a photodiode array detector (Waters 2998). A standard curve was created by rehydrating norbixin powder (45% (w/v), Chr. Hansen, Milwaukee, WI) in 2.5% (w/v) potassium hydroxide (BDH, VWR International, West Chester, PA) and then diluting in mobile phase. The maxima used for calculation was 482 nm. Norbixin concentration was calculated by total solids and correction for dilution during the extraction and SPE processes.

Descriptive Sensory Analysis

Sensory analysis was conducted on defatted fluid wheys, retentates, and rehydrated WPC80 (10% w/v) using a trained descriptive sensory panel and an established dairy flavor language (Drake et al., 2003, 2009). Panelists (n =8) each had more than 150 h of previous experience with the sensory analysis of fluid and dried whey products using the Spectrum™ descriptive analysis method (Meilgaard et al., 2007). All sensory testing was conducted in accordance with NCSU Institutional Review Board for Human Subjects guidelines.

Defatted fluid wheys, retentates, or reconstituted WPC80 (10% solids w/v) were evaluated by placing 30 mL in three-digit-coded 60 mL lidded cups (Solo Cup Company, Champaign, IL). Preparations were conducted with overhead lights off to avoid exposure to light. Samples were evaluated by each panelist in duplicate. Sensory data were collected on paper ballots or using Compusense™ five, release 4.8 (Compusense, Guelph, Canada).

Gas Chromatography Mass Spectrometry (GCMS)

Selected volatile compounds in defatted fluid wheys and WPC 80 powder were extracted by solid phase microextraction (SPME) using selective ion monitoring (SIM). Volatile compounds were selected from previous studies and were compounds that were relevant to flavor and/or bleaching (Campbell et al., 2012; Croissant et al., 2009; Listiyani et al., 2012; Jervis et al., 2012; Kang et al., 2012). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using a modified method of Liaw et al. (2010). Liquid samples were tested the day of manufacture and spray dried powders were reconstituted at 10 % solids (w/v) and evaluated within 7 days. All samples contained 10 % (w/v) sodium chloride (Fischer Scientific), and 10 ul internal standard

solution (2-methyl-3-heptanone in methanol at 81 ppm (Sigma Aldrich, Milwaukee, WI)) in 20 ml 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 MSD (Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1 cm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA) at 31 mm with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm.

The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 250°C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250°C. An Zb-5ms column (Zb-5ms 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) was used for all analyses at a constant flow rate of 1 ml/min. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°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 compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound. Retention indices were calculated using an alkane series (Sigma Aldrich, Milwaukee, WI) (Van den Dool and Kratz, 1963).

Statistical Analysis

All data was analyzed by a one-way analysis of variance (ANOVA) using a general linear model with Fisher's least significant difference for means separation (XLSTAT,

version 2009.1.02; Addinsoft Inc., New York, NY). Replication was designated as a random effect.

RESULTS

FLUID WHEY AND RETENTATE

Composition

Compositionally, samples were not different ($p>0.05$). Defatted fluid wheys averaged $6.71\pm 0.05\%$ solids, $0.89\pm 0.05\%$ protein, and $0.0\pm 0.01\%$ fat. Retentates (80% protein dry basis) averaged $10.6\pm 0.45\%$ solids, $8.80\pm 0.20\%$ protein, and $0.67\pm 0.05\%$ fat. All measurements are reported on a wet weight basis.

Norbixin and Color Analysis

Experiments were conducted to determine the optimum amount of HP needed to activate both the LP and EP systems. In fluid whey at 4C, 10 mg/kg HP provided the most bleaching in both LP and EP systems while 15 mg/kg HP was most efficient in retentate for both enzymatic systems (results not shown). The optimum level of HP to activate the LP system in fluid whey at 4C was very narrow (± 10 mg/kg) while those with EP added displayed a much wider range of HP addition for maximum bleaching activity (Figure 1). The same trend was observed for fluid whey at 20C and 35C, the range for HP addition was wider for EP than LP alone (results not shown). The optimum level of HP for retentate bleaching by LP or EP was 15 mg/kg. In retentate, both EP and LP alone exhibited effective bleaching ($>80\%$ norbixin destruction) over a wide range of HP addition (15 mg/kg-250 mg/kg) at either cold (4C) or warm (35C) temperatures (results not shown).

In fluid whey, bleaching with EP at 2 DBLU MB with the addition of 10 mg/kg HP for 1h at 4C yielded >80% bleaching. Bleaching using only endogenous enzyme (LP) under the same conditions was slower ($p<0.05$), requiring 6 to 24h to bleach >80% (results not shown) and variability in lactoperoxidase activity and optimum bleach times were observed. In fluid retentate, optimum bleach time at warm temperatures (35C) for LP and EP were not different ($p>0.05$) (5 min, >80% norbixin destruction; results not shown) nor were they distinct at 4C (30 min, >80% norbixin destruction; $p>0.05$) (results not shown).

Descriptive Sensory Analysis

Bleached fluid wheys, regardless of bleach treatment, had decreased sweet aromatic and cooked/milky flavors ($p<0.05$) (results not shown). Fluid wheys bleached using HP displayed a distinct sulfur flavor not present in enzymatically (LP or EP) bleached wheys. In retentates, similar to fluid wheys, all bleached samples, regardless of bleach treatment, exhibited decreased sweet aromatic and cooked/milky flavors ($p<0.05$). In addition, bleached retentates exhibited increased cardboard flavor intensities compared to control unbleached retentates ($p<0.05$). Similar to bleaching in fluid whey, HP bleached retentate exhibited a distinct sulfur note not detected in enzymatically (either HP or LP) bleached retentates.

Gas Chromatography Mass Spectrometry (GCMS)

Volatile compound analysis was consistent with descriptive analysis results. In fluid whey, enzymatically bleached wheys (EP or LP) were higher in aldehydes than chemically bleached wheys (HP 250 mg/kg) (results not shown). Bleached retentates, regardless of treatment, were higher in aldehydes including hexanal, heptanal, octanal, nonanal and

decanal compared to unbleached controls (results not shown). In addition, retentates bleached chemically (HP 250mg/kg) were higher in dimethyl trisulfide (DMTS) than other treatments ($p < 0.05$) (0.64 ± 0.05 ppb vs. 0.45 ± 0.02 ppb).

POWDER WPC80

Composition

Compositionally, WPC80 were not different ($p > 0.05$). Powdered WPC80 averaged $96.3 \pm 0.48\%$ solids, $77.6 \pm 2.1\%$ protein, and $3.7 \pm 0.4\%$ fat on a wet weight basis.

Phosphorus, potassium, calcium, magnesium, sulfur, and sodium were not different among treatments ($p > 0.05$). Iron was the only distinct mineral, the lactoperoxidase WPC80 was lower in iron than the control WPC80 ($p < 0.05$) (Table 1), and this observation has been previously documented in HP WPC80 (Jervis et al., 2012; Jervis and Drake, 2013).

Norbixin and Color Analysis

Consistent with and as expected from fluid whey results, enzymatic bleaching (LP and EP) removed more norbixin than traditional chemical bleaching (HP) ($p < 0.05$) (Figure 2). The addition of exogenous peroxidase increased the speed of the bleaching process; bleaching 92% in 1h while LP and HP bleached 97% and 38% respectively in 12h at 4C. $L^*a^*b^*$ values were consistent with norbixin values (data not shown).

Descriptive Sensory Analysis

Rehydrated WPC80 that were bleached, regardless of treatment, were characterized by a lack of sweet aromatic and buttery flavors and by increased cardboard flavor as compared to the unbleached control (Table 2). Hydrogen peroxide bleached WPC80 had a distinct oxidized/fatty flavor not detected in the other WPC80. Enzymatically bleached

WPC80 with exogenous peroxidase displayed a potato/brothy flavor but was also lower in cardboard flavor than enzymatically bleached WPC80 using lactoperoxidase alone (Table 2).

Gas Chromatography Mass Spectrometry

Volatile analysis results were consistent with descriptive analysis results. All four WPC80 were distinct in their volatile profile. Bleached WPC80, regardless of treatment, were higher in 1-octen-3-ol and 1-octen-3-one ($p < 0.05$) (Table 3). Among enzymatically bleached samples, lactoperoxidase WPC80 were higher in 2,3 octadienone, 2-pentyl fural, and hexanal than those with added EP ($p < 0.05$) (Table 3). Heptanal was higher EP bleached WPC80 than the control while dimethyl disulfide (DMDS) was higher in LP bleached WPC80 than the control ($p < 0.05$) (Table 3). Hexanal, a key volatile indicative of lipid oxidation, was highest in HP and LP treated WPC80.

DISCUSSION

Levels of HP needed to activate enzymatic systems at cold temperatures were in the range of values previously reported for other temperatures (Campbell et al., 2012; Bottomley et al., 1989). In agreement with previous research, chemical bleaching using HP (250 mg/kg) at cold temperatures was not very effective in fluid whey but was more effective in retentate (Listiyani et al., 2012; Fox, 2013). At 4C, enzymatic bleaching of fluid whey was more effective than traditional chemical bleach with HP. Furthermore, the addition of exogenous peroxidase in fluid whey increased the speed of bleaching at 4C. In an industrial setting, dosing the correct amount of hydrogen peroxide for enzymatic bleaching into a continuous fluid whey system can be difficult to do precisely. Seasonal

variations and processing deviations may also occur that alter the amount of HP needed to activate the LP system, further complicating matters. As such, it may be beneficial in industrial settings to add exogenous peroxidase to increase the speed of bleaching as well as robustness of the bleaching system.

In retentate, the addition of EP did not increase the speed of enzymatic bleaching ($p > 0.05$) but in fluid whey, the addition of EP greatly increased the speed of bleaching ($p < 0.05$, EP: 1h, LP: 12h). The decreased effect of the exogenous enzyme in retentate is likely because native lactoperoxidase is concentrated along with protein during ultrafiltration and is present at much higher levels in retentate than in fluid whey. As such, enzymatic activity is increased and the speed of bleaching in retentate increases compared to fluid whey. Like fluid whey, the LP system in retentate can be permanently inactivated if too much HP is dosed into the system. Fox (2013) demonstrated that in liquid whey protein retentate, 250 mg/kg HP destroyed more norbixin than 500 mg/kg HP suggesting that the LP range for hydrogen peroxide is much higher in retentate (up to 250 mg/kg) than in fluid whey (up to 20 mg/kg) which is also consistent with results from this study. Increases in enzyme levels, whether it be from membrane filtration or the addition of EP, increase the range at which HP can be dosed without permanently inactivating LP.

As expected, bleached fluid wheys and retentates, regardless of bleach treatment, had decreased sweet aromatic and cooked/milky flavors and increased cardboard flavor ($p < 0.05$). Increased cardboard flavor as a result of lipid oxidation in fluid whey, 34 and 80% protein retentate, and 34 and 80% protein spray dried powders have been extensively documented (Jervis et al., 2012; Campbell et al., 2012; Campbell et al., 2011; Kang et al.,

2012; Croissant et al., 2009; Listiyani et al., 2011; Listiyani et al., 2012). Fluid wheys and retentates that were chemically bleached with HP contained a distinct sulfur flavor not present in enzymatically (LP or MB) bleached wheys ($p < 0.05$, results not shown). Increased volatile sulfur compounds and distinct sulfur flavor in chemically HP bleached WPC80 has been previously reported (Jervis et al., 2012) and higher concentrations of DMTS were documented in fluid retentates that were bleached with HP in this study. Previous studies have also demonstrated functional differences between hydrogen peroxide bleached whey protein and unbleached controls suggesting hydrogen peroxide impacts protein integrity (Jervis et al., 2012; Campbell et al., 2013). Volatile sulfur compounds are formed from protein degradation of sulfur containing amino acids (Wright et al., 2006). While it does not directly influence cardboard flavor (Whitson et al., 2010), hexanal is considered a good indicator of lipid oxidation and cardboard flavors (Whitson et al., 2010). Hexanal relative abundance was higher in LP and HP bleached samples than unbleached or EP bleached WPC80. Higher relative abundance of this lipid oxidation compound is consistent with sensory results that LP and HP bleached WPC80 were higher in cardboard flavor than unbleached or EP bleached WPC80.

WPC80 from fluid whey bleached using exogenous peroxidase exhibited a distinct potato/brothy flavor. Potato flavor can be caused by a wide array of volatile compounds, but is mainly attributed to methional, an aroma compound formed from the degradation of the amino acid methionine (Jansky, 2010). Methional was not detected by headspace volatile compound analysis in EP WPC80 even though a distinct potato flavor was detected by trained sensory panelists. The threshold for methional is very low, less than 5 $\mu\text{g}/\text{kg}$

(Karagul-Yuceer et al., 2004). It is possible that methional was below instrumental headspace detection but still readily detected by trained panelists. It is also possible that another compound is responsible for the potato flavor in EP WPC80 documented by trained panelists. Methional has been detected previously by GC-O (gas chromatography olfactometry) and by solvent extraction with GCMS, but not by SPME GCMS in dried whey protein concentrates, isolates, and hydrolysates (Carunchia Whetstine et al., 2005; Leksrisonpong et al., 2010). Additional studies on the flavor chemistry of EP WPC80 would be required to confirm the source of this flavor.

CONCLUSION

It is imperative that dried dairy ingredients be colorless and bland in flavor to increase ingredient applications. Bleaching is required to eliminate color, but also produces undesirable flavors which can carry through into the finished product and influence consumer acceptance. Alternative bleaching agents, such as endogenous or exogenous peroxidases, can bleach effectively and can eliminate more color than traditional chemical bleaching agents in less time. These results demonstrate that off-flavors due to lipid oxidation are still present in enzymatically bleached WPC80 but are the same or lower in intensity than HP chemically bleached WPC80 and lower in lipid oxidation volatiles than HP chemically bleached WPC80. By bleaching at cold temperatures, manufacturers can minimize off-flavors and decrease membrane fouling. The addition of exogenous enzyme increased the speed of bleaching at 4C in fluid whey and also the range of HP allowed for enzymatic bleaching in fluid whey. As such, exogenous peroxidase may be beneficial in an

industrial setting where continuous dosing of a narrow range of hydrogen peroxide is difficult.

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TABLES AND FIGURES

Table 1. Select minerals in powdered WPC80 with different bleach treatments (Control=no bleach; HP=250 ppm hydrogen peroxide; LP= lactoperoxidase; EP= exogenous peroxidase (Maxibright™))

^{a-b} Means in the same column not sharing a common superscript are different ($P < 0.05$)

	Concentration P (% weight)	Concentration K (% weight)	Concentration Ca (% weight)	Concentration Mg (% weight)	Concentration S (% weight)	Concentration Fe (mg/kg)	Concentration Na (mg/kg)	Ash (% weight)
Control	0.37a	0.71a	0.52a	0.06a	0.99a	9.1a	2100a	3.3a
HP	0.37a	0.66a	0.55a	0.06a	0.98a	7.7ab	1900a	3.2a
LP	0.37a	0.72a	0.53a	0.06a	0.94a	7.4b	2200a	3.5a
EP	0.38a	0.67a	0.56a	0.06a	0.96a	8.0ab	2000a	3.4a

Table 2. Descriptive sensory profiles of WPC80 (Control=no bleach; HP=250 ppm hydrogen peroxide; LP= lactoperoxidase; EP= exogenous peroxidase (Maxibright™))
a–c Means in a row not sharing a common superscript are different ($P < 0.05$). Attribute intensities were scored on a 0 to 15 point universal intensity scale (Meilgaard et al., 2007). Most dried ingredient flavors fall between 0 and 4 (Croissant et al., 2009; Listiyani et al., 2011).

ND=Not Detected.

	Control	HP	LP	EP
Aroma Intensity	2.1b	2.5a	2.3a	2.4a
Sweet Aromatic	1.3a	ND	ND	ND
Cooked/Milky	2.4a	2.4a	2.3a	2.3a
Buttery	0.6a	ND	ND	ND
Cardboard	1.2c	2.5a	2.3a	1.9b
Potato/Brothy	ND	ND	ND	1.1a
Oxidized/Fatty	ND	1.2a	ND	ND
Astringent	1.3b	1.6a	1.4ab	1.4ab

Table 3. Relative abundance (ppb) of select volatile compounds in rehydrated spray dried WPC80 with different bleach treatments (Control=no bleach; HP=250 ppm hydrogen peroxide; LP= lactoperoxidase; EP= exogenous peroxidase (2 Dairy Bleaching Units Maxibright™))

^{a-b} Means in the same column not sharing a common superscript are different ($P < 0.05$).
 ND=Not Detected.

	Control	HP	LP	EP
1-hexen-3-one	0.367b	1.62a	0.783b	0.431b
1-octen-3-ol	ND	2.96a	3.83a	2.95a
1-octen-3-one	ND	2.95a	3.01a	1.97a
2,3 octadienone	0.213b	0.632b	1.507a	0.552b
2-methyl butanal	0.199a	0.206a	0.211a	0.231a
2-pentyl furan	1.467b	3.569b	9.385a	3.373b
3-methyl butanal	0.044a	0.059a	0.079a	0.061a
Decanal	0.099a	0.115a	0.128a	0.083a
Diacetyl	0.285a	0.249a	0.253a	0.246a
Dimentyl disulfide	0.174b	0.705ab	1.772a	0.650ab
Dimethyl sulfide	0.643a	0.484a	0.498a	0.539a
Heptanal	0.052b	0.194ab	0.456ab	0.634a
Hexanal	1.59b	7.69a	5.98a	3.54b
Nonanal	0.533a	0.569a	0.492a	0.331a
Octanal	0.555a	0.604a	0.565a	0.451a
p-xylene	0.294ab	0.401a	0.187b	0.187b
Toluene	2.91a	2.65a	2.99a	2.49a

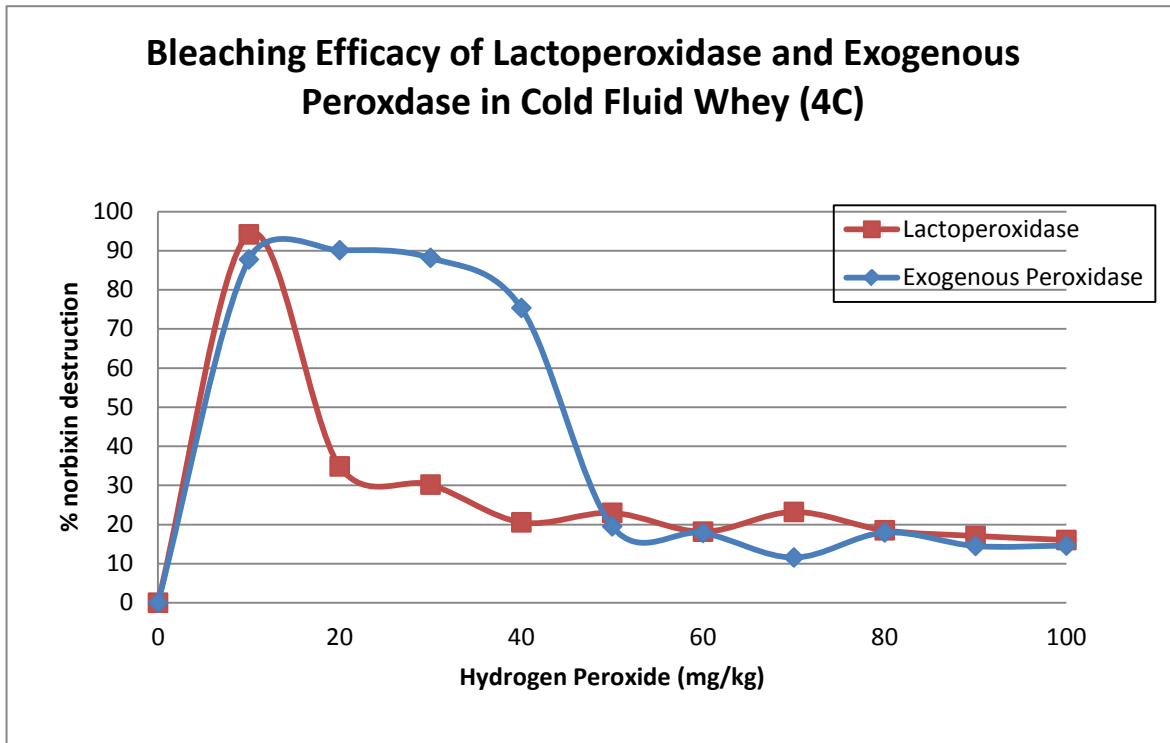


Figure 1. Bleaching efficacy of endogenous and exogenous enzyme, measured by percent norbixin destruction, in cold fluid whey (4C) at various levels of hydrogen peroxide after 24 hours; lines connecting points are for visual purposes only and do not imply correlation.

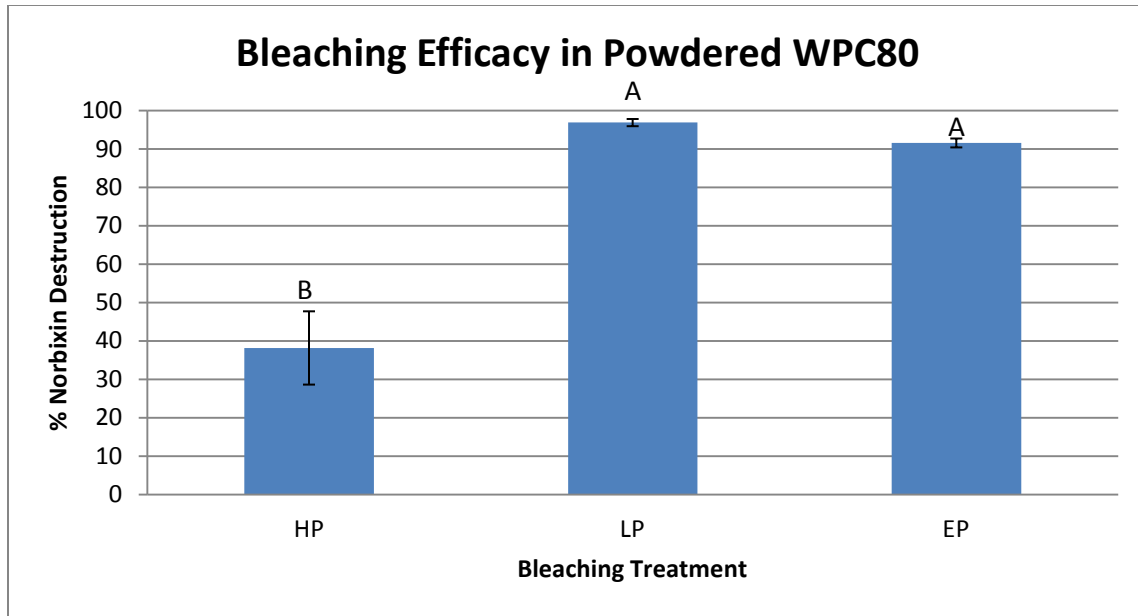


Figure 2. Bleaching efficacy, measured by percent norbixin destruction, in powdered WPC80 averaged among three replications (HP=250 ppm hydrogen peroxide; LP=lactoperoxidase; EP=exogenous peroxidase (2 Dairy Bleaching Units Maxibright™). HP and LP bleached at 4C for 12h and EP bleached at 4C for 1h.

