ABSTRACT

KANG, EY JUNG. Alternative Bleaching Methods for Cheddar Liquid Whey. (Under the direction of Dr. MaryAnne Drake.)

Annatto (Bixa orellana L.) is a natural colorant used to produce orange Cheddar cheese and is primarily composed of the carotenoid norbixin. The increasing demand for whey protein as an ingredient requires a bland-tasting and uncolored final product. Since a percentage of annatto remains in the whey, bleaching whey is a necessary step. Recent studies have highlighted the negative effect of bleaching on whey flavor while concurrently there is a dearth of current studies on bleaching conditions and efficacy. Recent international mandates have placed additional concern on the use of benzoyl peroxide as a bleaching agent.

The objective of this research was to evaluate alternative methods for bleaching liquid whey: ultraviolet radiation (UV), acid-activated bentonite (BT), and lactoperoxidase (LP). Two studies were conducted to investigate alternative bleaching methods for liquid Cheddar whey. For the first study, liquid whey was subjected to one of 4 treatments at 50°C: control (CT; 1 h), HP (250 mg/kg; 1 h), UV (1 min), or BT (0.5% w/w; 1 h). Norbixin elimination was 28, 79, and 39% for HP, BT, and UV treatments, respectively. The HP and BT WPC80 had higher fatty flavor compared to the CT WPC80 (P < 0.05), and the UV WPC80 had distinct mushroom/burnt and animal flavors. Volatile compound results were consistent with sensory results and confirmed higher relative abundances of volatile aldehydes in UV and HP WPC80 compared to CT and BT WPC80. Based on bleaching efficacy and flavor, BT may be an alternative to chemical bleaching of fluid whey.
The second study was based on recent studies that demonstrated that native milk lactoperoxidase (LP) was active in cheese whey and could be used to bleach colored fluid whey. The objective of this study was to investigate the impact of milk pasteurization temperature on the bleaching efficacy of fluid whey. Milk was not pasteurized, pasteurized at 63°C for 30 min (low heat; LH) or 78°C for 28 sec (high heat; HH) and colored Cheddar wheys were manufactured. The whey from raw milk was either unpasteurized (URW) or pasteurized at 63°C for 30 min (RW). Wheys from LH and HH treated milk were pasteurized at 63°C for 30 min (LW and HW). Hydrogen peroxide was then added at 250 ppm (chemical bleaching) or 25ppm (LP bleaching) at 35 or 50°C and aliquots were collected after 0, 3, 5, 7, 10, 15, 20, and 60 min. LW had a higher bleaching efficacy from 25ppm HP (90% norbixin destruction) than 250ppm HP (20% norbixin destruction) at 35°C and 50°C, consistent with LP activity. The norbixin content of unbleached HW was 18% lower than that of unbleached URW, and the protein content was decreased by 27% for unbleached HW compared to unbleached LW. Percent norbixin destruction in whey from HW was lower than LW in all treatments and there were no differences between 25 and 250ppm HP (22% norbixin destruction). Regardless of milk pasteurization temperature and bleaching agent, greater bleaching was observed with increased time. These results demonstrate the influence of milk heat treatment on bleaching efficacy and also suggest differences in norbixin association with whey components due to different milk heat treatments.

It is critical for the dairy industry to have alternatives to chemical bleaching methods which will not only be more effective in bleaching, but will also improve the flavor profile of dry whey ingredients. This research has established that BT is a possible alternative
bleaching agent and has also elucidated the role of milk and/or whey heat treatment on LP bleaching of whey.
Alternative Bleaching Methods for Cheddar Liquid Whey

by
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DEDICATION

To my loving parents, who have always encouraged me to follow my dreams.
BIOGRAPHY

Ey Jung Kang (Jamie) is from Seoul, South Korea. She is the second daughter of Dr. Kang and Mrs. Han. She graduated from Sookmyung Women’s University with a Bachelor of Science degree in food and nutrition. Intrigued by the story of the food scientists at NASA she decided to continue her studies in food science and technology at Oregon State University. Upon completing her Master of Science degree, she continued her Ph.D research under the direction of Dr. MaryAnne Drake at North Carolina State University.
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CHAPTER 1:

LITERATURE REVIEW

INVITED REVIEW: ANNATTO USAGE AND BLEACHING IN DAIRY FOODS

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ABSTRACT

Annatto is a yellow/orange colorant that is widely used in the food industry, particularly in the dairy industry. Annatto, consisting of the carotenoids bixin and norbixin, is most commonly added to produce orange cheese, such as Cheddar, to achieve a consistent color over seasonal changes. This colorant is not all retained in the cheese, and thus a percentage remains in the whey, which is highly undesirable. As a result, whey is often bleached. Hydrogen peroxide and benzoyl peroxide are the 2 bleaching agents currently approved for bleaching whey in the United States. Recent studies have highlighted the negative effect of bleaching on whey flavor while concurrently there is a dearth of current studies on bleaching conditions and efficacy. Recent international mandates have placed additional concern on the use of benzoyl peroxide as a bleaching agent. This review discusses the advantages, disadvantages, regulatory concerns, flavor implications, and optimal usage conditions of 2 widely used bleaching agents, hydrogen peroxide and benzoyl peroxide, as well as a few alternative methods including lipoxygenase, peroxidase, and lactoperoxidase systems.

Key words: annatto; bleach; flavor; whey
INTRODUCTION

Annatto is a yellow/orange carotenoid that is widely used in the food industry. Annatto comes from seeds of the tropical tree fruit Bixa orellana, which was named after Francisco de Orellana, a scientist and explorer of the upper Amazon (Giuliano et al., 2003). Clusters of the fruit, which is capsular shaped and covered in burrs, grow on this tree and there are about 10 to 50 small seeds inside the fruit (Ames and Hofmann, 2001). The seeds are covered in a bright red pulp and this pulp contains the annatto pigment (Giuliano et al., 2003). Latin America produces about 60% of the world's annatto, followed by Africa (27%) and Asia (12%) (Giuliano et al., 2003). Prices for annatto seeds depend on production and are proportional to the content of bixin, the major pigment (Giuliano et al., 2003). The United States and Europe are the 2 largest importers of annatto seeds, but the Japanese market has grown rapidly since the introduction of the colorant in 1963 (Ames and Hofmann, 2001).

Annatto as a colorant is not a new concept. The Aztecs used annatto extract as a dye for textiles, body dye (such as in lipsticks), and as a food colorant in the drink cacahuatl (Giuliano et al., 2003). In addition to being used to impart color in various items, annatto has been used as a spice. Native Americans commonly used ground bixa seeds in their cacao beverages to impart a slight red color and to add a musky flavor comparable to that of paprika or saffron (Miksicek et al., 1981; Norton, 2006). Today, annatto is still used as a spice, especially in Latin American dishes such as cochinita pibil, a pork dish with ground bixa seeds and bitter orange juice (Gerlach and Gerlach, 2002). It is also used in sausages, fish, margarine, snacks, dressings, sauces, and confections, but usage varies from country to country due to different food customs and legislations (Ames and Hofmann,
2001; Scotter, 2009). The major application of annatto in the United States is within the dairy industry, where it is used to color cheeses and other dairy products (Emerton, 2008).  

**EXTRACTION OF ANNATTO**  

Three commercial processes are used to extract the carotenoid pigment from dried annatto seeds: direct extraction into oil, direct extraction into aqueous alkali, or indirect extraction with solvents (Preston and Rickard, 1980). Extraction with oil or solvent yields a colorant that is mainly bixin (Mortenson et al., 2008). Extraction with aqueous alkali (the form used in the dairy industry, e.g., for cheese color) saponifies bixin's methyl group on bixin, yielding norbixin as the principal colorant (Giuliano et al., 2003; Mortenson et al., 2008). Both bixin and norbixin naturally occur in the cis form but can be converted to the trans form by light and heat (McKeown 1963, 1965). The trans forms of both bixin and norbixin are more red than their cis forms (Preston and Rickard, 1980; Scotter et al., 1994).  

**VOLATILE COMPOUNDS**  

Although annatto has been used in foods as a spice (flavoring), there is little information about how volatile compounds of annatto contribute to flavor. Galindo-Cuspinera et al. (2002) evaluated volatile profiles of oil and water-soluble annatto extracts and reported that β-humulene was the major volatile present. β-Humulene is described as having a woody, spicy aroma and a slightly bitter taste (Galindo-Cuspinera et al., 2002). Other important volatiles found in annatto extracts that might contribute to flavor were p-xylene, toluene, α- and β-pinene, γ-elemene, and spathulenol. Annatto extraction uses heat (Scotter et al., 2002), which means that different commercial products may experience varying degrees of thermal
degradation. Further studies also suggest that heat treatment of annatto in foods may result in additional flavor-contributing volatile compounds. Scotter (1995, 1998, 2000, 2001) studied the thermal degradation products of both bixin and norbixin and found that heat released the aromatic compounds m-xylene and toluene. As mentioned previously, annatto is a major colorant used in the dairy industry, often in cheese and in processed cheese that involves heat treatment. Volatiles from thermal degradation of annatto in actual dairy products (e.g., cheese) have yet to be studied. Studies conducted by Scotter et al. (2002) and Galindo-Cuspinera et al. (2002) indicate that there are numerous odorants in annatto, such as β-humulene, p-xylene, toluene, α- and β-pinene, γ-elemene, and sapathulenol, with potential to influence food aroma; however, more studies are needed in this area.