**Characterizing endogenous and exogenous peroxidase activity for bleaching of fluid
whey and retentate**

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INTERPRETIVE SUMMARY

After milk is colored with annatto for Cheddar cheese, a portion of the color remains in the fluid whey and must be bleached to remove the color. Enzymatic bleaching, both endogenous and exogenous, can be used to bleach fluid whey or retentate. Enzyme activity was characterized over a range of temperatures and pH and the relationship between enzyme activity and bleaching was determined.

ABSTRACT

The lactoperoxidase (LP) system may be used to achieve the desired bleaching of fluid whey with the addition of low concentrations (<50 ppm) of hydrogen peroxide to the system. The addition of an exogenous peroxidase enzyme to whey may also be used to aid in the bleaching of fluid whey when the LP system is not fully active. The objectives of this study were to monitor LP activity determined previously in refrigerated or frozen storage in milk, fluid whey, and whey retentate (10% solids) and to evaluate peroxidase activity in fluid whey and whey retentate (10% solids), with and without additional exogenous peroxidase enzyme, over a range of pH and temperatures. Subsequent experiments were conducted to determine the relationship between enzyme activity and bleaching efficacy. Raw and pasteurized milk, fat-separated pasteurized whey and whey retentate (10% solids) were evaluated for LP activity following 0, 24, 48 or 72 h storage at 4 or -20C using an established colorimetric method. A response surface model (RSM) was applied to evaluate both endogenous and exogenous peroxidases activity at various temperatures and pH in freshly manufactured whey and retentate.

Refrigerated or frozen storage at the parameters evaluated did not impact LP activity in milk, whey, or fluid retentate. Evaluating enzyme activity over a range of pH and temperatures demonstrated that in fluid whey, with and without added exogenous peroxidase, as pH decreased (to 5.5) and temperature increased (to 60C), peroxidase activity increased. Models of the data in fluid whey with and without added exogenous peroxidase were not different, however, added exogenous peroxidase did have a significant effect in retentate ($p < 0.05$). Retentate with additional peroxidase enzyme exhibited behavior similar

to that of fluid whey: as pH decreased and temperature increased, activity increased. However, in retentate without additional enzyme, as pH increased and temperature increased, activity increased. Subsequent experiments demonstrated that enzyme activity was negatively correlated to bleaching time (time for >80% norbixin destruction) in fluid whey ($p < 0.05$) but a linear relationship was not evident in retentate. When fluid whey is bleached enzymatically, if pH is decreased and temperature is increased, the rate of reaction increases (e.g. bleaching occurs in less time). When bleaching in retentate, a higher pH (pH 6.5 vs. pH 5.5) is desired for optimal bleaching by the LP system. Due to processing restraints, this may not be possible for all dairy producers to achieve and thus, addition of exogenous peroxidase could be beneficial to improve bleaching efficacy.

Key Words: Lactoperoxidase, whey, enzyme stability

INTRODUCTION

Lactoperoxidase (LP), a native enzyme found in milk, is often used to increase storage stability of fluid raw milk (Seifu et al., 2005). Lactoperoxidase is a member of the peroxidase family and when its activators, thiocyanate and hydrogen peroxide, are present, hypothiocyanate, a potent antimicrobial, is produced (Reiter and Harnulv, 1982). In addition to milk preservation, the lactoperoxidase system can be used to bleach fluid whey (Bottomley et al., 1989; Campbell et al., 2012). Norbixin, a natural orange colored carotenoid, is often added to Cheddar cheese milk to impart desired color (Kang et al., 2010). A portion of norbixin is retained in the fluid whey. The strong oxidizing capacity of hypothiocyanate results in the destruction of carotenoid conjugation and subsequent color loss of norbixin in cheese whey.

Using the LP system to bleach whey can be highly variable as levels of LP can vary depending on the lactation cycle of the cow, season, feeding regime, and breed (Kussendrager and van Hooijdonk, 2000). Like LP, thiocyanate concentration in milk and whey can vary widely due to feeding regime (Seifu et al., 2005). The third component of the LP system, hydrogen peroxide (HP), is not normally detected in raw milk and is typically added exogenously. HP can be generated endogenously by bacteria, although amounts sufficient to activate the LP system may not be generated (Seifu et al., 2005). Depending on the milk, any one of the three above components which make up the LP system could become the limiting factor to LP activity.

A commercial peroxidase is available to enhance fluid whey bleaching and can be added to fluid whey product in small quantities to help achieve desired and consistent

bleaching efficacy (Kang et al., 2010; Campbell et al., 2013; Szweda et al., 2013). This enzyme is derived from a mushroom, *Marasmius scorodoni* (Zorn et al., 2010). Very little is known about the enzyme mechanism in comparison to the lactoperoxidase mechanism, however, it is known that both of these enzymes require similar amounts of hydrogen peroxide to activate their respective systems (Bottomly et al., 1989; Zorn et al., 2010). Since the original patent for this enzyme was filed in 2006, several papers have addressed the bleaching capacity of *Marasmius scorodoni* extracts on beta-carotene in model systems (Scheibner et al., 2008; Puhse et al., 2009; Zelena et al., 2009), however, the activity and bleaching efficacy of this enzyme in conjunction with the natural LP system found in fluid whey or fluid whey retentate have yet to be fully investigated. Fluid whey is often further processed into a value added product, such as whey protein concentrate 34% or 80% or whey protein isolate (>90% protein). Dried whey products are commonly used as ingredients in other foods. Consumers and product manufactures alike demand dried whey ingredients to be colorless with a bland flavor (Kang et al., 2010). The objectives of this study were to monitor LP activity in refrigerated or frozen storage in milk, fluid whey, and whey retentate (10% solids) and to characterize peroxidase activity in fluid whey and whey retentate (10% solids), specifically activity with and without additional exogenous peroxidase, over a range of pH and temperatures by employing a response surface model (RSM) design.

MATERIALS AND METHODS

This experiment consisted of two distinct parts. The objective of experiment 1 was to monitor the stability of LP in raw milk, pasteurized milk, pasteurized fat-separated

Cheddar whey, and fluid Cheddar whey retentate (80% protein) during cold or frozen storage. The objective of experiment 2 was to monitor peroxidase activity in fluid Cheddar whey and fluid Cheddar whey retentate (80% protein) over a range of pH and temperatures. Experiments were subsequently conducted to confirm the relationship between enzyme activity and bleaching efficacy.

Experiment 1:

Lactoperoxidase activity was monitored in raw whole milk, pasteurized whole milk, pasteurized fat-separated fluid Cheddar whey, and fluid Cheddar whey retentate during cold or frozen storage (4°C or -20°C) over time (0h, 24h, 48h, or 72h) using an established colorimetric assay (Pruitt and Kamau, 1994). This experiment was replicated in triplicate.

Experiment 2:

Two factors were selected to optimize enzyme activity in fluid whey and retentate: pH and temperature. The evaluated ranges for pH and temperature were based on industrial dairy bleaching parameters (Table 1). Endogenous peroxidase (lactoperoxidase) as well as various levels of exogenous peroxidase (Maxibright™, DSM Food Specialties, The Netherlands) (2 DBLU (Dairy Bleaching Unit), 1 DBLU, and 0.5 DBLU) were evaluated.

The factors, pH and temperature, and levels of each factor were arranged into a central composite response surface design (RSM) (Myers and Montgomery, 1995), with two center points. Because levels of pH were randomized within levels of temperature for each replication, a mixed model approach to fitting was taken to accommodate this restriction on randomization. In addition to fixed effects, “replication” and “replication*temperature” were random effects in the model due to linear and quadratic terms for “temperature”, “pH”,

and “temperature*pH”. The experiment was replicated three times using three different lots of milk; both fluid whey and retentate were made from the same lot of milk within each replication. The steps between the different levels of each factor were the same, with the midpoint value falling in the middle of the range of the five levels.

In subsequent experiments, three points were selected along pH and temperature curves for each matrix (fluid whey or retentate) and each enzyme level (2 DBLU exogenous peroxidase, 1 DBLU exogenous peroxidase, 0.5 DBLU exogenous peroxidase, endogenous peroxidase (LP)) to further elucidate the relationship between peroxidase activity and bleaching time. Experiments were conducted in triplicate.

Production of whey and retentate (80% protein)

Raw clarified milk was obtained from the dairy at North Carolina State University (Raleigh, NC). Milk was batch pasteurized at 63°C for 30 min followed immediately by cheese manufacture. Cheddar cheese whey was manufactured from vat pasteurized whole bovine milk (195 kg) as described by Campbell et al. (2011). The whey was drained from the curds at pH 6.35 and a sieve was used to remove any remaining particles. The whey was immediately processed with a hot bowl cream separator (Model SI600E, Agri-Lac, Miami, FL) to reduce the fat content and then the whey was batch pasteurized at 63°C for 30 min.

To produce retentate, the fat-separated pasteurized whey was transferred into a 102 L stainless vat (FermentorTM, Blichmann EngineeringTM, Lafayette, IN) equipped with a coil heater (1/2” outer diameter, PAC Stainless LTD, Seattle, WA). The ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) was equipped with 5 polyethersulfone cartridge membrane filters (Model P2B010V05, 10 kDa nominal

separation cutoffs, 0.5 m² surface area, Millipore Inc., Billerica, MA). Each sample was run through a peristaltic pump (Model 77410-10) and the UF assembly using silicone tubing (Model 96440-73) that was connected to the vat. Pumps, pumpheads, and tubing were all obtained from Cole-Palmer (Vernon Hills, IL). This process continued until the retentate reached 80% protein (w/v) content confirmed by a SprintTM rapid protein analyzer (CEM, Matthews, NC). All treatments within each experiment were manufactured from the same lot of milk and the entire experiment was replicated in triplicate. For experiment 2, exogenous peroxidase (MaxibrightTM, DSM, Netherlands) was added at a rate of 2, 1 or 0.5 DBLU/mL (dairy bleaching units) to a portion of the wheys and retentates from each replication. Wheys and retentates were immediately pH adjusted using either lactic acid (85%; VWR International, West Chester, PA) or sodium hydroxide (1N; VWR International, West Chester, PA). A total of less than 0.5mL of sodium hydroxide or lactic acid was used to adjust the pH such that the protein concentration was not affected. The pH adjusted wheys and retentates were then placed in either a hot water bath or cold ice bath to equilibrate (30 min). Following pH adjustment and temperature equilibration, samples were immediately analyzed for peroxidase activity via spectrophotometry or norbixin content (bleaching) via HPLC (high performance liquid chromatography).

Compositional Analysis

Total solids of milk, liquid whey, and retentate were determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). Fat was quantified using the SmartTracTM (CEM, Matthews, NC). Protein was determined using the the SprintTM Rapid Protein Analyzer (CEM, Matthews, NC). All samples were measured in duplicate.

Enzyme Activity Measurement (Experiment 1 and 2)

LP measurements and calculations on fluid milks, wheys, and retentates were performed according to the International Dairy Federation method (Pruitt and Kamau, 1994). 2 mL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) stock solution (1mM ABTS (TCI, Tokyo, Japan) in 0.1mM phosphate buffer pH 6.0 (EMS, Hatfield, PA)) was placed in a disposable plastic cuvette (VWR International, West Chester, PA). To start the reaction, 0.10 mL of sample and 1.0 mL hydrogen peroxide solution (0.3 mM; Nelson Jameson, Inc., Marshfield, WI) was added simultaneously and mixed thoroughly. The absorbance was measured and recorded 10 times per s for 2 min at 412 nm (Cary 300 UV-Vis, Agilent, Cary, NC). Temperatures were controlled during measurement using a temperature control attachment to the spectrophotometer (TC125, Quantum Northwest, Liberty Lake, WA). To prevent any condensation, compressed nitrogen continuously flowed through the sample cell. The derivative of the line fitted to the data using a fifth order polynomial equation ($R^2 > 0.97$) was determined at time zero ($x=0$), which is presented as “slope”. The slope of the line may also be referred to as “activity”. The steeper the slope, the higher the enzyme activity and thus the faster the reaction proceeds.

Thiocyanate Measurement (Experiment 1)

Thiocyanate measurements of fluid milks, wheys, and retentates were performed according to the International Dairy Federation method (IDF, 1988). 4 mL of sample was mixed with 2.0 mL (w/v) of 20% trichloroacetic acid (TCA) solution (BDH, VWR International, West Chester, PA). The mixture was blended well and allowed to sit at room temperature for at least 30 min. The mixture was then centrifuged at $16,500 \times g$ for 10 min

(Model RC5B, Thermo Scientific, Waltham, MA). After centrifugation, 1.5 mL of the supernatant was mixed with 1.5 mL of ferric nitrate solution (16g Fe (NO₃)₃ (EMD Chemicals, VWR International, West Chester, PA) dissolved in 50 mL of 2M nitric acid (HNO₃) (BDH, VWR International, West Chester, PA) and then diluted with DI water to 100 mL). The measurement was performed at 460 nm (Cary 300 UV-Vis, Agilent, Cary, NC) within 10 min of ferric nitrate solution addition. The concentration of SCN (Sigma Aldrich, Milwaukee, WI) was determined by the use of external standard curves.

Bleaching (Experiment 2)

Liquid whey or retentate were first pH adjusted as previously described as necessary. Fluid wheys or retentates were then placed in amber glass jars and allowed to equilibrate to proper temperature in either a hot water bath (32 or 60°C) or an ice bath (4°C). Hydrogen peroxide (35% w/v, Nelson Jameson, Inc., Marshfield, WI) was added to activate the peroxidase system (4°C:10 mg/kg; 32 and 60°C:20 mg/kg). Aliquots of samples were removed at appropriate time points (0, 3, 5, 7, 10, 15, 20, 30, 40, 50 min, 1, 2, 4, 6, and 24 h) for norbixin extraction.

Norbixin Extraction and Quantification (Experiment 2)

Norbixin is the primary carotenoid in water soluble annatto extracts and was extracted and measured to determine percent annatto destruction and bleaching efficacy (Kang et al., 2010). Norbixin was extracted and quantified using HPLC by the method described by Campbell et al. (2012). To extract fluid wheys, 200ul was placed into a 2 mL microcentrifuge tube (VWR International, West Chester, PA). To this, 800ul of dilution solution (80% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/20%

water (EMD Chemicals, VWR International, West Chester, PA with 0.1% (w/v) formic acid (EMD Chemicals, VWR International, West Chester, PA)) was added. The solution was vortexed and centrifuged (14,000 x g, Microfuge® 18 Centrifuge, Beckman Coulter, Brea, CA). The supernatant was removed and placed into vials for quantification by HPLC. To extract retentate, 100ul of sample was placed into a 2 mL microcentrifuge tube. To this, 900ul dilution solution was added and the sample was vortexed and centrifuged as previously described. The supernatant was removed and placed into vials for quantification by HPLC. 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).

Quantification was conducted using HPLC (Waters 1525 Binary Pump, Waters, Milford, MA). Isocratic mobile phase (70% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/30% water (EMD Chemicals) with 0.1% (w/v) formic acid (EMD Chemicals) was used at a flow rate of 1mL/min pumped through a binary pump (Waters 1525, Waters, Milford, MA). 50 microliters of the sample was injected (Waters 2707 Autosampler) onto the column (Phenomenex Kinetex 2.6µm particle size, 10 cm length, 4.6mm inner diameter, 100Å pore size) which was heated to 40°C. The injector temperature was set to 4°C. Sample was sent through a photodiode array detector (Waters 2998). A standard curve was created by rehydrating norbixin powder (45% (w/v), Chr. Hansen, Milwaukee, WI) in 2.5% (w/v) potassium hydroxide (BDH, VWR International, West Chester, PA) and then diluting in mobile phase. The maxima used for calculation was

482 nm. Norbixin concentration was calculated by total solids and correction for dilution during the extraction.

Statistical Analysis

For experiment 1, a two way analysis of variance (ANOVA) comparing slopes was completed to compare LP and SCN in fluid milks, wheys and retentates with storage (XLStat 2010, Addinsoft, New York, NY). The main factors: storage condition and storage time were analyzed along with their potential interaction effects. Tukey's HSD was used as a post-hoc test.

For experiment 2, SAS 9.2 was used to analyze the slope data (SAS, Cary, NC). A mixed model (PROC MIXED) was used with replication and replication by temperature random effects in the model, in addition to fixed effects due to linear and quadratic terms for temperature and pH and the product of temperature and pH. Predicted values from the response fitted response surface model were plotted using PROC G3D. A mixed model (SAS 9.2, Cary, NC) was used to evaluate the bleaching experiment, which was conducted using a split-split plot design. Treatment was the whole plot factor, matrix was the subplot factor, and enzyme was the sub-subplot factor. Treatment, matrix, enzyme and all interactions were included in the model as fixed effects. Spearman's rank correlation coefficient (Proc Corr, SAS) was used to determine the correlation between activity level and bleach time.

RESULTS AND DISCUSSION

Experiment 1:

Composition of milk and wheys did not differ regarding the solids, protein and fat contents. Milks averaged $12.2\pm 0.2\%$ solids, $3.39\pm 0.01\%$ protein, and $3.70\pm 0.05\%$ fat. Fluid wheys averaged $6.71\pm 0.05\%$ solids, $0.072\pm 0.01\%$ protein, and $0.04\pm 0.01\%$ fat. Retentates averaged $12.7\pm 1.02\%$ solids, $10.4\pm 0.6\%$ protein, and $0.95\pm 0.09\%$ fat. All measurements are reported on a wet weight basis.

LP activity values for all samples (raw milk, pasteurized milk, fat separated pasteurized liquid whey, and fluid retentate (80% protein)) did not decrease with storage time (0, 24, 48, or 72 h) or storage conditions (refrigerated or frozen storage). Mullan et al. (1980) investigated the storage stability of lactoperoxidase in calf feed in order to reduce calf mortality and improve calf performance. These authors reported that LP stability was highly variable and in some cases, not stable in Cheddar cheese whey, losing as much as 50% activity over 3 days at 5°C, however, this behavior was not consistent. In some experiments, enzyme levels were stable for 3 days, consistent to results found in this study. The rate of decrease in enzyme activity may be due to several factors: qualitative and quantitative nature of microbial flora, coagulant enzyme used, storage temperature of whey, and pH (Mullan et al., 1980). Peroxidase enzyme activity levels in whey protein concentrates (11.29% solids, 9.32% protein) were stable for at least 4 months when stored at -30°C (Mullan et al., 1980), once again, in agreement with the present study.

Milk pasteurization did not impact LP activity (raw milk slope 0.214, pasteurized milk slope 0.213). The present study utilized LTLT pasteurization and thus it is expected

from previous studies that this pasteurization step would not affect LP activity in milk. The effect of heat treatment on bovine lactoperoxidase activity has been widely studied. Marin et al. (2003) found that when mild heat treatments were applied (68C for 15min or 72C for 2min), more than 90% of the LP activity was retained. LP is more resistant to long treatments at low temperatures than short treatments at high temperatures (Marin et al., 2003).

Fluid whey exhibited a higher LP activity slope than fluid milks, averaging 1.59 and 0.214 respectively (Figure 1). Previous studies have reported higher activity in wheys than milk (Seifu et al., 2005; Campbell et al., 2012). Retentate had the highest LP activity slope of all samples tested ($p < 0.05$) with slopes averaging 9.08 (results not shown). It is expected that retentates would have higher activity values as the LP is being concentrated during the ultrafiltration process.

Experiment 2:

Composition did not differ among replications. Fluid whey averaged $6.71 \pm 0.05\%$ solids, $0.071 \pm 0.01\%$ protein, and $0.03 \pm 0.01\%$ fat. Retentates averaged $10.6 \pm 0.45\%$ solids, $8.80 \pm 0.20\%$ protein, and $0.67 \pm 0.05\%$ fat. All measurements are reported in a wet weight basis.

Temperature, regardless of matrix, influenced LP activity. In fluid whey with and without added exogenous enzyme, and in retentate with added exogenous enzyme at the various levels tested, the temperature effect was more pronounced than the pH effect (Fig 2, 3, 4, 5, 6, 7, 8). In a previous study conducted in bovine milk and diluted whey, LP was optimally active at approximately 50°C (Ludikhuyze et al., 2001). Peroxidase activity in

retentate, without the addition of exogenous peroxidase, was more thermostable at more neutral pH (Figure 9). This behavior is very similar to horseradish peroxidase (HRP) which also exhibits more thermostability at neutral pH (Lemo et al., 2000). HRP is widely studied, arguable the most studied peroxidase enzyme, with numerous studies agreeing that optimum thermostability occurs at pH 7 (Lemos et al., 2000). The effect of pH and the medium all influence the thermal stability of the enzyme being studied. Both bovine LP and exogenous peroxidase are rather thermostable and highly resistant to pressure (Ludikhuyze et al., 2001; Puhse et al., 2009). Exogenous peroxidase (Maxibright™) has an optimal activity around 55-60°C (Puhse et al., 2009). Above 65°C, the enzyme begins to have conformational changes and above 78°C, enzyme activity decreases abruptly (Puhse et al., 2009). Lactoperoxidase, like exogenous peroxidase, experiences a sharp decline in activity around 78-80°C (Seifu et al., 2005; de Wit and van Hooydonk, 1996). The three main processes considered to be involved in thermal inactivation of peroxidases are 1) dissociation of the prosthetic (heme) group from the active enzyme system, 2) conformation change in the apoenzyme, and 3) modification or degradation of the prosthetic group (Lemos et al., 2000). Knowing the optimal activity temperature for both of these enzymes will help to ensure that optimal bleaching occurs during whey processing.

The pH optimum for the LP enzyme is highly dependent upon the particular electron donor (Pruitt et al., 1983). The most obvious choice of electron donor in the case of LP in bovine milk is SCN, as it is naturally present in milk. Previous studies have shown that myeloperoxidase (a peroxidase enzyme found in human mucus) and lactoperoxidase are similar in mechanism, with both enzymes able to carry out the peroxidation of SCN over a

broad pH range (Wever et al., 1982). The kinetics of the peroxidation catalyzed by LP are complex, with HP and SCN acting not only as substrates but also as competitive inhibitors with respect to each other (Wever et al., 1982). A study conducted by Wever et al. (1982) demonstrated that the position of the pH optima was a linear function of the logarithm of the ratio of [SCN]/[HP]. As the ratio increased, so did the pH optima. In a study conducted not long after Wever et al., Pruitt et al. (1983) confirmed that the LP system was highly dependent on both the relative and absolute values of SCN and HP concentrations. At varying equimolar concentrations of SCN and HP, Pruitt et al. (1983) demonstrated that as SCN and HP decreased, the pH optima increased. For example, when SCN and HP were at equimolar concentrations in the range of 0.5 to 2mM, the pH optimum was near 6.0. When SCN was in large excess (10mM), the pH optimum was near 6.7. Dependence of pH was similar for both the human saliva system and for the bovine LP system with the optimum in the pH range of 6.5-7 in PBS (phosphate buffered saline) buffer solution (Pruitt et al., 1983).

The pH optima of bovine LP in fluid whey and retentate were very different from each other. In fluid whey, the pH optimum was much lower than in retentate (Fig 5 and 9). Exact reasons for this are unknown, however, from previous literature, it can be speculated that SCN concentration and HP concentration heavily influence the pH maxima. As fluid whey is ultrafiltered and diafiltered to retentate, permeate consisting of lactose, minerals, and other small particles (less than 10Kda) is removed. Thiocyanate is among one of these constituents which is removed, thus decreasing the [SCN]/[HP] ratio and undoubtedly causing the pH optimum to shift. Thiocyanate levels in fluid whey averaged 4.7 ppm while retentate levels averaged 3.9 ppm ($p < 0.05$). When exogenous enzyme was added, the pH

optimums of fluid whey and fluid retentate were very similar (Figures 2, 3, 4, 6, 7, 8). Activity values in retentate without the addition of exogenous enzyme were opposite with regards to pH; as pH increased, activity increased (Figure 9). An increase in temperature also increased enzyme activity; however, pH had a much greater effect on enzyme activity than temperature (Figure 9).

Activity is a quick, easy, and inexpensive way to measure enzymatic activity, however, practically, it is important to understand if and how activity relates to norbixin destruction (bleaching based on time to >80% norbixin destruction). Three points were selected along each activity curve to further elucidate the relationship between bleaching, enzyme activity and bleach time (Figure 10).

Across all treatments, retentate or liquid whey and various levels of EP, activity increased with increasing temperature. Norbixin analysis demonstrated that the speed of norbixin destruction (bleaching based on time to >80% norbixin destruction) also increased with increasing temperature. In liquid whey, the speed of bleaching increased with the addition of EP at all temperatures (4°C, 32°C, 60°C) (Figure 10). In retentate, EP increased the speed of bleaching only at 4°C and 32°C and these effects were only observed at the higher levels of added EP (1 and 2 DBLU). Increased activity did not reflect increased bleaching with the addition of EP in retentate. In fluid whey, activity and bleaching time were negatively correlated ($r = -0.73$; $p < 0.05$), thus, as activity increased, the time needed to bleach (>80% norbixin destruction) decreased. In retentate, there was no correlation found between activity and bleaching ($r = -0.23$). This result suggests that the relationship between enzymatic activity and bleaching is not linear in retentate. However, retentate, even without

the addition of EP, bleached much faster than fluid whey (for example, at 32°C, >80 percent norbixin was destroyed in 37 min in fluid whey versus 7 min in retentate) and thus it may not be possible to accurately measure bleaching in such a short time frame (<3 min) and a correlation may exist but due to equipment limitations, it may not be possible to detect.

CONCLUSION

LP activity following 0, 24, 48 or 72 h storage at 4 or -20°C, regardless of matrix (milk, whey, or retentate) did not decrease. In fluid whey, regardless of added exogenous enzyme, as pH decreased (from 6.5 to 5.5) and temperature increased (from 4 to 60 °C), enzyme activity increased. In fluid retentate, added exogenous enzyme made a significant difference in the optimal parameters for enzyme activity. LP activity in retentate without additional enzyme increased as pH (from 5.5 to 6.5) and temperature increased (from 4 to 60 °C). Enzyme activity behavior in retentate with the addition of exogenous enzyme demonstrated that as pH decreased (from 6.5 to 5.5) and temperature increased (from 4 to 60 °C), so did enzyme activity. Enzymatic bleaching is complex and an increase in peroxidase activity did not necessarily correlate to an increase in bleaching. Knowing the proper parameters can help dairy processors further optimize enzymatic bleaching in fluid whey and retentate.

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TABLES AND FIGURES

Table 1: Response surface model design using two factors (pH and temperature).

Data Point	pH	Temperature (C)
1	5.50	32
2	5.65	12
3	5.65	52
4	6.00	4
5	6.00	32
6	6.00	32
7	6.00	60
8	6.35	12
9	6.35	52
10	6.50	32

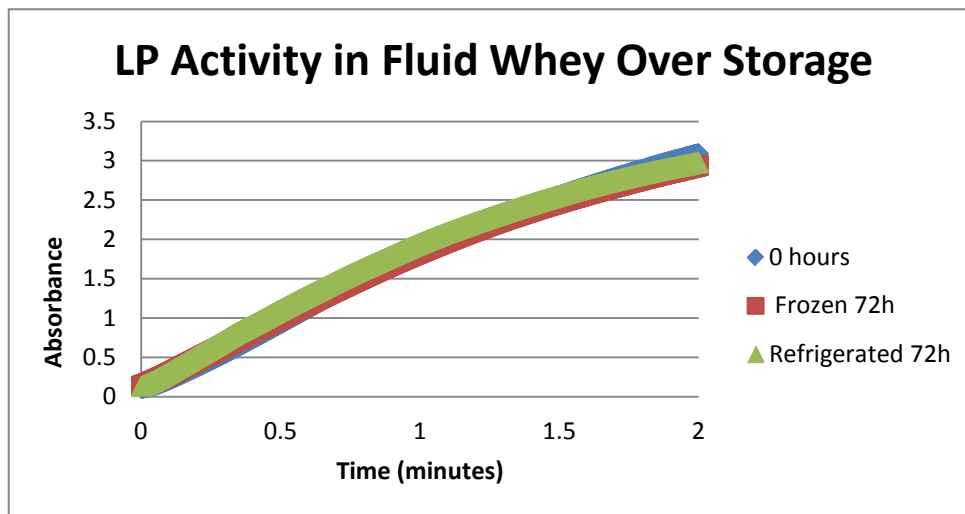


Figure 1: Lactoperoxidase (LP) activity in fluid whey over 72h of refrigerated or frozen storage using IDF method (Pruitt and Kamau, 1994) to determine activity.

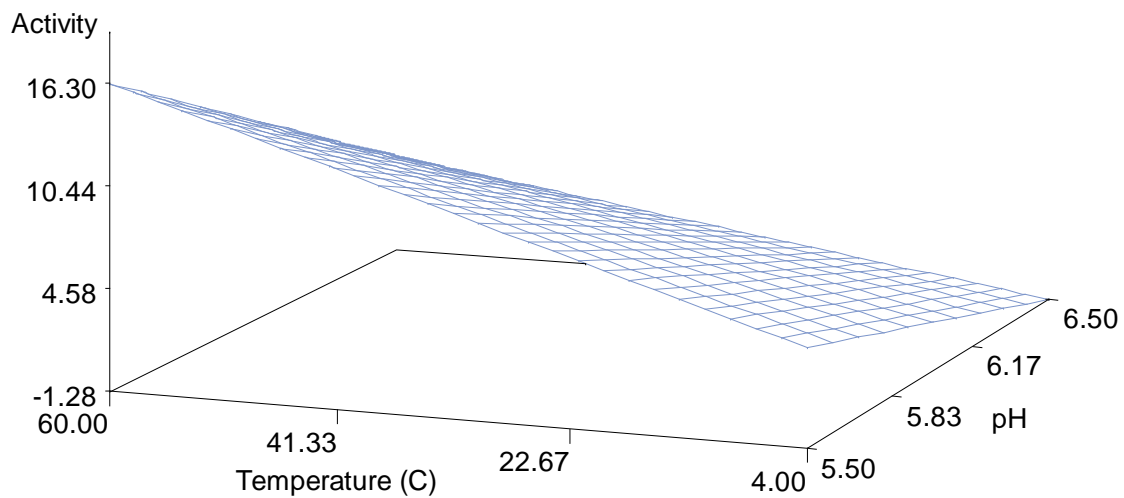


Figure 2: Three dimensional model displaying the effects of temperature and pH on fluid whey with added exogenous enzyme (2 Dairy Bleaching Units) on enzyme activity

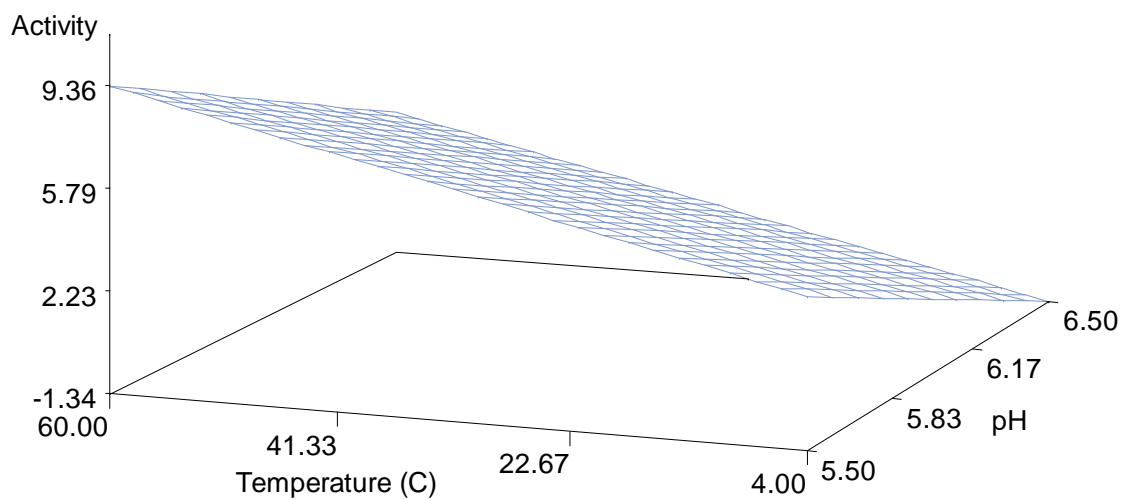


Figure 3: Three dimensional model displaying the effects of temperature and pH on fluid whey with added exogenous enzyme (1 Dairy Bleaching Unit) on enzyme activity

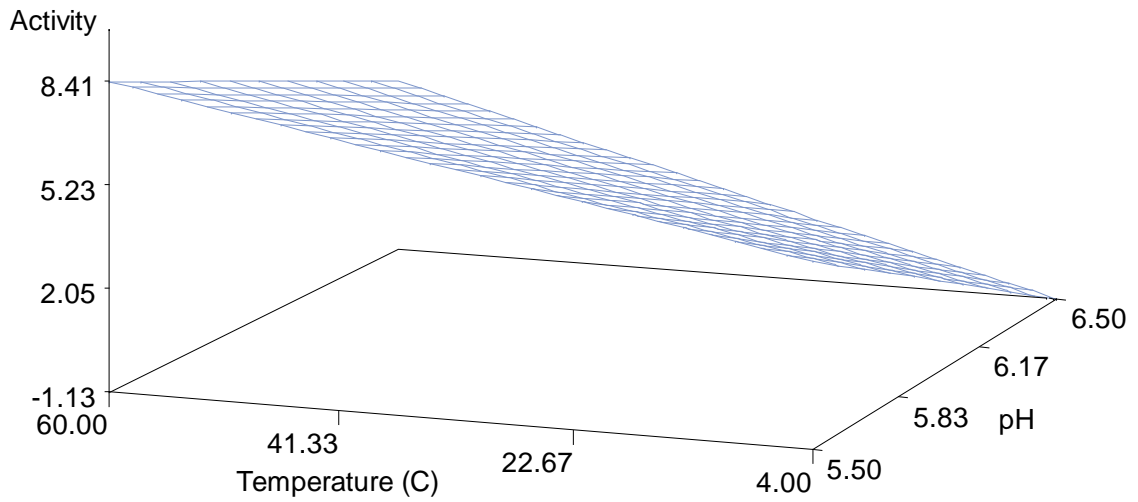


Figure 4: Three dimensional model displaying the effects of temperature and pH on fluid whey with added exogenous enzyme (0.5 Dairy Bleaching Units) onenzyme activity

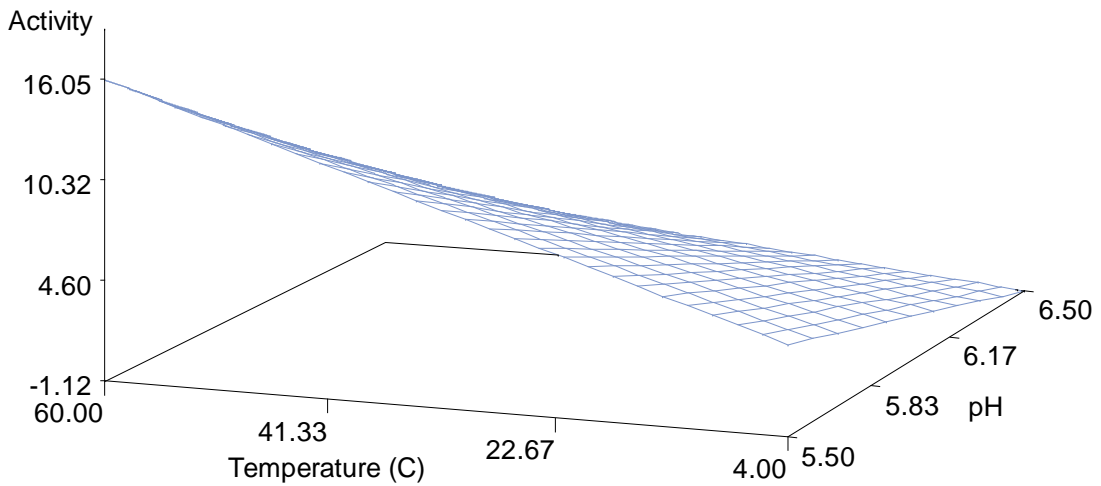


Figure 5: Three dimensional model displaying the effects of temperature and pH on fluid whey without added exogenous enzyme (lactoperoxidase only) on enzyme activity

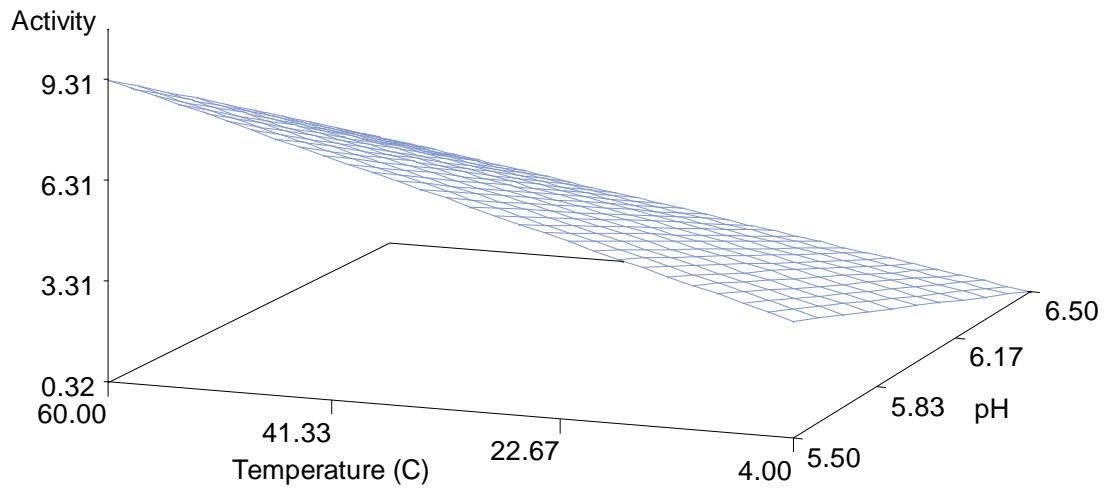


Figure 6: Three dimensional model displaying the effects of temperature and pH on retentate with added exogenous enzyme (2 Dairy Bleaching Units) on enzyme activity

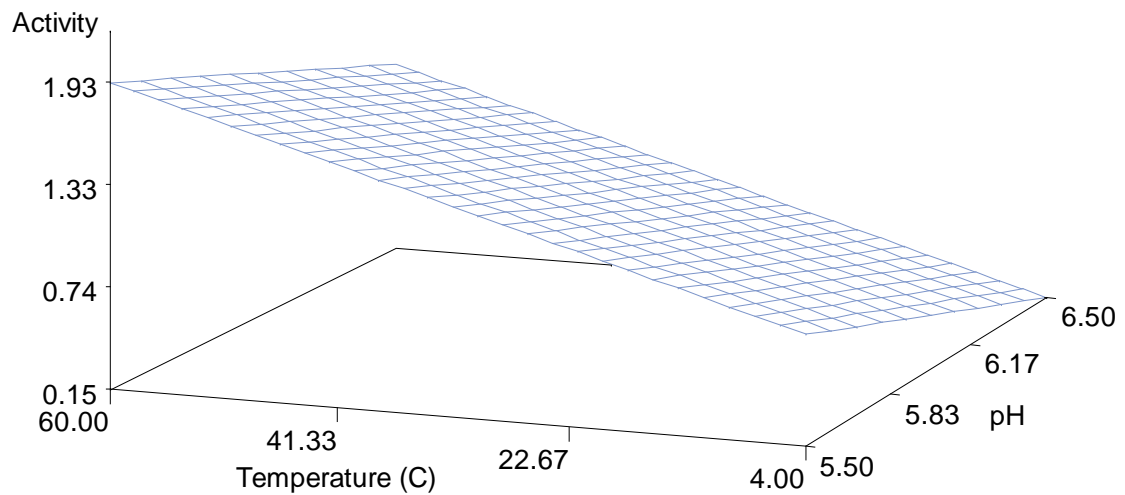


Figure 7: hree dimensional model displaying the effects of temperature and pH on retentate with added exogenous enzyme (1 Dairy Bleaching Unit) on enzyme activity

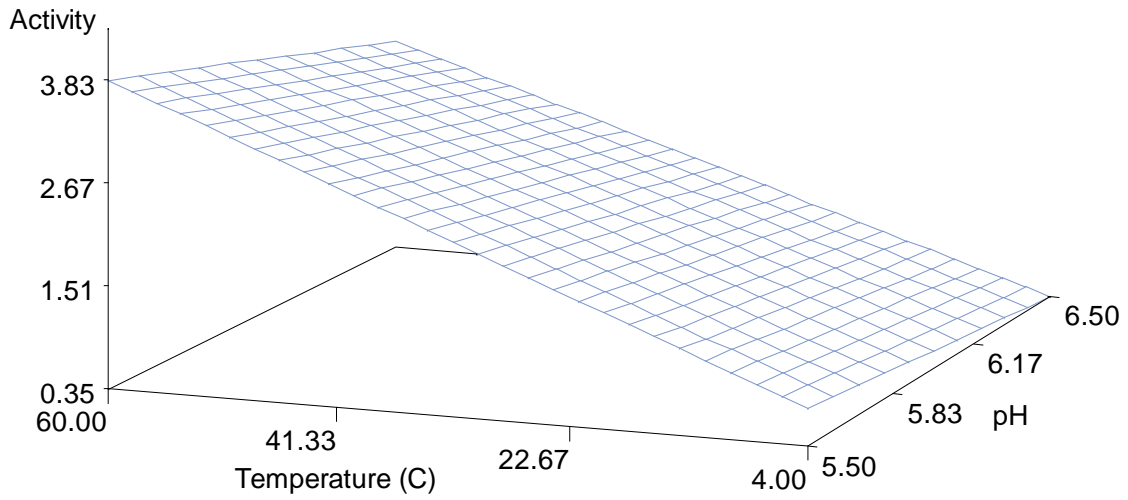


Figure 8: Three dimensional model displaying the effects of temperature and pH on retentate with added exogenous enzyme (0.5 Dairy Bleaching Units) on enzyme activity

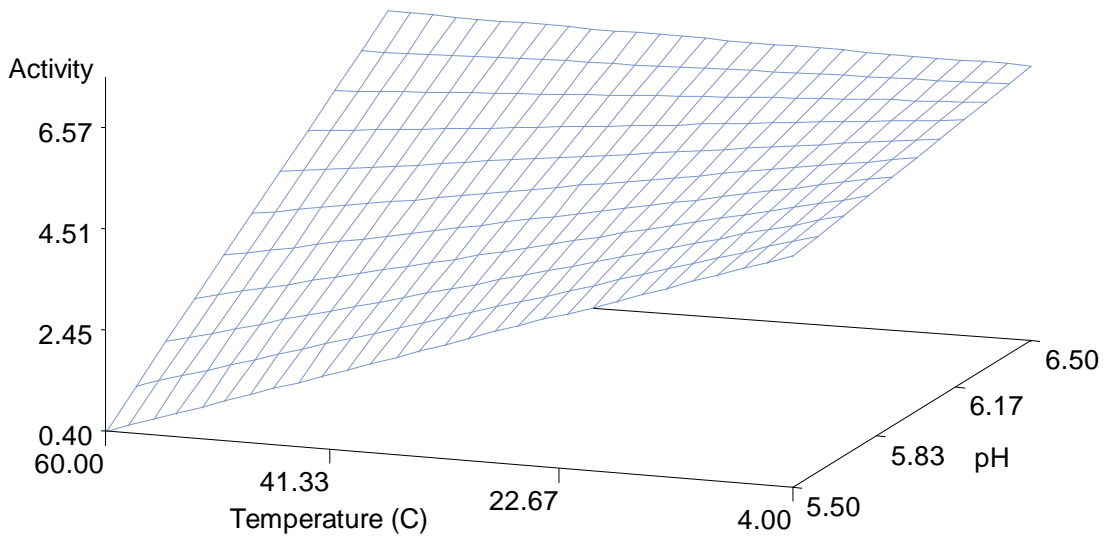


Figure 9: Three dimensional model displaying the effects of temperature and pH on retentate without added exogenous enzyme (lactoperoxidase only) on enzyme activity

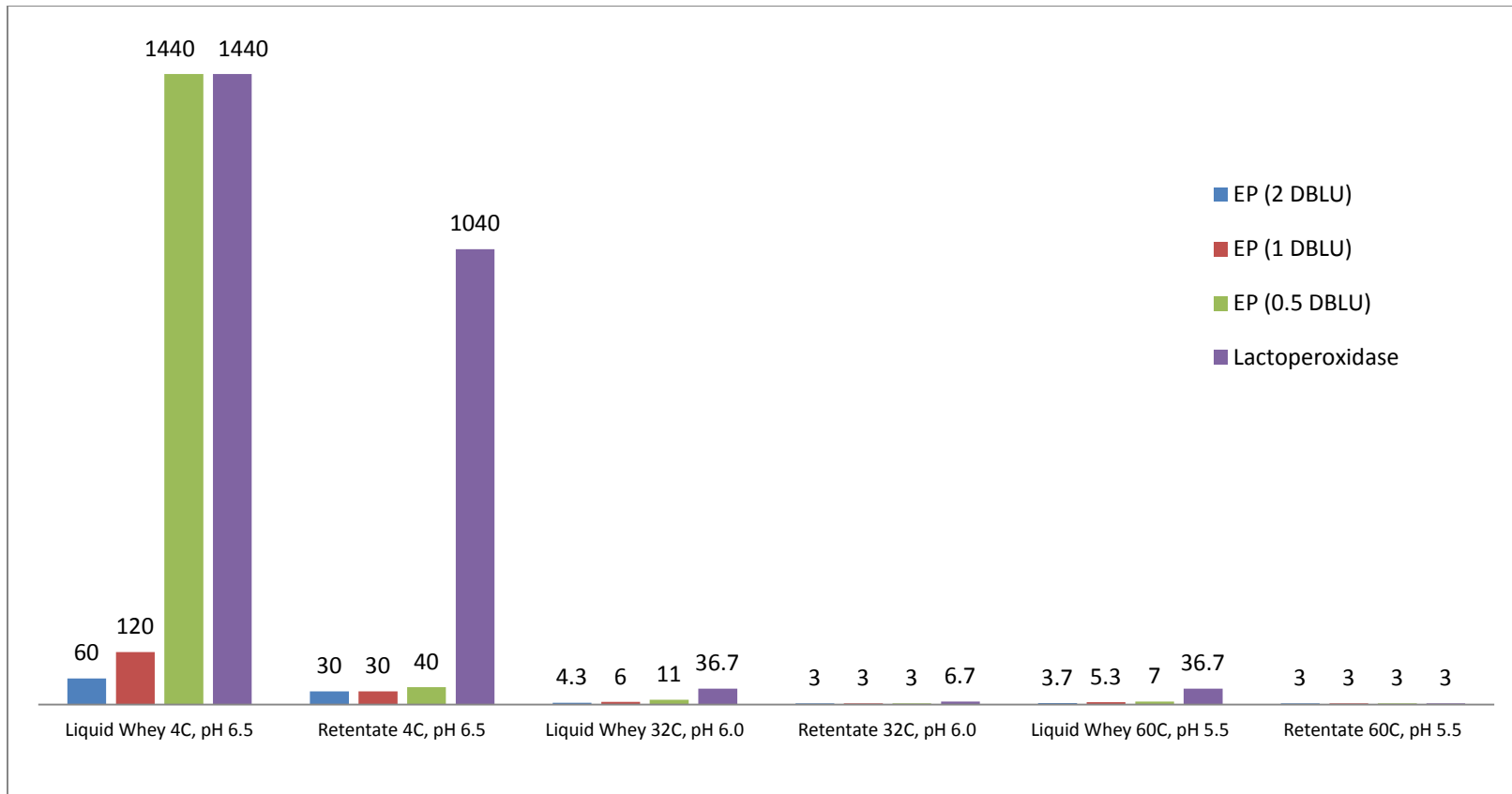


Figure 10: Amount of time in min (y axis) to bleach >80% norbixin using lactoperoxidase with or without added exogenous peroxidase (EP) in fluid whey or retentate (10% solids, 80% protein) at various temperatures and pH

Short Communication

Development of a Novel Method for the Extraction of Norbixin from Whey and its Subsequent Quantification via High Performance Liquid Chromatography

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ABSTRACT

Norbixin is the primary carotenoid in annatto coloring which imparts desired orange color in Cheddar cheese. A portion of the colorant remains in the cheese whey and is undesirable. A bleaching step is often applied. There are also legal limits for norbixin concentrations in products destined for infant formula. As such, evaluation of norbixin concentrations in whey and whey ingredients is desirable. Current extraction methods are laborious and require solvents that are banned in many countries. The objective of this study was to develop a fast and inexpensive norbixin extraction and quantitation technique using approved solvents with similar sensitivity to current established methods.

Instead of solvent extraction and column purification, acetonitrile was added directly to fluid wheys, retentates, and rehydrated whey protein concentrates. An isocratic mobile phase (70% acetonitrile/30% water with 0.1% (w/v) formic acid) was used and to increase sensitivity, a large volume (50 μ l) was injected onto the column. The column used was a C18 column, with a particle size of 2.6 μ m and column length of 10 cm. The column inner diameter was 4.6 mm and the pore size was 100 Å . All of the previously described conditions allowed the run time to be only 4 min. Sample was sent through a photodiode array detector and quantified at 482nm. Norbixin was quantified using external standard curves. The developed method had a >90% norbixin recovery percentage in both milk and whey (9.39 μ g/L – 2.35 mg/L). The limit of detection (LOD) of norbixin in fluid whey was 2.7 ppb and the limit of quantitation (LOQ) was 3.5 ppb, both of which are significantly lower than in previously described methods. The extracts were stable over 30 min at room temperature and stable over 24 h in the injector at 4C ($p > 0.05$). Repeatability and precision

of the method had relative standard deviations of less than 13%. The developed method provides time and cost savings for evaluation of norbixin concentration in whey and whey products.

Key Words: Norbixin, annatto, whey, HPLC

INTRODUCTION

Cheddar cheese is often colored with annatto, a yellow-orange carotenoid primarily comprised of norbixin, to impart the desired orange color (Kang et al., 2010). A portion of the colorant remains in the cheese whey and is undesirable (Kang et al., 2010). In order to remove the color, a bleaching step is often applied. In the United States there are two chemicals approved for the bleaching of whey: hydrogen peroxide and benzoyl peroxide. Due to increasing regulations regarding these two bleaching agents, various alternative bleaching agents have been investigated (Kang et al., 2012; Campbell et al., 2012). In addition, there are also legal limits for norbixin concentrations in products destined for infant formula. As such, evaluation of norbixin concentrations in whey and whey ingredients is desirable.

High performance liquid chromatography (HPLC) is a highly sensitive technique used to separate and subsequently quantify non-volatile components. Carotenoids are often measured using a photodiode array (PDA) detector which measures absorbance of eluent components at a certain wavelength. The maximum wavelength for norbixin absorbance is 482nm (Kovary et al., 2001). Since HPLC does not require large sample volumes, it is a very versatile technique which can be applied to a wide array of products from food to pharmaceuticals to environmental contaminants.

Current extraction methods for norbixin are time intensive, laborious, and expensive (Lancaster and Lawrence, 1995; Scotter et al., 2002; Croissant et al., 2009) (Table 1). In addition, current extraction methods require solvents that are banned in many countries. The objective of this study was to develop a fast and inexpensive extraction technique using

approved solvents with similar sensitivity to current established methods. Linearity, stability, and repeatability were measured to insure the new extraction and quantification method were equally robust as current methods.

METHODS

Production of colored milk, fluid whey, fluid retentate and WPC80

Cheddar whey was manufactured from vat pasteurized whole bovine milk (195 kg) as described by Campbell et al. (2011). To color the milk, annatto was added at a rate of 15 mL/454 kg milk (Danisco, St. Louis, MO; 3% norbixin w/v). The whey was drained from the curds at pH 6.35 and a sieve was used to remove any remaining particles. The whey was immediately processed with a hot bowl cream separator (Model SI600E, Agri-Lac, Miami, FL) to reduce the fat content. The whey was pasteurized at 63°C for 30 min. A portion of fluid whey was removed for bleaching. Two different bleaching treatments were applied: hydrogen peroxide (HP) and lactoperoxidase (LP). For hydrogen peroxide (HP) chemical bleaching, 250 ppm HP were added to liquid whey and allowed to react for 1 h at 50°C with gentle agitation. The concentration of HP was selected since it represents the mid-range of the legally allowed amount of HP for traditional chemical bleaching of whey and also represents a concentration that is generally applied by industry (Kang et al., 2010; Listiyani et al., 2011). Catalase (20 mg/kg, FoodPro CAT, Danisco, New Century, NJ) was added at a rate of 20 ppm to remove excess HP and stop the bleaching process. For lactoperoxidase (LP), 20 ppm HP was added to fluid whey to activate the enzymatic system and allowed to bleach for 1h at 50°C with gentle agitation. Catalase was added to remove any excess HP and stop the enzymatic bleaching process.

The remainder of the fluid whey (unbleached) was transferred into a 102 liter stainless vat (FermentorTM, Blichmann EngineeringTM, Lafayette, IN) equipped with a coil heater (1/2" outer diameter, PAC Stainless LTD, Seattle, WA) and cooled to 50°C before ultrafiltration and diafiltration commenced as described by Campbell et al. (2012). This process continued until the retentate reached 80% protein (w/v) content confirmed by a SprintTM rapid protein analyzer (CEM, Matthews, NC). The retentate was then collected and spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark).

Norbixin Extraction Procedure

Cheese milk

To extract norbixin from milk, 1 gram of colored cheese milk (15 mL annatto/454 kg milk) was added to into a 25 mL volumetric flask and filled to volume with dilution solution (80% acetonitrile and 20% water (EMD Chemicals, VWR International, West Chester, PA) with 0.1% (w/v) formic acid (EMD Chemicals). A magnetic stirrer was added and the solution was allowed to mix in the dark for 10 min. Two mL of the sample was then transferred into an Eppendorf tube (VWR International, West Chester, PA) and placed in the microcentrifuge at 14,000 x g for 5min at room temperature. The clear supernatant was removed and placed into an amber vial for injection (Phenomenex, Torrance, CA).

Whey before bleaching

To extract fluid whey, 2 grams of fluid whey was added to into a 25 mL volumetric flask and filled to volume with dilution solution (80% acetonitrile and 20% water (EMD Chemicals) with 0.1% (w/v) formic acid (EMD Chemicals). The extraction was then carried out as previously described.

Whey after bleaching

To extract fluid whey after bleaching, 2 grams of bleached whey was added to into a 10 mL volumetric flask and filled to volume with dilution solution (80% acetonitrile and 20% water (EMD Chemicals) with 0.1% (w/v) formic acid (EMD Chemicals). The extraction was then carried out as previously described.

WPC80 powder

One gram of powder was first weighed into a 10 mL volumetric flask. HPLC grade water was added to volume (EMD Chemicals). A magnetic stirrer was added and the solution was allowed to mix in the dark for 10 min. Two grams of this whey solution was placed in another 10 mL volumetric flask and filled to volume with dilution solution (80% acetonitrile and 20% water (EMD Chemicals) with 0.1% (w/v) formic acid (EMD Chemicals). The extraction was then carried out as previously described.

Chromatographic Conditions and Norbixin Quantitation

The liquid chromatographic system (Breeze HPLC, Waters, Milford, MA) used consisted of a 1525 binary pump, 2707 automatic injector, 1500 series column heater and a 2998 photodiode-array detector. Isocratic mobile phase (70% acetonitrile (EMD Chemicals)/30% water (EMD Chemicals) with 0.1% (w/v) formic acid (EMD Chemicals) was used at a flow rate of 1mL/min pumped through a binary pump (Waters 1525, Waters, Milford, MA). Fifty microliters of the sample were injected (Waters 2707 Autosampler) onto the column (Phenomenex Kinetex 2.6 μ m particle size, 10 cm length, 4.6mm inner diameter, 100 \AA pore size) which was heated to 40°C. The injector temperature was set to 4°C. Sample was sent through a photodiode array detector (Waters 2998). The maxima

used for calculation was 482 nm. Sample run time was 3min. An external standard (Double Strength Annatto (3% Norbixin), Danisco, St. Louis, MO) was used for calibration. Calibration curves were obtained by plotting peak areas of norbixin in solvent (70% acetonitrile (EMD Chemicals)/30% water (EMD Chemicals) with 0.1% (w/v) formic acid (EMD Chemicals) against concentrations of norbixin injected. Norbixin eluted at approximately 2 minutes and an example chromatogram can be seen in Figure 2.

Limit of Detection and Limit of Quantitation

There are several different approaches to determine limit of detection (LOD) and limit of quantitation (LOQ). In this study, LOD and LOQ were determined based on the norbixin concentration level that generated a signal to noise (S/N) ration of 3 and 6, respectively, by adding decreasing concentrations of norbixin to fluid whey samples. The samples were injected 6 times and the relative standard deviation (RSD) was below 10%.

Linearity

Norbixin was quantitated in dilution solution (blank), fluid whey, and milk matrices over a wide range (0-120 ug/L) of concentrations. Linearity was determined using linear regression.

Repeatability/Intermediate Precision

On 6 different days, with two different technicians, norbixin was extracted from cheese milk, whey before bleaching, whey after bleaching and WPC80 retentate and quantified to ensure that the method and results were consistent. Extraction and quantitation were carried out as described previously.

Extraction Efficacy

Three different levels of norbixin were spiked into either milk or fluid whey (Table 3) and extracted as described previously to determine norbixin recovery during extraction. Recovery was measured in quadruplicate.

Sample Stability

Sample solution stability at room temperature: Sample extracts for cheese milk, whey before and after bleaching, and WPC80 powder were prepared as previously described. Small aliquots were removed at an interval of 5 min for 30 min under continuous stirring in the dark at room temperature. Aliquots were then centrifuged at 14,000 x g for 5 min. The supernatant was removed and placed on the HPLC for injection.

Sample stability in injector: Sample extracts for cheese milk, whey before and after bleaching, and WPC80 powder were prepared as described previously. Samples were injected for 24 h with an interval of 4 h.

Statistical Analysis

Linear regression was calculated using Excel (Microsoft Excel, Redmond, WA). Analysis of variance (ANOVA), which was used to calculate the repeatability and intermediate precision. Other statistical analyses, including relative standard deviation, were conducted using Statgraphics (StatPoint Technologies, Inc., Warrenton, VA).

RESULTS AND DISCUSSION

Limit of Detection and Limit of Quantitation

The limit of detection of norbixin in fluid whey was 2.7 ppb and the limit of quantitation of norbixin in fluid whey was 3.5 ppb. Determination of LOD and LOQ were

conducted in fluid whey as matrix will affect the clarity of the chromatogram slightly. LOD and LOQ of norbixin determined in dilution solution were 2.0 and 3.0 ppb respectively and were lower compared to the LOD and LOQ determined in matrix ($p < 0.05$). The LOD and LOQ reported in this study were lower than current methods by about 50 fold. A previous norbixin quantification method using HPLC (Scotter et al., 2002) reported a LOD in analyte of 10 ppb but depending on the food commodity analyzed, the LOD was about 100 ppb. The LOQ calculated from an analyte peak was 100 ppb (Scotter et al., 2002). Another published method determining annatto via HPLC in high-fat dairy products such as cheese and butter reported an LOQ of 100 ppb to 1 ppm depending on the dairy product (Lancaster and Lawrence, 1995).

Linearity

Norbixin was linear in both the dilution solution (blank) and in fluid whey and milk matrices over a wide range (0-120 ug/L) (Figure 3). Linearity was determined using linear regression and R^2 values were >0.99 indicating norbixin can be extracted over a range of values.

Repeatability/Intermediate Precision

The repeatability and precision of the method was determined by between day and within day analysis expressed by the relative standard deviation (Table 2). The experiment was conducted over 6 days with one set of samples (cheese milk, fluid whey, retentate, and bleached wheys) evaluated each day for a total of 6 replications. The method precision was $RSD < 13\%$ for norbixin in all matrices. The repeatability within day ($RSD < 3\%$) and between days ($RSD < 13\%$) was acceptable in all matrices. Previous studies determining

norbixin in various matrices reported standard deviations of 1% (corn snack products; de Oliveira Rios and Mercadente, 2004), 6% (processed foods; Breithaupt, 2004), and 13% (meat, Noppe et al., 2009).

Extraction Efficiency

Recovery of norbixin from spiked milk and whey was >90% over a range of 9.39 ug/L to 2.35 mg/L (Table 3). Recovery percentage for norbixin from both spiked milk and whey were similar. The recovery percentage was well within those previously reported. Previous studies found a wide range of norbixin recoveries depending on the method and matrix used. Noppe et al. (2009) was able to recover between 99 and 102% of norbixin in meat tissue while de Oliveira Rios and Mercadante (2004) reported 97% recovery of annatto in extruded corn snack products. In dairy products, recovery rates of 80-100% (Bareth et al., 2002), 93.2% (Lancaster and Lawrence, 1995), and 75-96% (Scotter et al., 2002) have been reported. It is important to note that these methods did not measure norbixin in milk or whey but rather in other dairy products such as yogurt, cheese, custard, ice cream, and butter. In milk, the quantification of beta-carotene, a carotenoid similar to norbixin, has been widely studied. Recovery of beta-carotene from milk has been reported to be anywhere from 70% (Chauveau-Duriot et al., 2010) to 92.6% (Liu et al., 1998).

Sample Stability

At room temperature, milk, whey and powder extracts were stable over 30 min ($p>0.05$; results not shown). The stability of milk, whey and powder extracts were also tested over 24 h in the sample injector. The extracts remained stable over 24 h further proving the robustness of the method ($p>0.05$; results not shown).

CONCLUSION

The developed method provides a fast and inexpensive norbixin extraction and quantitation technique with better sensitivity than current methods. In addition, solvent waste is greatly reduced compared to current methods and only approved solvents are used so the method can be used in countries with stricter chemical regulations.

ACKNOWLEDGEMENTS

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TABLES AND FIGURES

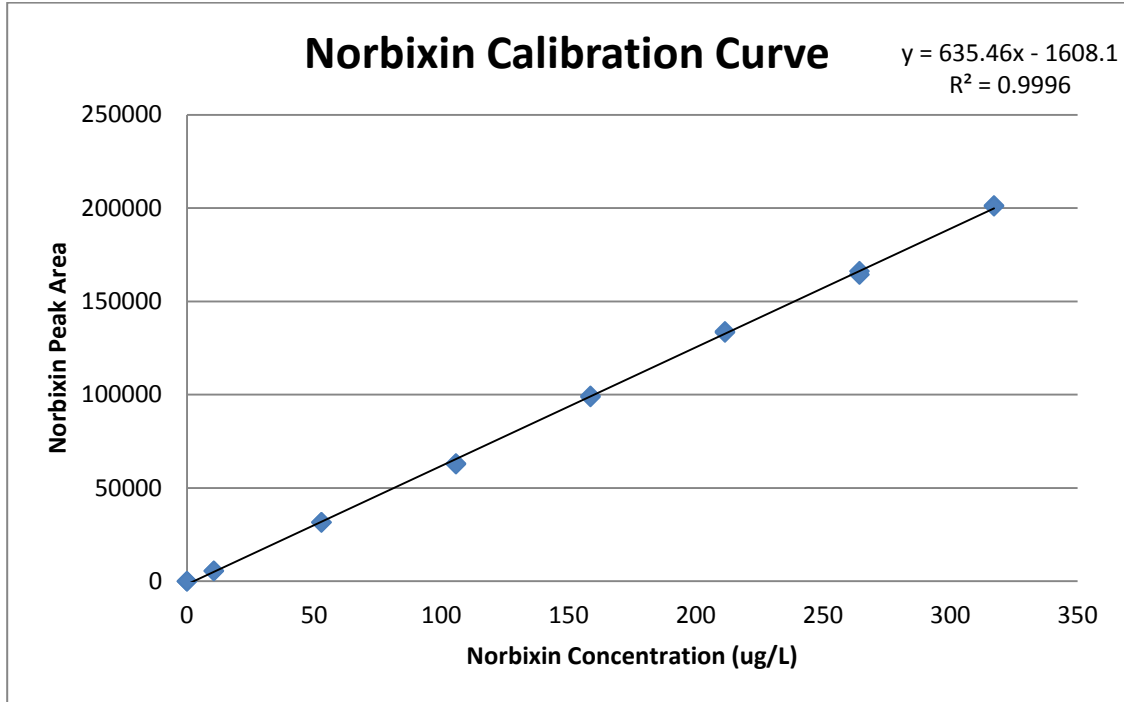


Figure 1. Example calibration curve for norbixin

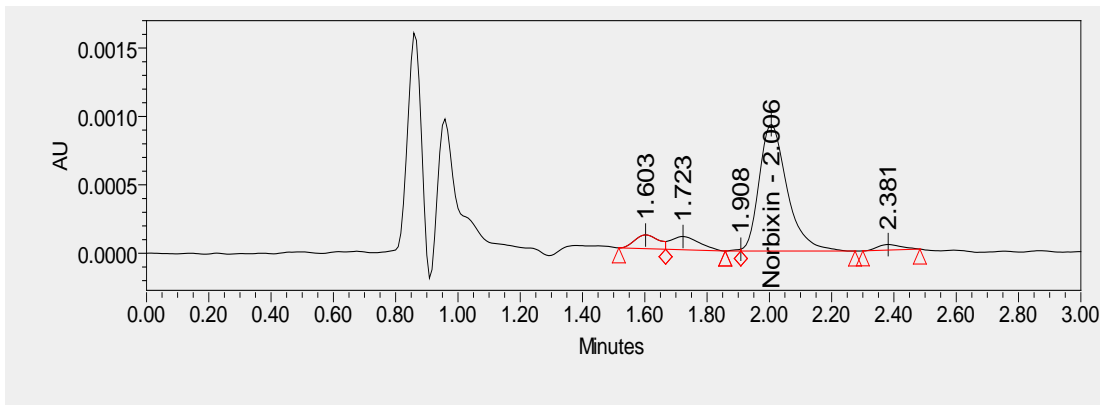


Figure 2. Example chromatogram of norbixin extracted from fluid whey

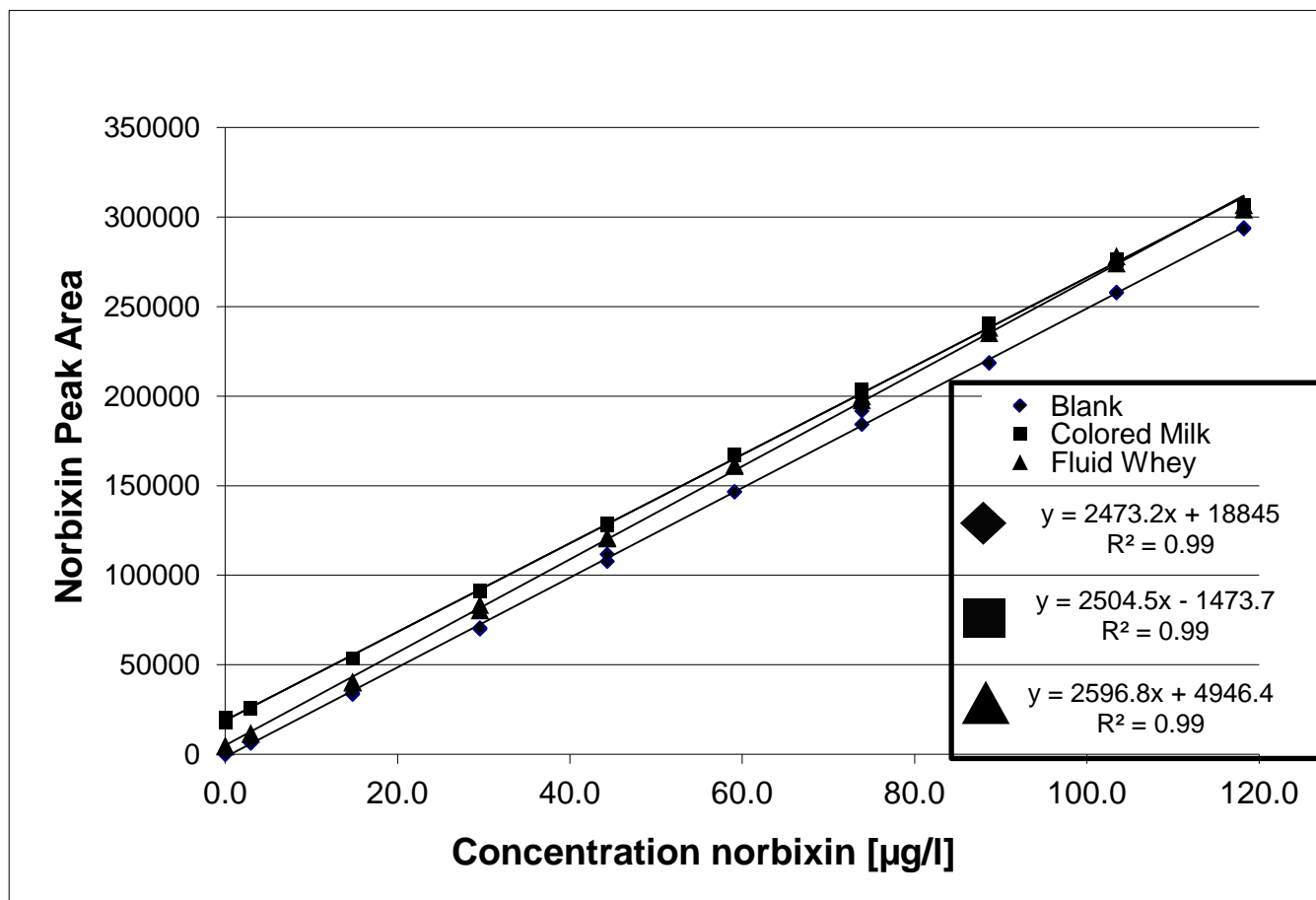


Figure 3. Linearity of norbixin in blank, colored milk, and fluid whey from 0-120 ug/l

Table 1. Cost and Time Analysis of Various Annatto Extraction Methods (prices calculated using Fischer Scientific as the sole supplier of all materials)

	Scotter et al. (2002)	cost	Lancaster and Lawrence (1995)	cost	Croissant et al. (2009)	cost	Proposed Method	cost
Solvents	100 mL hexane	12.04	3g celite	0.43	6 mL ethanol	0.23	24 mL acetonitrile: water (80:20)	3.21
	3g celite	0.43	75 mL ethanol:water:ammonium hydroxide (100:35:15)	1.09	6 mL chloroform	0.78		
	3 mL 0.1% BHT (in methanol)	0.05	100 mL petorelum ether	11.4	2 mL acetic acid (1%)	0.07		
	40 mL ethanol: water: ammonia (100:35:15)	0.58	100 mL acetic acid (11%)	7.22	7mL hexane	0.84		
	50 mL acetic acid (11%)	3.61	120 mL chloroform: glacial acetic acid (98.5:1.5)	15.57	5 mL hexane: diethyl ether (1:1)	0.59		
	25 mL methanol	1.34	10 mL chloroform	1.3	1mL acetone	0.11		
	75 mL chloroform: acetic acid (98.5:1.5)	9.73	55 mL methanol	3.81	3 mL methanol: glacial acetic acid (7:3)	0.21		
Disposable Goods	Glass wool	1.47	Glass wool (x2)	2.94	SPE Strata NH2 column	4.08		
Total Cost		\$27.78		\$40.82		\$6.91		\$3.21
Total extraction time	2.5 h		2.5 h		2 h			15 min
Total Waste	293 ml		460 ml			30 ml		24 ml

Table 2. Repeatability and intermediate precision within days and between days of norbixin from various matrices (n=6)

	Cheese Milk		Fluid Whey		Retentate		Bleached Whey ¹		Bleached Whey ²		Bleached Whey ³	
	Mean (ug/kg)	RS D [†] (%)	Mean (ug/kg)	RS D (%)	Mean (ug/kg)	RS D (%)	Mean (ug/kg)	RS D (%)	Mean (ug/kg)	RSD (%)	Mean (ug/kg)	RSD (%)
Repeatability within day	779.6	1.6	117.0	1.3	321.9	1.2	Below LOQ	n/a	Below LOQ	n/a	35.3	2.8
Repeatability between days	779.6	4.0	117.0	6.9	321.9	12.3	Below LOQ	n/a	Below LOQ	n/a	35.3	9.8
Intermediate Precision Single*	779.6	4.3	117.0	7.0	321.9	12.4	Below LOQ	n/a	Below LOQ	n/a	35.3	10.2
Intermediate Precision Duplicate*	779.6	4.1	117.0	7.0	321.9	12.3	Below LOQ	n/a	Below LOQ	n/a	35.3	10.0

*Intermediate precision for respectively a single and a duplicate analysis on an arbitrary day

[†] Relative Standard Deviation

¹ Bleached using lactoperoxidase, 20 ppm hydrogen peroxide, 50C for 1h

² Bleached using Maxibright™ (2 Dairy Bleaching Units), 20 ppm hydrogen peroxide, 50C for 1h

³ Bleached using 250 ppm hydrogen peroxide, 50C for 1h

Table 3. Recovery (%) of norbixin from spiked samples in milk (n=4) and whey (n=4)

Spiked Level in Milk	78.2 ug/L	780 ug/L	2.35 mg/L
Recovery in Milk	105% ± 4.1%	99% ± 0.6%	96% ± 10%
Spiked Level in Whey	9.39 ug/L	56.3 ug/L	312.9 ug/L
Recovery in Whey	102% ± 3.0%	97% ± 7.0%	90% ± 5.7%

Enzymatic Bleaching in Commercial Retentates

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ABSTRACT

The objective of this study was to evaluate the effect of enzymatic bleaching (lactoperoxidase (LP) or exogenous peroxidase (EP)) on the color and flavor of commercially produced whey protein concentrates (34% or 80%). Optimum levels of added hydrogen peroxide and optimum bleaching times were determined in commercial retentates by quantifying norbixin destruction. Retentates were then bleached and sensory and volatile analyses were conducted. In some retentates, LP bleaching was not observed, however, EP bleaching was effective under all conditions. Enzymatic bleaching (both LP and EP) occurred faster at 35C than at 4C ($p < 0.05$). Solids level also affected the speed of bleaching with lower solids bleaching in less time than higher solids ($p < 0.05$). Bleached retentates, regardless of treatment, were higher in aroma intensity and cardboard flavor and were also higher in aldehydes ($p < 0.05$). LP activity and subsequent bleaching of commercial retentates was variable while EP bleaching was consistently effective.

KEY WORDS: Whey, lactoperoxidase, flavor, bleach

INTRODUCTION

In the United States, it is highly desirable for Cheddar cheese to have an orange color and thus annatto, a natural orange colored carotenoid comprised primarily of norbixin, is often added to the cheese milk. Colored whey streams are also utilized in other countries from various cheeses with added annatto. A fraction of this color is retained in the fluid whey (Kang et al., 2010), and must be bleached. Fluid whey, the by-product of cheese manufacture, is often further processed into value added products such as whey protein concentrate (WPC) 34% or 80% or whey protein isolate (>90% protein). Typical whey processing steps include fat separation, pasteurization, bleaching and a variety of membrane processing steps such as microfiltration, ultrafiltration and diafiltration. The high protein retentate is then spray dried into powder.

The flavor of fluid whey carries through into the final spray dried products (Croissant et al., 2009), and consumers and product manufacturers demand that dried whey ingredients be colorless with a bland flavor (Kang et al., 2010). Off-flavors in dried whey proteins associated with bleaching, either with benzoyl peroxide (BP) or hydrogen peroxide (HP), have been well documented in the literature (Croissant et al., 2009; Listiyani et al., 2011, 2012; Jervis et al., 2012). Due to the increased demand for bland, colorless whey ingredients, international concerns with the use of BP, and increasing concerns with HP, chemical bleaching alternatives are desirable (Kang et al., 2012; Campbell et al., 2012). Campbell et al. (2012) recently demonstrated that as little as 10 ppm HP was sufficient for greater than 80% norbixin destruction by endogenous lactoperoxidase (LP) in fluid whey at 35C. Exogenous peroxidase (EP), which gained recent FDA approval as a bleaching agent

in dairy products, has also been shown to efficiently bleach fluid whey at cold temperatures (Campbell and Drake, 2013). Enzymatic bleaching, either utilizing the native lactoperoxidase system (LP) or by adding an exogenous peroxidase (EP) has yet to be explored in whey protein retentate.

Traditionally, lactoperoxidase (LP) was used to increase storage stability of fluid milk and reduce the loss of fresh milk due to microbial spoilage. LP is a native peroxidase enzyme found in fluid milk and when in the presence of thiocyanate and hydrogen peroxide at sufficient levels, a potent antimicrobial (hypothiocyanate) is produced (Reiter and Harnulv, 1982). In addition to milk preservation, the lactoperoxidase system can be used to bleach whey (Bottomley et al., 1989; Campbell et al., 2012; Campbell and Drake, 2013). The strong oxidizing capacity of hypothiocyanate will result in the destruction of the conjugated bonds in norbixin and thus effectively remove the color in cheese whey. Using the LP system to bleach whey can be highly variable as levels of LP can vary depending on the lactation cycle of the cow, season, feeding regime, and breed (Kussendrager and van Hooijdonk, 2000). In addition, dairy processing steps such as heat treatment of milk or whey can also influence LP levels. Like LP, thiocyanate concentration in milk and whey can vary widely due to feeding regime (Seifu et al., 2005). In processing whey into value-added products such as high protein retentates, thiocyanate can pass through the membrane during the ultrafiltration step and thus would not stay in the retentate. If the thiocyanate in the retentate is too low, enzymatic bleaching using the LP system will not occur. The third component of the LP system, hydrogen peroxide, is not normally detected in raw milk and is typically added exogenously. HP can be generated endogenously by bacteria, although

amounts sufficient to activate the LP system may not be generated (Seifu et al., 2005). Depending on the milk and the processing of the whey, any one of the three components which make up the LP system could limit LP activity in whey protein retentate.

In order to facilitate enzymatic whey bleaching, a commercial exogenous peroxidase (EP), Maxibright™ (MB), is available and can be added to fluid whey or retentate in small quantities to help achieve desired and consistent bleaching efficacy. Both LP and EP require similar amounts of hydrogen peroxide to activate their respective systems (Bottomley et al., 1989; Zorn et al., 2003) and while LP requires thiocyanate for bleaching to proceed, EP does not require this component for enzymatic bleaching (Zorn et al., 2003). Since the original patent was filed in 2006, several studies have addressed the bleaching capacity of EP on beta-carotene in model systems (Scheibner et al., 2008; Puhse et al., 2009; Zelena et al., 2009), however, the bleaching efficacy and subsequent effects of enzymatic bleaching (LP or EP) compared to traditional chemical bleaching (hydrogen peroxide (HP) 250 ppm) in commercial whey protein retentates has yet to be investigated. Additionally, pilot plant manufactured whey or whey protein retentates may not fully represent challenges to LP bleaching encountered by industry. The objective of this study was to evaluate the effect of enzymatic bleaching (LP or EP) on the color and flavor of commercially produced whey protein retentates (34% or 80%) at various temperatures (4C or 35C), solids levels (9%-32%), and hydrogen peroxide levels (0-250 ppm).

METHODS

Experimental Design Overview

Retentates, WPC34 and WPC80, were received in duplicate frozen via overnight shipping from 4 different manufacturers across the United States and Canada. Upon arrival, contents were inspected for damage, frozen status confirmed, and were placed in frozen storage (-20C). Previous studies have confirmed that LP activity is not affected by frozen storage (Campbell et al., 2013a). All experiments were conducted within 5 d of sample receipt and prior to experiments each day, enzymatic (LP) activity was tested to verify no enzymatic degradation occurred. Thiocyanate was measured in each retentate. Optimum HP levels to activate the LP and EP systems were first determined. Then, optimum bleaching times, defined as >80% norbixin destruction, for both LP and EP were determined. The hydrogen peroxide level and times with the greatest percentage of norbixin destruction and the fastest bleaching times were then selected for further analyses and subjected to both descriptive analysis and volatile compound analysis. Chemically bleached whey, hydrogen peroxide 250 ppm, was included as a control.

Compositional Analysis

Total solids of retentates were determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). Fat was quantified using the CEM Smart Trac rapid fat analyzer (CEM, Matthews, NC). Protein was determined using the Sprint™ Rapid Protein Analyzer (CEM, Matthews, NC). All samples were measured in triplicate.

Lactoperoxidase (LP) Measurement

LP measurements and calculations were performed according to the International Dairy Federation method (Pruitt and Kamau, 1994). Two mL of ABTS stock solution (1mM ABTS (TCI, Tokyo, Japan) in 0.1mM phosphate buffer pH 6.0 (EMS, Hatfield, PA)) was placed in a disposable plastic cuvette (VWR International, West Chester, PA). To start the reaction, 0.10 mL of sample and 1.0 mL hydrogen peroxide solution (0.3 mM; Nelson Jameson, Inc., Marshfield, WI) were added simultaneously and mixed thoroughly. Absorbance readings at 412 nm (Cary UV Vis, Agilent Technologies Inc, Santa Clara, CA) were recorded 10 times per second until the reaction reached a plateau (2 min). Calculations were determined using the least squares regression of the absorbance data and Beer's law (Pruitt and Kamau, 1994).

Thiocyanate (SCN) Measurement

Thiocyanate measurements were performed according to the International Dairy Federation method (IDF, 1988). Four mL of retentate was mixed with 2.0 mL (w/v) of 20% trichloroacetic acid (TCA) solution (BDH, VWR International, West Chester, PA). The mixture was blended and allowed to sit for at least 30 min. The mixture was then centrifuged at $16,500 \times g$ for 10 min (Model RC5B, Thermo Scientific, Waltham, MA). After centrifugation, 1.5 mL of the supernatant was mixed with 1.5 mL of ferric nitrate solution (16g Fe (NO₃)₃ (EMD Chemicals, VWR International, West Chester, PA) dissolved in 50 mL of 2M nitric acid (HNO₃) (BDH, VWR International, West Chester, PA) and then diluted with DI water to 100 mL. Measurements were performed at 460 nm within 10 min

of ferric nitrate solution addition. The concentration of SCN (Sigma Aldrich, Milwaukee, WI) was determined by the use of external standard curves.

Activation of LP or EP System

The optimum level of HP to activate the LP or EP system was determined in each retentate by adding 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, or 250 mg/kg HP to each pasteurized fat-separated liquid Cheddar retentate (Campbell et al., 2012). Bleaching was then conducted as described below. The concentration of HP which resulted in the most bleaching (norbixin destruction determined via HPLC), which varied according to solids level, manufacturer, and lot, was selected for further trials.

Optimum Bleaching Time

Optimum bleaching times were determined according to Campbell and Drake (2013). Briefly, retentate was placed in amber glass jars at the appropriate temperature (either 35C or 4C) and allowed to equilibrate. To activate the LP system, the selected concentration of HP (Table 1) (35% w/v, Nelson Jameson, Inc., Marshfield, WI) was added and allowed to bleach with gentle agitation. To activate the EP system, 2 DBLU MB (dairy bleaching unit; DSM, Delft, Netherlands) was added to the retentate and agitated gently. To that, HP was added and allowed to bleach with gentle agitation (Table 1). Chemical bleaching was conducted by adding 250 ppm HP (35% w/v, Nelson Jameson, Inc.) and allowed to bleach with gentle agitation. Aliquots of samples were removed at appropriate time points (35C: 0h, 0.5h, 1h, 1.5h, 2h, 3h, 4h, 6h; 4C: 0h, 1h, 2h, 4h, 6h). Peroxide test strips (EMD Chemicals,

VWR International, West Chester, PA) were used to determine if any HP remained after the bleaching treatment. If so, catalase (FoodPro CAT, Danisco, New Century, NJ) was added at a rate of 20 mg/kg to deactivate HP. Norbixin measurements were performed immediately.

Norbixin Extraction and Quantification

Norbixin is the primary carotenoid in water soluble annatto extracts and was extracted and measured to determine percent annatto destruction and bleaching efficacy (Kang et al., 2010). Norbixin was extracted and quantified using HPLC by the method described by Campbell et al. (2013b). To extract retentates, 100 ul was placed into a 2 mL microcentrifuge tube (VWR International, West Chester, PA). To this, 900 ul of dilution solution (80% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/20% water (EMD Chemicals, VWR International, West Chester, PA with 0.1% (w/v) formic acid (EMD Chemicals, VWR International, West Chester, PA)) was added. The solution was vortexed and centrifuged (14,000 x g, Microfuge® 18 Centrifuge, Beckman Coulter, Brea, CA). The supernatant was removed and placed into vials for quantification by HPLC. 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).

Quantification was conducted using HPLC (Waters 1525 Binary Pump, Waters, Milford, MA). Isocratic mobile phase (70% acetonitrile (EMD Chemicals, VWR International, West Chester, PA)/30% water (EMD Chemicals, VWR International, West

Chester, PA) with 0.1% (w/v) formic acid (EMD Chemicals, VWR International, West Chester, PA)) was used at a flow rate of 1mL/min pumped through a binary pump (Waters 1525, Waters, Milford, MA). Fifty microliters of the sample was injected (Waters 2707 Autosampler) onto the column (Phenomenex Kinetex 2.6µm particle size, 10 cm length, 4.6mm inner diameter, 100Å pore size) which was heated to 40°C. The injector temperature was set to 4°C. Sample was sent through a photodiode array detector (Waters 2998). A standard curve was created by rehydrating norbixin powder (45% (w/v), Chr. Hansen, Milwaukee, WI) in 2.5% (w/v) potassium hydroxide (BDH, VWR International, West Chester, PA) and then diluting in mobile phase. The maxima used for calculation was 482 nm.

Descriptive Sensory Analysis

Sensory analysis was conducted on retentates using a trained descriptive sensory panel and an established dairy flavor language (Drake et al., 2003, 2009). Panelists (n =8) each had more than 150 h of previous experience with the sensory analysis of fluid and dried whey products using the SpectrumTM descriptive analysis method (Meilgaard et al., 2007). All sensory testing was conducted in accordance with NCSU Institutional Review Board for Human Subjects guidelines.

Retentates (30 mL) were evaluated in three-digit-coded 60 mL lidded cups (Solo Cup Company, Champaign, IL). Preparations were conducted with overhead lights off to avoid exposure to light. Samples were evaluated by each panelist in duplicate. Sensory data were collected using CompusenseTM five, release 4.8 (Compusense, Guelph, Canada).

Gas Chromatography Mass Spectrometry (GCMS)

Selected volatile compounds in retentates were extracted by solid phase microextraction (SPME) using selective ion monitoring (SIM). Volatile compounds were selected from previous studies and were compounds that were relevant to flavor and/or bleaching (Campbell and Drake, 2013; Campbell et al., 2012; Croissant et al., 2009; Listiyani et al., 2012; Jervis et al., 2012; Kang et al., 2012). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using a modified method of Liaw et al. (2010). All samples were tested the day bleaching experiments were conducted. All samples contained 10 % (w/v) sodium chloride (Fischer Scientific), and 10 ul internal standard solution (2-methyl-3-heptanone in methanol at 81 ppm (Sigma Aldrich, Milwaukee, WI)) in 20 ml 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 7820N GC with 5975 inert MSD (Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1 cm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA) at 31 mm with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm.

The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 250°C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250°C. An Zb-5ms column (Zb-5ms 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) was used for all analyses at a constant flow rate of 1 ml/min. Purge

time was set at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and source at 250°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 compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound. Retention indices were calculated using an alkane series (Sigma Aldrich, Milwaukee, WI) (Van den Dool and Kratz, 1963).

Statistical Analysis

Data was analyzed by a one-way analysis of variance (ANOVA) using a general linear model with Fisher's least significant difference for means separation (XLStat, version 2009.1.02; Addinsoft Inc., New York, NY) or by using a mixed model with replication designated as a random effect (SAS 9.2, Cary, NC). Principal component analysis was conducted to visualize differences among treatments.

RESULTS AND DISCUSSION

Retentate Composition

Protein composition of commercial retentates ranged from 35.7 to 72.9% (dry w/w) (Table 1). Solids upon receipt ranged from 11.3 to 29.9% (Table 1). Retentates with higher solids levels (>20%) were diluted to lower levels to provide a more meaningful comparison. Lactoperoxidase activity varied greatly within manufacturer (Figure 1). Previous pilot plant studies demonstrated high and consistent LP activity in WPC80 retentates (Campbell et al., 2013a) and thus we expected that retentates with the highest protein content would have the highest LP activity since LP is concentrated during the filtration process. However,

Manufacturer B, which had the highest protein content, had the lowest LP activity. Manufacturers A and C exhibited the most LP activity with their initial slopes being 10.9 and 11.0, respectively ($p < 0.05$). Manufacturer B had the lowest LP activity ($p < 0.05$; slope: 0.001) (Figure 1). Many factors may contribute to LP activity variability including initial LP activity in fluid milk, heat treatment of milk and whey and/or fluid storage (Seifu et al., 2005). Manufacturer D, which had high LP activity (slope: 8.77), had the lowest SCN content (Table 2) ($p < 0.05$). In subsequent experiments, manufacturer D had >90% norbixin destruction using the LP system alone, demonstrating that SCN was not a limiting factor. Previously, Seifu et al. (2005) reported that concentrations of 15 ppm or greater were required for the activation of the LP system in fluid bovine milk to take advantage of the natural microbial system. Campbell et al. (2012) reported consistent high bleaching (>80% norbixin destruction) of fluid whey with SCN levels of 3.8 ppm, consistent with these results.

Retentate Bleaching

The average amount of hydrogen peroxide necessary to activate enzymatic bleaching in retentates (either LP or EP) varied greatly with manufacturer (20-250 ppm; Table 1) consistent with the variability in native LP activity and SCN. In some cases, more HP was required for optimum bleaching with EP compared to LP and in some cases it was not. In addition, quantity of HP necessary for enzymatic bleaching varied from lot to lot within manufacturer (5-100 ppm difference between lots from same supplier; Table 1). The variability in native LP activity (and SCN) and the subsequent large range of optimum HP addition for LP bleaching demonstrates the potential challenges for utilizing this system

industrially. If too little HP is added, bleaching may occur but is not optimal, if too much is added, LP is irreversibly oxidized and no bleaching occurs. Campbell and Drake (2013) previously demonstrated that the application of EP in fluid whey expanded the range of optimal HP suggesting that the use of EP may be appealing industrially not only for consistency (due to variability in LP activity) but also due to expanding the tolerance range for HP addition which may be difficult to control in a continuous flow industrial setting.

Bleaching with the addition of EP resulted in faster bleaching than LP bleaching, particularly at low temperatures (6 h vs. 2 h; Table 1). Previously, Campbell and Drake (2013) reported that with the addition of EP to fluid whey at 4C, time for sufficient enzymatic bleaching (>80% norbixin destruction) was decreased from 12 h to 1 h. Retentate from manufacturer B, which had the lowest LP activity, bleached very poorly (17.0% norbixin destruction) using only the native LP system (Table 3). However, with the addition of EP, 76.6% norbixin destruction was observed. At high levels of hydrogen peroxide (250 ppm), which is commonly used in the dairy industry to chemically bleach whey, norbixin destruction ranged from 18.7 to 34.5% depending on manufacturer in this study (Table 3). These results are in agreement with previous literature which reported norbixin destruction ranging from 11.4 to 43% depending on HP chemical bleaching parameters (Kang et al., 2012; Jarvis et al., 2012; Fox et al., 2013). At this level of HP, the native LP enzyme system is irreversibly inactivated (Kussendrager and Hooijdonk, 2000).

Flavor Analysis

Bleaching, regardless of treatment, increased cardboard flavor in retentates and aroma intensity and decreased sweet aromatic flavor in retentates ($p < 0.05$) compared to

unbleached retentates (results not shown). The oxidative process of bleaching either chemically or enzymatically results in increased lipid oxidation and off flavors (Croissant et al., 2009; Jervis et al., 2012; Campbell et al., 2012). Bleaching at 35C increased off-flavors compared to bleaching at 4C (Figure 2) and corresponding increases in aldehydes, also consistent with previous studies in fluid whey (Listiyani et al., 2011). As with bleaching efficacy, solids level did not have a significant impact on flavor (total aldehyde load: 292.4 ppm (high solids) and 290.0 ppm (low solids)) ($p>0.05$) (Figure 2). All bleached retentates had low but distinct levels of fatty flavor (absent in unbleached retentates), but retentates bleached with LP or EP were higher in fatty and potato flavors than retentates which were bleached chemically (HP 250 ppm) (Figure 2). Chemically bleached samples were higher in sulfur flavor than enzymatically bleached retentates (Figure 2). Previous studies have documented similar off-flavors in HP and LP bleached whey (Listiyani et al., 2011; Campbell et al., 2012; Kang et al., 2012). Enzymatically bleached retentates, either EP or LP, were higher in hexanal (284.6 ppm and 293.9 ppm respectively) compared to the control or HP bleached retentates (170.8 ppm and 210.8 ppm respectively) ($p<0.05$). While hexanal does not contribute directly to cardboard flavor, it is a good indicator of lipid oxidation (Whitson et al., 2010). Campbell et al. (2012) also documented differences in lipid oxidation compounds between LP and HP bleached fluid wheys (LP bleached wheys had higher concentrations than HP fluid wheys). They concluded that these differences were related to the amount of bleaching (bleaching efficacy) in that more bleaching resulted in more oxidation. Enzymatic bleaching results in greater norbixin destruction (more bleaching) than HP chemical bleaching (average percent norbixin destruction 76.7% vs.

26.4%, respectively) and also results in more oxidation products generated. In WPC80 from fluid wheys bleached with LP or HP, cardboard and fatty flavors were not different.

CONCLUSION

In contrast to pilot plant manufacture studies, the bleaching efficacy of native lactoperoxidase alone varied greatly by manufacturer and within manufacturer ($p < 0.05$). In contrast, bleaching with the addition of EP, similar to previous pilot plant manufacture studies, was consistently effective. The addition of EP may be necessary to achieve consistent desired color loss by enzymatic bleaching. Enzymatic bleaching (either LP or EP) destroyed more norbixin than traditional chemical bleaching (hydrogen peroxide at 250 ppm) ($p < 0.05$). Bleached retentates, regardless of treatment, were higher in aroma intensity and cardboard flavor and lower in sweet aromatic flavors ($p < 0.05$), and were also higher in aldehydes ($p < 0.05$). Enzymatic bleaching (both LP and EP) occurred faster at 35C than at 4C ($p < 0.05$), although increased off-flavors were observed at 35C as opposed to 4C. Enzymatic bleaching in retentates is a feasible option for dairy processors to remove color from wheys, however, due to inherent variability in LP activity, the addition of EP is advised for consistent bleaching.

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TABLES AND FIGURES

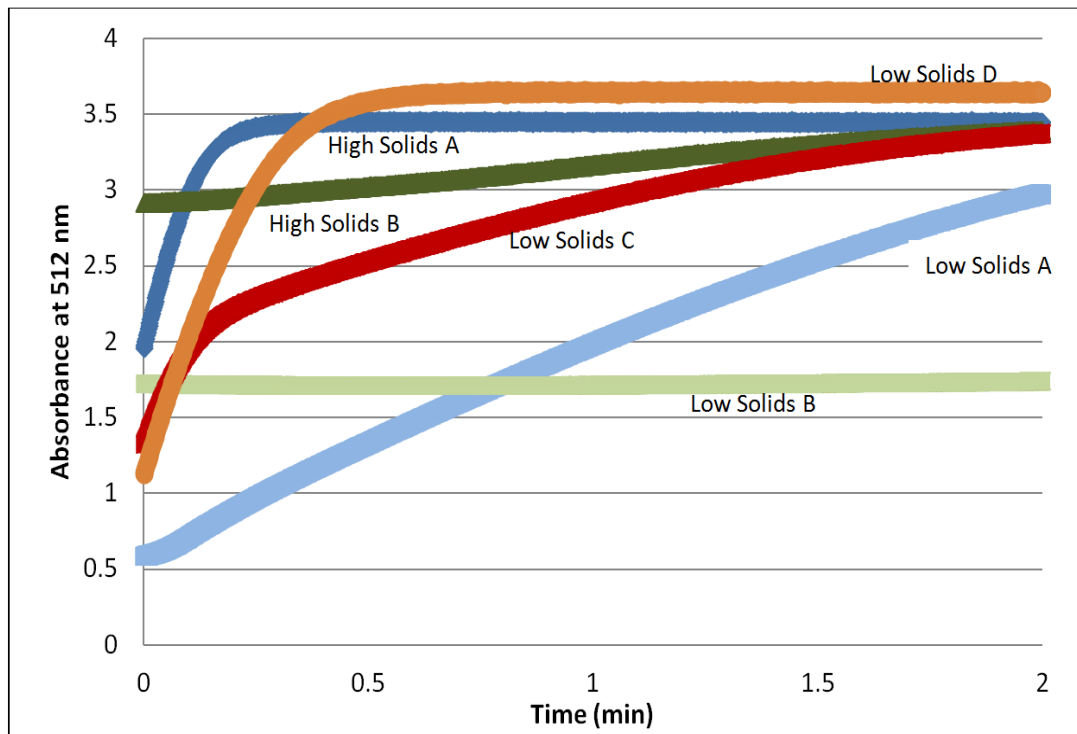


Figure 1. Lactoperoxidase activity in whey retentates from various manufacturers (A, B, C, D) at high (approx. 30%) solids and low (approx. 10%) solids. Initial slopes: high solids A (10.9^a), low solids A (1.03^c), high solids B (0.001^d), low solids B ($<0.001^d$), low solids C (11.1^a), low solids D (8.77^b). ^{a-d} means not sharing a common superscript are different ($p < 0.05$).

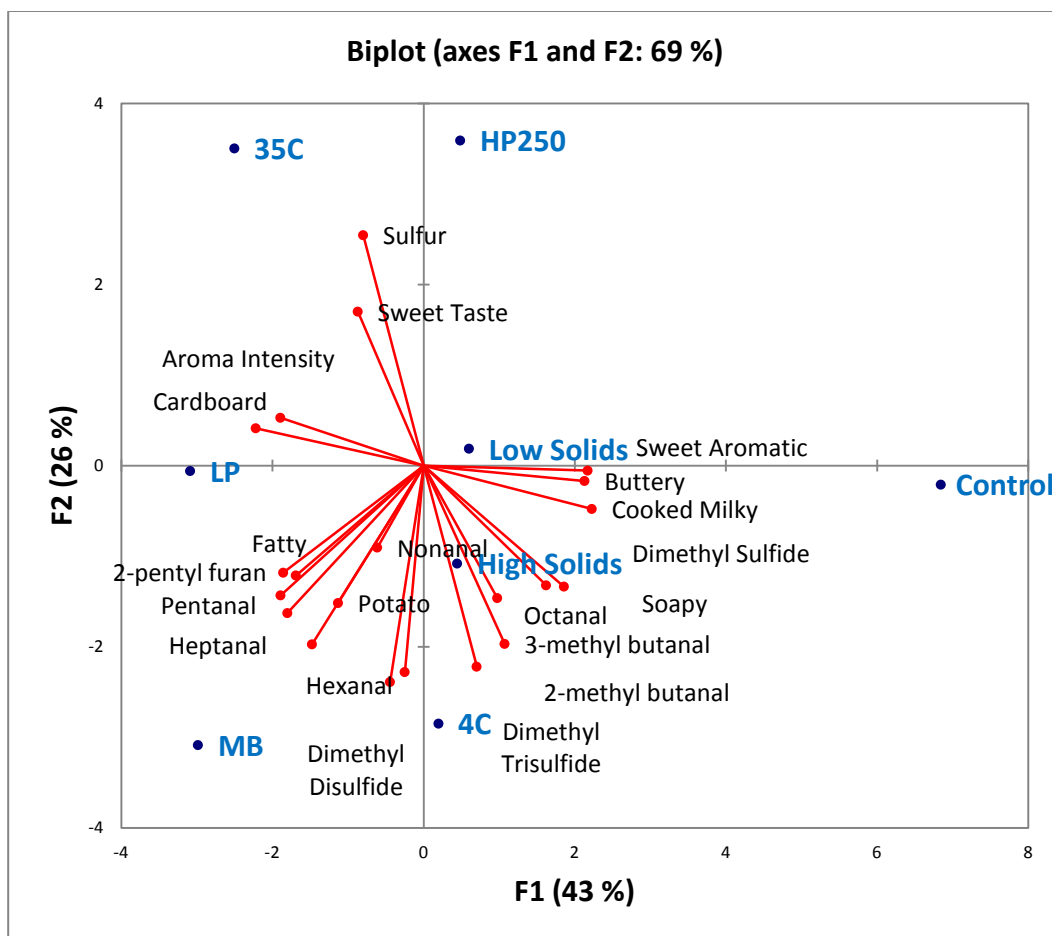


Figure 2. Principle component analysis (PCA) biplot of sensory attributes and volatile compounds of retentates bleached at various temperatures (4C or 35C), solids levels (high solids and low solids), and with various bleaching agents (lactoperoxidase (LP), exogenous peroxidase (EP), hydrogen peroxide 250 ppm (HP250), and no bleaching (Control)).

Table 1. Proximate analysis, optimal hydrogen peroxide levels (ppm), and bleaching times for enzymatic bleaching of whey retentates from duplicate samples from 4 manufacturers.

	Solids *†	Fat* (w/w dry basis)	Protein* (w/w dry basis)	4C				35C			
				Lot 1		Lot 2		Lot 1		Lot 2	
				LP‡	EP+	LP	EP	LP	EP	LP	EP
Manufacturer A	23.9±0.29	2.42±0.02	35.7±0.83	60 ppm; 6h	90 ppm; 6h	80 ppm; 6h	150 ppm; 6h	80 ppm; 6h	150 ppm; 6h	70 ppm; 4h	250 ppm; 4h
Manufacturer A	9.28±0.01	2.42±0.02	35.7±0.83	20 ppm; 6h	30 ppm; 6h	20 ppm; 3h	30 ppm; 2h	25 ppm; 6h	70 ppm; 6h	25 ppm; 1.5h	70 ppm; 1.5h
Manufacturer B	29.9±1.2	4.47±0.15	72.9±1.8	40 ppm; 6h	40 ppm; 4h	30 ppm; 6h	30 ppm; 6h	30 ppm; 6h	30 ppm; 4h	40 ppm; 6h	60 ppm; 6h
Manufacturer B	8.93±0.11	4.47±0.15	72.9±1.8	40 ppm; 6h	40 ppm; 6h	25 ppm; 6h	25 ppm; 6h	30 ppm; 6h	30 ppm; 3h	15 ppm; 6h	60 ppm; 6h
Manufacturer C	11.3±2.7	2.21±0.19	53.8±10.2	25 ppm; 6h	25 ppm; 2h	30 ppm; 6h	30 ppm; 6h	20 ppm; 1h	25 ppm; 30 min	40 ppm; 30 min	60 ppm; 30 min
Manufacturer D	12.3±0.25	1.09±0.52	42.9±2.54	25 ppm; 2h	40 ppm; 1h	25 ppm; 6h	25 ppm; 2h	25 ppm; 30 min	30 ppm; 30 min	30 ppm; 30 min	90 ppm; 30 min

* Averaged over both lots

† Retentates with higher solids levels (Manufacturer A and B) were diluted to lower solids levels to provide a more meaningful comparison

‡Lactoperoxidase

+Exogenous peroxidase

Table 2. Thiocyanate (SCN) levels (ppm) for commercial whey retentates from 4 manufacturers.

Manufacturer	SCN (ppm)
A	10.8b
B	27.5a
C	5.22c
D	2.59d

*each lot obtained in duplicate

^{a-d} Means in the same column not sharing a common superscript are different ($P < 0.05$).

Table 3. Percent norbixin destruction in whey retentate by manufacturer across temperature (35, 4C), bleach treatment (lactoperoxidase (LP), exogenous peroxidase (EP), hydrogen peroxide 250 ppm (HP)), and solids level (high, low).

Main Effects				
<i>Temperature</i>	A	B	C	D
35	65.2a	46.5a	79.8a	84.8a
4	65.1a	28.4b	64.9a	64.9b
<i>Treatment</i>				
LP	87.5a	17.0b	93.5a	94.9a
EP	86.9a	76.6a	92.0a	95.1a
HP	21.1b	18.7b	31.4b	34.5b
Interactions				
<i>Temperature*Treatment</i>				
35*LP	96.8a	26.9c	98.6a	98.2a
35*EP	97.2a	87.4a	96.4a	98.5a
35*HP	34.9c	25.2cd	44.3ab	57.8c
4*LP	78.2b	7.05e	88.5a	91.6b
4*EP	76.5b	65.8b	87.6a	91.8b
4*HP	7.26d	12.3de	18.6b	11.2d
<i>Solids*Treatment</i>				
High*LP	87.5a	21.8c	n/a	n/a
High*EP	81.9a	64.9b	n/a	n/a
High*HP	26.2b	19.4c	n/a	n/a
Low*LP	87.5a	12.1c	n/a	n/a
Low*EP	91.9a	88.2a	n/a	n/a
Low*HP	15.9b	18.1c	n/a	n/a

^{a-e} Means in the same column not sharing a common superscript are different ($P < 0.05$).

APPENDIX

Impact of Bleaching Permeate from Microfiltered Skim Milk on 80% Serum Protein Concentrate*

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* Use of names, names of ingredients, and identification of specific models of equipment is for scientific clarity and does not constitute any endorsement of product by authors, Cornell University North Carolina State University, the Northeast Dairy Foods Research Center or the Southeast Dairy Foods Research Center

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ABSTRACT

Whey proteins that have been removed prior to the cheese making process are referred to as “native” whey proteins or milk serum proteins. Because serum proteins isolated directly from milk are not exposed to the cheese making process they are free of functional or sensory effects from this process. Whey proteins used in food and beverage applications are largely derived from annatto-colored Cheddar cheese. Some of the annatto is left in the whey and this color is converted to a colorless compound by bleaching. The effect of bleaching serum proteins on flavor and functionality of spray dried protein provides a platform to investigate the effect of bleaching free from the confounding effects of the cheese manufacture. The objective of this study was to characterize and compare the sensory and functional properties of 80% milk serum protein concentrate (SPC80) produced from bleached and unbleached microfiltration (MF) permeate made from skim milk with and without added annatto color. Colored and uncolored MF permeates were bleached with benzoyl peroxide (BP) or hydrogen peroxide (HP), ultrafiltered, diafiltered, and spray-dried. The SPC80 from unbleached colored and uncolored MF permeates were manufactured as controls. All treatments were manufactured in triplicate. All SPC80 were evaluated by sensory testing, instrumental analyses, functionality, color, and proximate analysis. The HP-bleached SPC80 was higher in lipid oxidation compounds than other bleached or unbleached SPC80, specifically hexanal, heptanal, nonanal, decanal, and 2,3 octadienone. The HP treatments were higher in aroma intensity, cardboard and fatty flavors compared with the unbleached and BP bleached SPC80. The SPC80 bleached with BP had lower norbixin concentrations compared to SPC80 bleached with HP. Functionality testing demonstrated

that HP treatments had more soluble protein after 10 min of heating at 90°C at pH 4.6 and pH 7 than the no bleach and BP treatments regardless of additional color. Foams generated from bleached SPC80 were more stable than those from unbleached SPC80. Those bleached with HP were lower in yield stress than other SPC80. Overall, HP bleaching destroyed less norbixin and caused more lipid oxidation and subsequent off flavors than BP bleaching. However, heat stability of SPC80 was enhanced by HP bleaching compared to control or BP bleached SPC80.

Key Words: whey, flavor, bleaching, milk serum proteins, native whey protein

INTRODUCTION

Whey proteins that have been removed prior to the cheese making process have been referred to as “native” whey proteins or milk serum proteins (Drake et al., 2009; Evans et al., 2009, 2010). Milk serum proteins are a valuable milk fraction and are not exposed to the cheese making process, thus leaving them free of any functional or sensory effects from this process. It may be advantageous to remove the milk serum proteins prior to cheese making for several reasons, namely to produce more mild-tasting and consistent value-added whey protein products. If milk serum proteins were removed before the manufacture of cheese, the cheese composition would be the same because the majority of these proteins are not retained in the cheese (Nelson and Barbano, 2005a). Nelson and Barbano (2005b) went on to demonstrate that a high quality cheese could be made from milk with low serum protein content.

Recent studies have shown that gel strength, foaming, and emulsification properties of milk serum protein powders (24 to 37% protein) were significantly stronger than whey protein powders (24 to 37% protein) made from cheese whey (50% Gouda, 50% Emmental) and that these differences could be explained by denaturation rate, fat residues, and protein composition, respectively (Heino et al., 2007). The serum protein concentrate (SPC) powders used by Heino et al. (2007) had lower fat content than corresponding whey protein concentrate (WPC) powders. The higher fat residues present in conventional whey protein powders led to decreased foam stability and reduced foam volume compared to SPC. Increased functionality (foaming, gelation, and emulsification) is not the only benefit to serum whey proteins versus conventionally processed whey proteins. Serum whey proteins

are purported to have a higher biological activity than traditional whey proteins (Marcelo and Rizvi, 2007). Whey proteins can be used in a variety of applications and serum whey proteins may be of particular interests to the pharmaceutical and biomedical industries as these proteins are still in their “native conformation” (Marcelo and Rizvi, 2007).

Lipid oxidation is initiated during the cheese making process (Campbell et al., 2011b) and subsequent steps in whey processing increase volatile oxidation products (Croissant et al., 2009; Campbell et al., 2011a; Whitson et al., 2011). Serum protein concentrates are lower in fat than conventionally processed whey protein concentrates and previous work has demonstrated that SPC were also lower in lipid oxidation compounds and lipid oxidation-associated off flavors compared to whey proteins (Evans et al., 2010). These differences were attributed not only to decreased fat content but also to the lack of residual effects from the cheese making process.

Traditional whey often contains residual annatto color (norbixin) that was added to the cheese milk to impart a desirable yellow/orange color in the cheese. Once the whey is removed, the whey is often bleached to make it more acceptable for use in a wide range of food products (Kang et al., 2010). Currently, there are two approved chemical bleaching agents in the United States: benzoyl peroxide (BP) and hydrogen peroxide (HP) (Kang et al., 2010). Both of these bleaching agents have a negative impact on the flavor of whey protein products (Croissant et al., 2009; Listiyani et al., 2011; Jervis et al., 2012). The effect of bleaching on serum protein flavor and functionality has not been investigated, and provides a way to compare the effects of bleaching and bleaching agent without the confounding effects of the cheese make process. The objective of this study was to characterize and

compare the sensory and functional properties of 80% milk serum protein concentrates (SPC80) produced from bleached and unbleached MF permeate made from skim milk with and without added annatto color.

MATERIALS AND METHODS

Experimental Design

For SPC80 manufacture, a 3 X 2 full factorial design with 3 levels of bleaching (no bleach, 50 ppm BPO, and 500 ppm **HP**) and 2 levels of coloring (no annatto and 0.066 mL annatto/kg of milk) was employed. The experiments were replicated 3 times, resulting in 18 total MF processing runs for SPC80 manufacture. Each individual processing run was conducted over 3 consecutive days in a week.

Microfiltration Processing of Skim Milk

Pre-Microfiltration Processing. On the first day of processing, raw whole milk (about 1150 kg) was pasteurized with a plate heat exchanger (Model 080-S, AGC Engineering, Manassas, VA) at 72°C for 16 s, cooled to 50°C, and separated with a centrifugal cream separator (Model 619, DeLaval, Inc., Kansas, MO). After separation, the skim milk (about 1060 kg) was kept at 50°C and processed with a UF system in batch recirculation mode using a PES SW membrane (Model 3838, GEA NIRO Inc., Hudson, WI) with a molecular weight cutoff of 10,000 Da. Before processing, the UF membrane was cleaned following the procedure described by Evans et al. (2009). The skim milk was ultrafiltered for about 4 h to achieve a CF of approximately 2.2X. After UF, the retentate was diluted back to the original TP content of the skim milk as determined by IR. For the treatments with added annatto, the colorant (annatto cheese color - 2X, P/N 70741, Chr

Hansen, Inc., Milwaukee, WI) was added (0.066 mL/kg of milk) to the diluted UF retentate. The diluted UF retentate was then cooled using a plate heat exchanger and stored overnight at $\leq 4^{\circ}\text{C}$. The UF system was then cleaned as described by Evans et al. (2009). This UF step was conducted to reduce the lactose content of the MF feed so that the MF retentate produced in this study could be used in another study. In practice, SPC80 could be produced without ultrafiltering the skim milk prior to MF.

Microfiltration. The next day, the diluted UF retentate was microfiltered in a continuous feed-and-bleed 3X process using a pilot-scale system. The day before processing, the MF system was cleaned as described by Zulewska et al. (2009). The MF system (Tetra Alcross M7, TetraPak Filtration Systems, Aarhus, Denmark) was equipped with ceramic Membralox graded permeability (GP) membranes (Pall Corporation, Cortland, NY, nominal pore diameter = 0.1 μm , surface area = 1.7 m^2). Seven tubular, 19-channel ceramic membranes were housed in the tubular stainless steel MF module. The MF system consisted of a feed pump (type LKH 10/110 SSS 1.75 kW) and a retentate recirculation pump (type LKH 20/125 SSS 6.3 kW), both from Alfa Laval, (Kansas City, MO). The retentate recirculation pump was equipped with a variable frequency drive (MC Series, Model M12100C, Lenze AC Tech, Uxbridge, MA) and a magnetic flow transmitter (I/A Series, IMT25, Foxboro, Foxboro, MA) so that the cross-flow velocity could be controlled and monitored, respectively. More complete details of the MF processing conditions are provided by Adams et al. 2012 (submitted to JDS). After processing, the MF system was cleaned as described by Zulewska et al. (2009). If the MF permeate was not bleached, it was cooled to 4°C with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$.

SPC Manufacture

Bleaching of MF Permeate. If the MF permeate was going to be bleached, it was recirculated through a plate heat exchanger in a large stainless steel tank to heat it to 66°C. The bleaching process and conditions for MF permeate were identical to those applied to fluid whey by Jervis et al. (2012) to allow for direct comparison of results. Two different bleaches were used, benzoyl peroxide (BP) at 50 ppm (Oxylite Type XX Benzoyl Peroxide 32% by weight, Nelson Jameson, Marshfield, WI) or hydrogen peroxide (HP) at 500 ppm (35% w/w HP, FCC grade, Columbus Chemical Industries, Inc., Columbus, WI). When bleaching with BP, the powdered bleach was mixed with about 30 kg of MF permeate using a high shear mixer then added to the remainder of the MF permeate. The permeate was held for 30 min at 66°C with agitation then cooled with a plate heat exchanger and held overnight at 4°C. When bleaching with HP, the liquid bleach was added to about 30 kg of MF permeate, mixed with the remainder of the MF permeate, and agitated for 30 min at 66°C. The HP concentration in the liquid bleach was diluted from 35% to 10% w/w concentration. The 10% w/w concentration was verified with a 10% HP test strip (Indigo Instruments, Niagara Falls, NY). If the HP concentration was lower than 10%, then the actual concentration was calculated and the amount of HP was adjusted to achieve a level of 500 ppm in the MF permeate. After 30 min at 66°C, the MF permeate was cooled with a plate heat exchanger to 50°C, liquid catalase enzyme derived from *Aspergillus niger* (FoodPro CAT, PD 216626-2.0EN Danisco, Madison, WI) was added at 20 ppm, and the MF permeate was mixed for 10 min. The MF permeate was then cooled with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$.

Ultrafiltration of MF Permeate. The following day, approximately 530 kg of MF permeate was weighed into a vat, heated to 50°C using a plate heat exchanger, and processed with a UF system in batch recirculation mode using a PES SW UF membrane (Model 3838, GEA NIRO Inc., Hudson, WI; nominal molecular weight cutoff: 10,000 Da, surface area: 13.6 m²). Before processing, the UF membrane was short cleaned following the procedure described by Evans et al. (2009). The initial clean water flux was about 53 L/m² h. During processing, the system was operated in a constant pressure mode with 276 kPa of retentate inlet pressure, 103 kPa of retentate outlet pressure, and no backpressure on the permeate side of the membrane. The MF permeate was ultrafiltered for about 90 min. Every 15 min during processing, flux was measured and samples of the permeate and the retentate were taken for composition analysis using IR to monitor the process. Ultrafiltration was continued until the protein content of the retentate was 51% protein as a percentage of lactose plus fat plus protein, as determined by IR. The corresponding CF was about 3.8X. After UF, the retentate was diluted with pasteurized RO water at 50°C to bring the weight back to the original total weight of the starting MF permeate for DF. The membrane was not cleaned before proceeding to the DF stage. The mixture was recirculated through the membrane for 5 min to ensure complete mixing, then the DF process was started. Diluted UF retentate was DF for about 120 min. Diafiltration was continued until the protein content of the retentate measured by IR was 92% protein as a percentage of lactose plus fat plus protein in the retentate. The corresponding CF was about 11.2X. After producing the liquid SPC80, the UF system was cleaned as described by Evans et al. (2009). The fouled water flux before cleaning was, on average, 36% of the initial clean water flux (19 vs. 53 L/m² h)

and the clean water flux after cleaning was similar to the clean water flux prior to processing (about 53 L/m² h).

Spray Drying. The 80% SPC were spray-dried using a spray dryer (model 1, Niro Atomizer Inc., Columbia, MD). The feed material (about 40 kg) was kept at or below 7°C. The spray dryer was equipped with an FU11 atomizer rotating at 23,000 rpm and the feed rate was 16 kg/h. The inlet temperature was 200°C and the outlet temperature at the bottom of the spray dryer cone was 95°C. The powder from the first 10 min of the run was discarded. Powder exited the bottom of the dryer cone, went through a cyclone to separate the powder from the hot air, and was collected. The powder in the collection vessel was about 30 to 35°C. The dried product was collected, mixed, and packaged every 30 min. The total time of the drying run was approximately 3.5 h. The 80% SPC for sensory and functional property testing was packaged in polyethylene terephthalate (Mylar) zip-lock bags (Sorbent Systems, Los Angeles, CA) and shipped to North Carolina State University (Raleigh). Upon receipt, samples were at -80°C.

Chemical Analyses

The MF permeate was analyzed for total solids (TS), fat, total nitrogen (TN), nonprotein nitrogen (NPN), and noncasein nitrogen (NCN) content using forced air oven drying (AOAC, 2000; method 990.20; 33.2.44), ether extraction (AOAC, 2000; method 989.05; 33.2.26), Kjeldahl (AOAC, 2000; method 991.20; 33.2.11), Kjeldahl (AOAC, 2000; method 991.21; 33.2.12), and Kjeldahl (AOAC, 2000; method 998.05; 33.2.64) methods, respectively. Crude protein (CP) was calculated by multiplying TN by 6.38. The pH of MF permeate was measured with a solid polymer electrode (HA405-DXK-S8/120, Mettler-

Toledo, Bedford, MA) and an Accumet 915 pH meter (Fisher Scientific, Pittsburgh, PA) that was calibrated at 50°C using standard pH 4 and 7 buffer solutions (Fisher Scientific, Pittsburgh, PA).

The SPC80 powders were reconstituted to 10% solids and the liquids were analyzed for fat and TN by the methods indicated above. The pH was measured with an electrode (model Electrolyte 9823, Mettler Toledo) that was standardized at pH 7.01 and 4.00 at 22°C. The reconstituted samples were analyzed for total solids content by forced air oven drying (AOAC, 2000; method 990.20; 33.2.44) and moisture content of the powder was calculated. Mineral analysis was determined by the NCSU Analytical Services Laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009).

Color Analysis of Liquid and Spray Dried SPC80

The Hunter L (lightness), a (red-green), b (yellow-blue) values for the fresh SPC80 powders and reconstituted liquids (10% solids w/v) were determined in duplicate with a MacBeth Color-Eye spectrophotometer (Model 2020; Kollmorgen Instruments, Corp., Newburgh, NY) with Optiview software from the same company. Methods were identical to those used in Jervis et al. (2012).

Norbixin Extraction and Quantification

Norbixin extraction and quantification by UV-VIS spectroscopy from SPC80 was conducted using the method from Campbell et al. (2011a) and applied by Jervis et al. (2012). Extractions were performed under lights covered with premium full-spectrum F885 flat sheet filters (Ergomart, Dallas, TX) to minimize norbixin isomerization and degradation

(Mercadante, 2008). Norbixin in SPC80 (1 g) was solvent extracted followed by column solid phase extraction purification.

The concentration of norbixin was determined spectrophotometrically using a UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Durham, NC). A 0.7 mL aliquot of each sample was transferred to a 1 cm 28Q10 Spectrosil quartz cuvette (Starna Cells Inc., Atascadero, CA) and measured at 458 nm, the maxima for norbixin. A five point standard curve was created within the concentration range of 50 µg/kg to 10 mg/kg norbixin. Norbixin powder (45% w/w, Chr. Hansen, Milwaukee WI) was rehydrated in 2.5% potassium hydroxide solution (VWR International) and then diluted in methanol:glacial acetic acid [7:3 (vol/vol)]. Norbixin concentration was calculated by total solids and correction for dilution during the extraction and SPE processes and by an external standard curve. Measurements on the spectrophotometer were completed in duplicate.

Descriptive Sensory Analysis

Sensory analysis was conducted on rehydrated SPC80 (10% w/v) using a trained descriptive sensory panel and an established dairy flavor language (Drake et al., 2003, 2009) previously applied to SPC34 and SPC80 (Evans et al., 2009, 2010). Panelists (n =8) each had more than 150 h of previous experience with the sensory analysis of fluid and dried whey products using the SpectrumTM descriptive analysis method (Meilgaard et al., 1999). All sensory testing was conducted in accordance with the NCSU Institutional Review Board for Human Subjects guidelines.

Reconstituted SPC (10% solids w/v) was evaluated by placing 30 mL in three-digit-coded 60 mL lidded cups (Solo Cup Company, Champaign, IL). Preparations were

conducted with overhead lights off to avoid exposure to light. Samples were evaluated by each panelist in duplicate. Sensory data were collected on paper ballots or using Compusense™ five, release 4.8 (Compusense, Guelph, Canada).

Gas Chromatography Mass Spectrometry (GCMS)

Selected volatile compounds in SPC80 powder were extracted by solid phase microextraction (SPME) using selective ion monitoring (SIM). Compounds were selected based on previously identified flavor-contributing compounds in serum and whey proteins (Evans et al., 2009, 2010; Whitson et al., 2011; Jervis et al., 2012). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using the method described by Jervis et al. (2012). Spray dried powders were reconstituted at 10 % solids (w/v). All samples contained 10 % (w/v) sodium chloride (Fischer Scientific, Fairlawn, NJ) and 10 µl internal standard solution (2-methyl-3-heptanone in methanol at 81 ppm (Sigma Aldrich, Milwaukee, WI)) in 20 ml 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 MSD (Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1 cm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA) at 31 mm with 4 sec pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm. An Zb-5ms column (Zb-5ms 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex) was used for all analyses at a constant flow rate of 1 ml/min. Compounds were identified using the NIST 2005 library of spectra and

comparison of spectra of authentic standards injected under identical conditions.

Compounds were quantitated using external standard calibration curves for selected compounds. Retention indices were calculated using an alkane series (Sigma Aldrich, Milwaukee, WI) (Van den Dool and Kratz, 1963).

Quantification of selected volatile compounds in samples was executed using five point external standard curves ranging from 0.50 $\mu\text{g}/\text{kg}$ to 2 mg/kg (depending on selected compound) and integrated to an internal standard (81ppm 2-methyl-3-heptanone). External standard curves were prepared by pipetting 4.0 mL of water into 20 mL autosampler vials with steel screw top lids lined with silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA) with spiked amounts of target compounds and 10 μL internal standard. HPLC grade water (VWR International) was used to bring the total solution volume to 5.0 mL. Sodium chloride (Fischer Scientific, Fairlawn, NJ) was added to the vials prior to the addition of water so that the total solution was 10% (w/v) sodium chloride.

Functional Properties

Foam Generation : . The method for foam measurement was previously described by Jervis et al. (2012). Briefly, an Artisan Kitchen Aid Mixer (Kitchen Aid, St. Joseph's, MI, USA) with a 4.5 quart stationary bowl and a rotating wire beater was used for foam formation. Solutions of protein (10% w/v of protein) were rehydrated for 6 h at room temperature (20 to 24°C) on a stir plate set to 200 rpm. Solutions were refrigerated overnight at 4°C and tempered to 25°C. Solutions were then adjusted to pH 7 using 1N NaOH (VWR International). Solutions were prepared in triplicate. A 200 mL sample of

solution was whipped at speed 10 (beater rpm of 752) for 19 min and 36 sec (Davis and Foegeding, 2007).

Foam Stability. Foam drainage was measured using the methods of Phillips et al. (1990) and Luck et al. (2001). Foam stability was measured by the time it took for half of the pre-foam mass to drain through a 6-mm hole in a whipping bowl. The starting time for these measurements was taken immediately after foam formation (Davis and Foegeding, 2007). A longer drainage half-life corresponds to greater foam stability.

Foam Yield Stress. Yield stress was conducted previously as described by Jervis et al. (2012).

Foam Overrun. Overrun measurements were conducted immediately following yield stress measurements. Foam was removed from the bowl using a rubber spatula in a circular pattern and gently filling a weighing dish (100 mL) and taking the weight of the foam. This was done ten times and the mean value was used to calculate overrun and air phase fraction using the following equations:

$$\text{Overrun} = \frac{(\text{wt. 100mL solution}) - (\text{wt. 100mL foam})}{\text{wt. 100mL foam}}$$

$$\text{Air phase fraction}(\varphi) = \frac{\% \text{overrun}}{(\% \text{overrun} + 100)}$$

All experiments were measured in triplicate (Davis and Foegeding, 2007; Wilde, 2000; Dickinson, 1999).

Solubility. Solubility measurements were conducted as described by Jervis et al. (2012). The SPC80 was rehydrated to 10% protein (w/v) as described for foam generation.

Solutions were then adjusted using 1N HCl or 1N NaOH to pH 3, 4, 5, 6, or 7 and brought to 100 mL total volume with deionized water resulting in 10% w/v protein solutions. Turbidity and solubility were measured and recorded on samples both before and after centrifugation. Turbidity was measured using a Turbidimeter (2100AN, Hach Company, Loveland, CO) and solubility was measured using the micro bicinchoninic acid assay. After these measurements were complete, solutions were then centrifuged at 16,500 x g for 10 min (model RC5B, Thermo Scientific). Turbidity and solubility of the samples was measured once again, but this time on the supernatants.

Heat Stability. Heat stability measurements were conducted as described by Jervis et al. (2012). The SPC80 was rehydrated to 5% (w/v) and 10% protein (w/v) as described for foam generation. Solutions were then adjusted using 1N NaOH to pH 7 and brought to volume. Solutions were placed in a heated water bath at 90°C for either 0, 10, 20 or 30 min. Turbidity and micro BCA measurements of solubility were taken before and after centrifugation at room temperature and calculated as described previously. The supernatant was then collected and brought to pH 4.6 using 1N HCl. Turbidity and solubility were measured on the pH 4.6 supernatant before and after centrifugation. All solutions were measured in quadruplicate.

Statistical Analyses

All data was analyzed by ANOVA using the Proc GLM (general linear model) procedure of SAS (SAS version 8.02, 1999-2001, SAS Institute Inc., Cary, NC). Tukey's honestly significant difference was conducted as a post hoc test. To determine if there were differences ($P < 0.05$) in composition or pH among color and bleaching treatments for MF

permeates, the GLM was dependent variable = bleach + color + replicate + bleach*color + error. The same GLM was used to determine if there were differences ($P < 0.05$) in composition or Hunter color values among color and bleaching treatments for SPC80 powders. Principal component analysis was applied to the correlation matrix of sensory and volatile component data to visualize how bleaching agent differentiated SPC80 based upon sensory attributes and/or volatile components.

RESULTS

Chemical and Mineral Analyses

No differences in moisture, protein, and fat were detected among treatments ($P > 0.05$) with moisture 4.81 ± 0.24 , protein (dry basis) 83.17 ± 0.42 , and fat (dry basis) 0.526 ± 0.05 percent (Table 1, 2). No differences in potassium were detected between treatments ($P > 0.05$) (Table 3). A significant bleach effect ($P < 0.05$) was found in all other minerals with higher amounts of sulfur and sodium found in HP samples compared to BP and unbleached SPC80 (Table 3). Hydrogen peroxide treated SPC80 contained lower amounts of phosphorus, calcium, magnesium, and iron compared to the other treatments (BP and control; Table 3) ($P < 0.05$). Lower amounts of iron in HP treated samples as compared to all other treatments was previously noted in WPC80 (Jervis et al., 2012).

Color Analysis of Liquid and Spray Dried SPC80

The addition of color (annatto) had a significant effect on L, a, and b values, as expected ($P < 0.05$) (Table 4). Bleaching, regardless of added color or bleach treatment, increased the whiteness of the powders (L-value) ($P < 0.05$). Hydrogen peroxide treatments, with and without added annatto color, had higher L-values than BP treatments (with and

without added color) ($P < 0.05$). The yellowness of SPC80 bleached with either HP or BP were lower than annatto with no bleach in liquids and powders ($P < 0.05$). Benzoyl peroxide-bleached liquid and powdered SPC80 with added annatto color had lower b-values in both liquid and powder forms compared to HP and no bleach treatments with added color ($P < 0.05$) (Table 4). These findings were consistent with a previous study with WPC80 (Jervis et al., 2012).

Norbixin Extraction and Quantification

Both bleach treatments, either BP or HP, reduced norbixin concentration ($P < 0.05$) (Table 5). Benzoyl peroxide-bleached SPC80 with added annatto contained less residual norbixin than those bleached by HP with added annatto ($P < 0.05$). Regardless of whether annatto was added, the amount of residual norbixin in BP-bleached SPC80 was not different from the control with no added annatto ($P > 0.05$). Hydrogen peroxide reduced norbixin by 50% compared to the control with added annatto while benzoyl peroxide reduced norbixin by 92% compared to the control with added annatto. Previous studies with fluid whey bleaching have also demonstrated that BP bleaching resulted in greater norbixin destruction than HP bleaching (Jervis et al., 2012; Listiyani et al. 2011, 2012).

Descriptive Sensory Analysis

Sensory profiles of SPC80 were distinct (Table 6). Hydrogen peroxide -bleached SPC80, regardless of added annatto, were higher in aroma intensity, cardboard, and fatty flavors ($P < 0.05$) than those bleached with BP or those receiving no bleach treatment. Sweet aromatic and cooked/canned corn flavors were higher and cardboard flavor was lower

in those SPC80 which received no bleach treatment, regardless of added annatto, compared to those which were bleached (either HP or BP) ($P < 0.05$).

Gas Chromatography Mass Spectrometry (GCMS)

Among bleach types, SPC80 was distinct in volatile compound profiles (Figure 1). SPC80 bleached with HP, either colored or uncolored, contained higher concentrations of lipid oxidation compounds than those bleached with BP ($P < 0.05$). Compounds found at higher amounts in HP treated SPC80 compared to either BP-bleached or unbleached SPC80 were hexanal, heptanal, nonanal, benzaldehyde, and 2,3 octadienone ($P < 0.05$) (Table 7). Bleached SPC80, either BP or HP bleached, were higher in octanal and 2-pentyl furan than SPC80 that was not bleached.

Functional Properties

Foam Stability. Differences in foam stability were detected among bleach treatments ($P < 0.05$; Table 8). Foams from bleached SPC80 (BP or HP) were more stable than the unbleached controls ($P < 0.05$) and foams from BP-bleached SPC80 were more stable than those bleached with HP ($P < 0.05$). Foams from unbleached SPC80 were more stable with added annatto than without ($P < 0.05$).

Foam Yield Stress. Color and bleach impacted yield stress of foams (Table 9). The SPC80 with added annatto, regardless of bleach treatment, had lower yield stress compared to those without added annatto ($P < 0.05$). The SPC80 treated with HP had lower yield stress than BP and control SPC80, regardless of color ($P < 0.05$).

Foam Overrun. Average overrun was $1233.2\% \pm 40.7\%$ and no differences were detected among SPC80 treatments ($P > 0.05$; $R^2:0.28$; SE: 61.4) (data not shown).

Solubility. Solubility of SPC80 with no heat treatment differed among bleach treatments within a specific pH value ($P < 0.05$) (Figure 2). At pH 3, unbleached SPC80, colored and uncolored, were less soluble than those bleached by either HP or BP ($P < 0.05$). At pH 4, unbleached SPC80 and those bleached with BP, either colored or uncolored, were less soluble than those bleached by HP ($P < 0.05$). At pH 5, bleached SPC80, either HP or BP, regardless of color were less soluble than unbleached SPC80 ($P < 0.05$). No differences in solubility were detected at pH 6 or 7 ($P > 0.05$).

Heat Stability. Serum protein powders bleached with HP, both colored and uncolored, at 10% (w/v) protein were more soluble after 10 min at 90°C than colored or uncolored BP bleached SPC80 at pH 4.6 ($P < 0.05$) (Figure 3). All SPC80, whether colored or uncolored, bleached or not bleached, gelled after 20 min of heating and pH adjustment to 4.6. No differences in heat stability were observed at 5% (w/v) protein at pH 7 and pH 4.6 ($P > 0.05$) (results not shown).

DISCUSSION

Flavor

Compositionally, all SPC80 powders were similar; however, visually their appearance differed. Consistent with previous studies, BP bleached better than HP. Norbixin values and b values were in agreement: as norbixin increased, so did b values. Flavor effects of BP and HP bleaching have been consistently reported in the literature for WPC34 and WPC80 (Listiyani et al., 2012; Jervis et al., 2012), and similarly, BP and HP had effects on flavor of SPC80.

The flavor of dried dairy ingredients is expected to be bland and flavorless so as to not to influence product flavor and influence product acceptability (Drake et al., 2006). In the United States, a large volume of whey comes from Cheddar cheese in which annatto, an orange carotenoid pigment comprised of bixin and norbixin, is added to the milk to give the cheese a characteristic orange color (Kang et al., 2010). Some of this colorant is present in the whey and the whey must be bleached to remove the color (Kang et al., 2010). Bleaching of fluid whey increases lipid oxidation, volatile compounds, and produces off-flavors that carry into whey protein concentrates thus influencing consumer acceptability of final products (Croissant et al., 2009; Listiyani et al., 2012; Jervis et al., 2012). Serum protein concentrates are lower in fat (0.53% vs 4.67% on a dry weight basis) than WPC (Evans et al. 2009, 2010) because the fat remaining in skim milk is retained in the retentate of the MF process, while the serum proteins pass into the permeate. During WPC production the fat not removed from whey is concentrated by UF and DF in the WPC retentate. The high content of fat in WPC has a negative effect on flavor and provides a reservoir for increased lipid oxidation compounds compared to SPC (Liaw et al., 2010; Evans et al., 2010). Further, the starter culture contributes to elevated lipid oxidation compounds in fluid whey and subsequent increased lipid oxidation during processing (Campbell et al., 2011a, 2011b; Liaw et al., 2010, 2011). Serum proteins contain lower amounts of volatile compounds and lower intensities of flavors commonly found in WPC due to the absence of starter culture and cheese making residuals. However, bleaching significantly impacts flavor in SPC.

Consistent with WPC80 (Jervis et al., 2012), SPC80 bleached with HP were higher in fatty and cardboard flavors than those bleached with BP or those receiving no bleach

treatment ($P < 0.05$). Cardboard and fatty flavors have been attributed to increased volatile lipid oxidation compounds (Wright et al., 2009; Whitson et al., 2010; Whitson et al., 2011; Jervis et al., 2012). HP-bleached SPC80 were higher in hexanal, heptanal, nonanal, decanal, 2-pentyl furan, 2,3 octadienone and benzaldehyde the other SPC80 treatments ($P < 0.05$) (Figure 1). In HP-bleached SPC80, the volatile compound with the highest concentration was hexanal. While hexanal is not directly attributed to cardboard off-flavors, it is a good indicator of lipid oxidation (Whitson et al., 2010). Hexanal can be created by multiple pathways during lipid oxidation (Frankel, 2005). Benzoyl peroxide -bleached SPC80 contained lower levels of hexanal than HP-bleached SPC80 ($P < 0.05$); however, lower still were those SPC80 which received no bleach treatment. The BP has a lower oxidizing potential than HP (Listiyani et al., 2012; Jervis et al., 2012). The increase in dimethyl disulfide (DMDS) and the presence of cabbage/sulfur aroma in HP-treated proteins was the key distinguishing characteristic in BP vs. HP WPC80 (Jervis et al., 2012). Volatile compound types and relative differences between BP and HP were the same between WPC80 and SPC80. Increased volatile sulfur compounds along with higher elemental sulfur in HP-treated SPC80 suggest sulfur amino acid catabolism. Further research is needed to determine amino acid profiles of HP and BP-treated proteins to confirm these effects. The SPC80 bleached with BP were lower in sweet aromatic and higher in cardboard flavors compared to unbleached SPC80 ($P < 0.05$) due to increased lipid oxidation. The bleaching mechanism of HP and BP are different, so it is expected that the products and off-flavors from these pathways would also be distinct, as previously noted by Jervis et al. (2012). Hydrogen peroxide bleaching may also result in Fenton reactions (Jervis et al., 2012). The

decreased iron observed in HP bleached SPC80 could be due to iron catalyzed HP decomposition, thus allowing for more radical peroxidation and increased concentrations of hydroxyl radicals that can lead to increased lipid oxidation. This mechanism was previously suggested by Jervis et al. (2012) for HP bleaching of WPC80.

Functionality

The WPC80 with similar manufacture and treatment to SPC80 manufactured in this study did not produce any foams (Jervis et al., 2012). However, all SPC80 in the current study foamed, likely due to the lower fat content compared to WPC80 (0.52% vs 4.67% on a dry basis, respectively). Properties of foaming, such as overrun, foam stability, and yield stress, depend upon the ability of proteins to unfold and orient at the air-water interface (Schmidt et al., 1984). Foam stability in SPC80 was significantly affected by bleaching. The foams of SPC80 bleach treatments were more stable than those not bleached. Previous studies have indicated that foaming is enhanced by oxidizing agents, such as both BP and HP, suggesting a potential role of sulfhydryl-disulfide mediated protein conformational reactions in the foaming process (Schmidt et al., 1984). This suggests that BP and HP cause protein denaturation and that these effects on foaming previously were not detected in WPC80 due to their high fat content.

The solubility of SPC80 also varied depending on bleach treatment and pH. Samples without bleach were highly insoluble at pH 3 and 4. Benzoyl peroxide -bleached samples were highly insoluble at pH 4 and 5 while HP-bleached samples were only highly insoluble at pH 5. No differences in solubility at pH 6 or 7 were detected among samples. No differences among samples at various pH values were observed in WPC80 with different

bleach treatment (Jervis et al., 2012). It is possible that the elevated fat content obscured the significant differences among WPC80 and without this obscuring factor, differences in solubility in SPC80 due to bleaching were more discernible than in WPC80. As protein denatures, it is more likely to precipitate thus leading to decreased solubility. Similar trends in heat stability among SPC80 were also observed among WPC80 (Jervis et al., 2012). Hydrogen peroxide-bleached SPC80 did not gel after 10 min of heating while both the BP and the unbleached samples did form gels. This same result was seen in WPC80 (Jervis et al., 2012). Jervis et al. (2012) hypothesized that HP bleaching damaged proteins differently than BP and unbleached WPC80. Hydrogen peroxide may attack other conjugated double bonds leading to increased free radicals, which would increase protein degradation or amino acid damage. However, BP appears to be more specific, attacking primarily norbixin (Jervis et al., 2012). These differences in oxidation and oxidation products may also be due to differences in solubility of the bleaching agents themselves. Oxidation of proteins leads to cleavage of peptide bonds producing free amino acids and amino acid residues that are highly susceptible to oxidation (Stadtman and Levine, 2003). Oxidation of free amino acids may also be due to the Fenton reaction system (Stadtman and Levine, 2003). Consistent with WPC80 results, HP-bleached SPC80 contained decreased amounts of elemental iron, suggesting that the Fenton reaction had occurred (Jervis et al., 2012).

Hydrogen peroxide -bleached SPC80 may contain smaller fractions of protein than BP-bleached SPC80 which were unable to align quickly and gel within the 10 min of heating. Smaller fragments would take more time to aggregate and align to form a gel than intact proteins and have been shown to increase the denaturation temperature of β -LG

(Barbeau et al., 1996). Jervis et al. (2012) noted that HP appeared to improve the heat stability temperature of the whey protein which may enhance functionality. However, it was unclear if there was damage to the amino acids which would then affect the nutritional value. Future work comparing HP and BP bleaching should include amino acid compositional analysis compared to unbleached controls.

CONCLUSIONS

Without the confounding effect of the cheese making procedure, the contribution of bleaching on protein flavor and functionality were confirmed and clarified. Functionality differences were more prevalent in bleached SPC80 compared to previous studies conducted with WPC80, suggesting that bleaching impacts functional properties as well as flavor. Benzoyl peroxide exhibited higher bleaching efficiency and created less off flavors in SPC80 than hydrogen peroxide, which is consistent with previous studies with WPC80. Hydrogen peroxide may improve the heat stability of rehydrated SPC80, potentially enhancing functionality when used as an ingredient in heat treated products. Understanding more about how each step contributes to the overall flavor, without confounding effects, is critical to producing consistent, flavorless dairy protein ingredients which can be used in a variety of applications without influencing consumer acceptability. These results confirm that bleaching impacts flavor and functionality of WPC and SPC80 and further suggests the need for alternative bleaching agents.

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Table 1. Mean (n = 3) composition (% by weight) and pH of the microfiltration (MF) permeate with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (BP) (50 ppm) or bleaching with hydrogen peroxide (HP) (500 ppm) before spray drying.

MF Permeate				
Treatment	Total solids	Fat	CP	pH
No color	3.27	0.001	0.73	6.71
No color + BP	3.17	0.004	0.72	6.66
No color + HP	3.22	0.003	0.70	6.71
Annatto	3.25	0.004	0.72	6.72
Annatto + BP	3.31	0.005	0.72	6.71
Annatto + HP	3.33	0.007	0.73	6.73
R - square	0.71	0.48	0.49	0.057
SEM	0.084	0.003	0.029	0.036

No differences in means within the same column were detected ($P > 0.05$).

CP = crude protein (total nitrogen x 6.38);

SEM = Standard error of the means.

Table 2. Mean (n = 3) composition (% by weight) of spray dried 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (BP) (50 ppm) or bleaching with hydrogen peroxide (HP) (500 ppm) calculated on a dry and wet basis.

Treatment	Dry basis			Wet basis	
	Moisture	Fat	CP	Fat	CP
No color	4.96	0.62	83.07	0.59	78.95
No color + BP	5.07	0.52	83.35	0.49	79.12
No color + HP	4.73	0.52	83.89	0.50	79.91
Annatto	4.52	0.53	82.88	0.51	79.13
Annatto + BP	5.02	0.45	82.71	0.43	78.55
Annatto + HP	4.54	0.51	83.09	0.49	79.31
R - square	0.25	0.73	0.78	0.72	0.70
SEM	0.326	0.043	0.308	0.041	0.342

No differences in means within the same column were detected ($P > 0.05$).

CP = crude protein (total nitrogen x 6.38).

SEM = Standard error of the means.

Table 3. Mean (n = 3) mineral composition (mg/kg) of spray dried 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (BP) (50 ppm) or bleaching with hydrogen peroxide (HP) (500 ppm) calculated on a dry basis.

Treatment	P	Ca	K	Mg	S	Na	Fe
	-wt%-	-wt%-	-wt%-	-wt%-	-wt%-	mg/kg	mg/kg
No Color	0.407 a	0.577 a	0.697 a	0.070 a	1.13 bc	1800 ab	9.06 a
No Color + BP	0.377 b	0.543 bc	0.667 a	0.070 a	1.12 c	1693 d	8.23 ab
No Color + HP	0.347 c	0.493 d	0.707 a	0.060 b	1.14 a	1803 ab	6.97 bc
Annatto	0.390 ab	0.567 ab	0.677 a	0.070 a	1.12 bc	1727 cd	8.98 a
Annatto + BP	0.387 ab	0.567 ab	0.693 a	0.070 a	1.13 ab	1764 bc	8.65 a
Annatto + HP	0.363 bc	0.520 cd	0.723 a	0.067 a	1.15 a	1851 a	6.17 c
R - square	0.94	0.97	0.86	0.93	0.98	0.97	0.95
SE	0.007	0.007	0.019	0.001	0.004	13.7	0.33

^{a-d} Means in the same column not sharing a common superscript are different ($P < 0.05$).

SE = Standard error

Table 4. Mean (n = 3) color (L, a, and b-values) of liquid (10% w/v powders) and spray dried 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (BP) (50 ppm) or bleaching with hydrogen peroxide (**HP**) (500 ppm).

Treatment	L-value	a-value		b-value	
	Powder	Liquid	Powder	Liquid	Powder
No color	88.6 ^d	5.15 ^{abc}	1.59 ^c	13.9 ^b	7.21 ^{cd}
No color +BP	89.3 ^c	3.23 ^{bc}	1.16 ^{cd}	9.9 ^b	6.48 ^d
No color + HP	91.2 ^a	3.20 ^{bc}	0.75 ^d	10.4 ^b	6.00 ^d
Annatto	87.7 ^c	8.13 ^a	3.36 ^a	23.3 ^a	14.7 ^a
Annatto + BP	89.3 ^c	2.29 ^c	1.42 ^c	9.8 ^b	8.24 ^c
Annatto + HP	89.9 ^b	5.82 ^{ab}	2.54 ^b	21.8 ^a	12.8 ^b
R - square	>0.99	0.85	0.98	0.93	0.99
SEM	0.083	0.673	0.102	1.286	0.299

^{a-c} Means in the same column not sharing a common superscript are different ($P < 0.05$).

SEM = Standard error of the means.

Table 5. Mean (n = 3) norbixin recovery (mg of norbixin/kg of total solids) from 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (BP) (50 ppm) or bleaching with hydrogen peroxide (HP) (500 ppm).

No Color	No Color + BP	No Color + HP	Annatto	Annatto + BP	Annatto + HP	R - squared	SE
1.62 cd	1.69 c	0.69 d	15.03 a	1.26 cd	7.38 b	0.98	0.33

^{a-d} Means in the same row not sharing a common superscript are different ($P < 0.05$).

SE = Standard error

Table 6. Mean (n = 3 replicates with 10 panelists) sensory attributes¹ of 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (**BP**) (50 ppm) or bleaching with hydrogen peroxide (**HP**) (500 ppm).

	Aroma Intensity	Sweet Aromatic	Cooked/Canned Corn	Fatty	Cardboard	Bitter	Astringency
No Color	2.2 c	1.7 a	3.3 a	ND	0.6 d	0.6 a	2.2 d
No Color BP	2.3 b	0.5 b	2.0 bc	1.5 b	1.6 c	0.6 a	2.6 a
No Color HP	2.6 a	ND	2.0 bc	1.8 a	2.5 b	0.7 a	2.6 a
Annatto	2.3 b	1.8 a	3.1 a	ND	0.7 d	0.6 a	2.3 cd
Annatto BP	2.0 c	0.9 b	2.0 bc	1.5 b	1.9 c	0.8 a	2.4 bc
Annatto HP	2.6 a	ND	1.7 c	1.9 a	2.8 a	0.6 a	2.5 ab
R- squared	0.66	0.92	0.90	0.94	0.93	0.78	0.77
SE	0.11	0.12	0.11	0.11	0.12	0.07	0.05

^{a-d} Means in the same column not sharing a common superscript are different ($P < 0.05$)

ND=Not Detected.

¹ Intensities were scored on a 0 to 15 universal scale where 0 = none and 15 = very high intensity (Meilgaard et al., 1999). Dried whey ingredient intensities usually fall between 0 and 4 on this scale (Drake et al., 2003; Wright et al., 2009). SE = Standard error.

Table 7. Mean (n = 3) concentration* ($\mu\text{g}/\text{kg}$) or relative abundance ($\mu\text{g}/\text{kg}$) of selected volatile compounds ($\mu\text{g}/\text{L}$) of spray dried 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (BP; 50 ppm) or bleaching with hydrogen peroxide (HP; 500 ppm) isolated using solid-phase microextraction.

	Annatto No Bleach	Annatto BP	Annatto HP	No Color No Bleach	No Color BP	No Color HP	R- square	SE
2,3 Octanedione	1.09c	3.69b	11.50a	0.90c	4.69b	9.73a	0.78	0.70
2,4 Nonadienal	0.986a	1.05a	0.961a	0.798a	0.664a	0.758a	0.05	0.30
2,6 Nonadienal	2.05a	2.44a	2.90a	2.28a	1.69a	1.86a	0.06	0.12
*2-Pentyl furan	9.97b	172a	191a	6.99b	149a	156a	0.51	15
*3-Methylbutanal	28.1a	20.4ab	26.6a	13.5b	20.4ab	17.2b	0.28	2.9
Alpha Pinene	6.78a	5.63a	4.82a	5.97a	5.85a	5.60a	0.04	0.80
*Benzaldehyde	11.8d	11.9d	23.0b	11.9d	14.5c	25.7a	0.87	2.3
*Decanal	3.23b	5.78ab	7.83a	6.27ab	3.56b	5.14ab	0.23	0.71
Decane	32.8a	28.9ab	19.2ab	33.4a	25.4ab	12.7b	0.17	0.36
Diacetyl	1.06a	1.06a	1.79a	0.927a	0.706a	0.936a	0.06	0.15
DMTS	1.57a	1.36a	1.64a	1.24a	1.26a	1.40a	0.06	0.19
*Heptanal	4.59d	9.35cd	34.6a	3.97d	10.7c	24.7b	0.79	1.9
*Hexanal	2.59c	78.0b	308a	2.93c	104b	290a	0.78	19
Limonene	1.12a	1.79a	2.21a	2.14a	1.89a	1.98a	0.10	0.25
Methional	2.60a	1.85ab	1.43b	1.49ab	1.85ab	1.95ab	0.13	0.25
*Nonanal	4.34d	11.0bc	21.8a	7.13cd	9.44cd	15.2b	0.50	1.6

*Octanal	3.92ab	4.73ab	5.16a	2.69b	4.72ab	4.95a	0.14	0.61
Toluene	8.44b	7.88b	7.02b	5.51b	16.6a	7.25b	0.39	1.2

^{a-d} Means in same row not sharing a common superscript are different ($P < 0.05$).

* Quantified using external standard curves to yield concentration

SE = Standard error.

Table 8. Mean (n = 3) foam stability (minutes) from 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with **(BP)** benzoyl peroxide (50 ppm) or bleaching with **(HP)** hydrogen peroxide (500 ppm).

No Color	No Color + BP	No Color + HP	Annatto	Annatto + BP	Annatto + HP	R - squared	SE
24.9 c	31.8 a	30.2 a	27.5 b	30.1 a	27.5 b	0.89	0.74

^{a-c} Means in the same row not sharing a common superscript are different ($P < 0.05$).

SE = Standard error

Table 9. Mean (n = 3) yield stress (Pa) of foams generated from 80% serum protein concentrate (SPC) with and without annatto color added to the milk followed by either no bleaching, bleaching with **(BP)** benzoyl peroxide (50 ppm) or bleaching with **(HP)** hydrogen peroxide (500 ppm).

No Color	No Color + BP	No Color + HP	Annatto	Annatto + BP	Annatto + HP	R - squared	SE
38.9 b	43.9 a	38.4 b	42.9 ab	38.5 b	29.8 c	0.84	1.60

^{a-c} Means in the same row not sharing a common superscript are different ($P < 0.05$).

SE = Standard error

Figure 1. Principal component biplot of relative abundance of volatile compounds found in 80% serum protein concentrate (SPC80) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm).

F1 and F2 = principal components 1 and 2; HP= hydrogen peroxide, BP= benzoyl peroxide

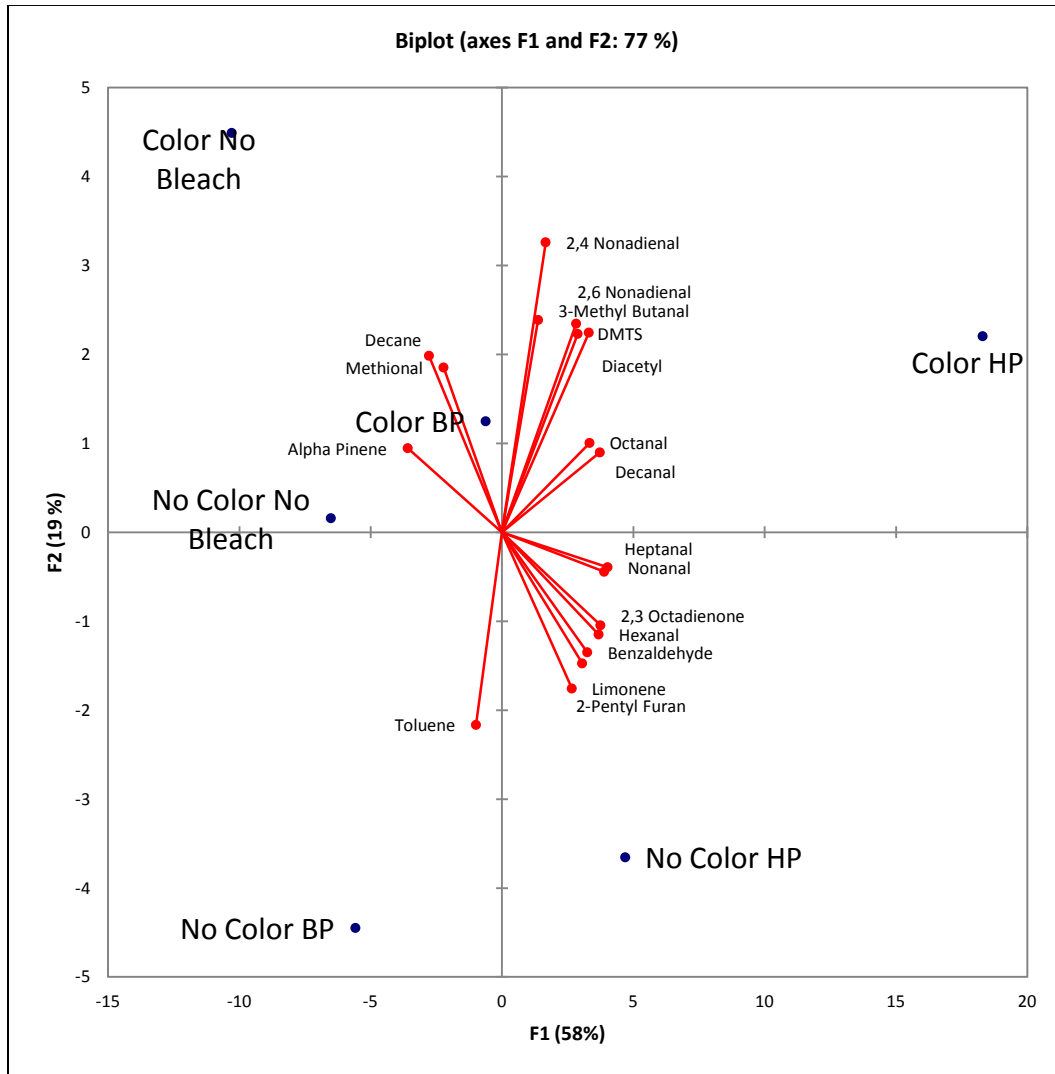


Figure 2. Mean percent insolubility of 80% serum protein concentrate (SPC80) at 10% (w/v) protein using turbidity loss (low turbidity loss indicates high solubility). **The SPC80** was manufactured with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (**BP**) (50 ppm) or bleaching with hydrogen peroxide (**HP**) (500 ppm).

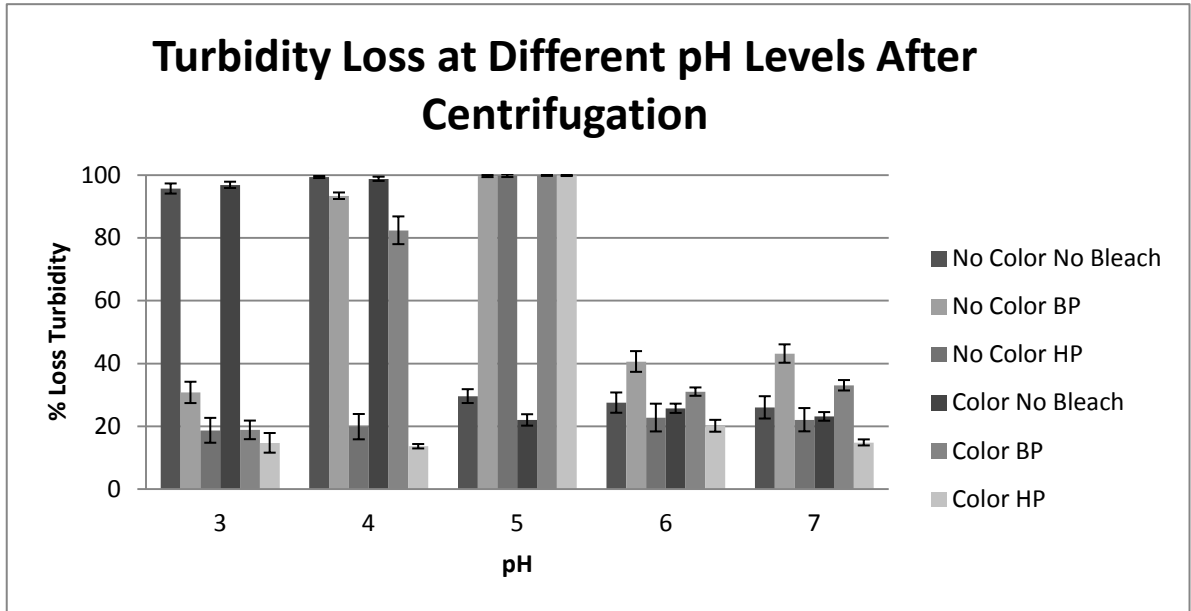


Figure 3. Mean percent insolubility of 80% serum protein concentrate (SPC80) at pH 4.6 and 10% (w/v) protein heated for 0, 10, 20, and 30 min at 90°C measured using turbidity loss (low turbidity loss indicates high solubility). SPC were manufactured with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm).

*Solutions not represented gelled and therefore could not be measured