**DISCOLORATION – OXIDATION**

Annatto has been used in dairy products since the 1800s to standardize the color of cheese, which varies due to seasonal feed variations in the milk. Chr. Hansen Inc., a major, modern-day supplier of annatto to the dairy industry, opened their first factory in 1874 (Kristin Schneider, Chr. Hansen Inc., Milwaukee, WI; personal communication). Over the years, an orange color has become expected in many cheeses, requiring addition of annatto to cheesemilk. According to 21CFR73.30 (US FDA, 2009a), annatto extract may be used for coloring foods as long as good manufacturing practices (GMP) are followed. Therefore, in the United States, there is no “maximum level” of usage for annatto; however, Scotter et al. (2002) reported that the maximum level of annatto addition in commercial Red Leicester cheese in the United Kingdom was 50 mg of norbixin/kg of cheese and the estimated range
of annatto in Red Leicester was 23.7 to 37.5 mg of norbixin/kg of cheese. For ripened orange, yellow, and broken white cheese, and flavored processed cheese in the United Kingdom, the maximum level of annatto addition is 15 mg/kg. The estimated range of annatto is 0.2 to 9.6 mg/kg \((n = 16)\) and 0.2 to 21.4 mg/kg \((n = 8)\) for ripened orange, yellow, and broken white cheese, and for flavored processed cheese, respectively. Currently in the United States, no study has been conducted to determine the amounts of annatto typically found in cheese. In the United States, annatto is approved as a color additive in foods at GMP, meaning that the “Annatto extract may be safely used for coloring foods generally, in amounts consistent with good manufacturing practice…” (Hallagan et al., 1995; Giuliano et al., 2003; US FDA, 2009a). Although annatto is used to standardize the color of cheese, some studies have reported the usage of annatto leading to pinking or discoloration of cheeses (Hood and White, 1929; Moir, 1933; Morgan, 1933; Barnicoat, 1937, 1950; Govindarajan and Morris, 1973). Bixin and norbixin have highly conjugated structures making these compounds susceptible to both oxidation and reduction. Oxidation is important to the fluid whey industry because oxidation leads to color loss, which is the primary goal of bleaching and will be discussed later in this review. Also important, a study suggested that norbixin might be able to bind with proteins such as β-casein or β-lactoglobulin and form a stable complex that can help prevent oxidation and color loss (Govindarajan and Morris, 1973). This reaction has not been proven; it may be desirable in some products such as cheese but reduce color removal during bleaching of whey.
CAROTENOIDS IN MILK

Carotenoids are lipophilic molecules that are found in milk fat. The diet of the cow can influence the color of cheese (Carpino et al., 2004). A wide variety of carotenoids and their degradation products are present in the forage that cows eat, but only a small number of different carotenoids can be identified in milk. This is because carotenoid transfer from diet to milk is low and carotenoids can be broken down to colorless compounds in the gastrointestinal tract (Noziere et al., 2006). Some of these colorless compounds may be transferred to the milk and have effects on the sensory profile even though they do not impart any color to the final product. Carotenoids identified in milk include lutein, violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, all-trans β-carotene, α-carotene, and 13-cis β-carotene. Among those, lutein and β-carotene are the most quantitatively important (Noziere et al., 2006) with β-carotene comprising about 90% of total carotenoids present in cow's milk (Hulshof et al., 2005).

Carotenoids can influence the sensory properties of milk either indirectly through their antioxidant properties by stabilizing oxidizable compounds or directly through their yellowing properties. β-Carotene can be converted to vitamin A, but incomplete conversion leaves a yellow color in the milk (Patton et al., 1980). The main components in milk are riboflavin (vitamin B$_2$), a green compound present in the aqueous phase, and β-carotene in the lipid phase (Noziere et al., 2006). Riboflavin and carotenoid contents vary widely in milk and are influenced by diet, breed, and season (Schröder, 2003; Noziere et al., 2006). Croissant et al. (2007) reported that milk from pasture-fed Holstein and Jersey cows exhibited more yellow color than that from their similar counterparts fed a conventional,
TMR diet. These findings were expected because pasture-fed cows ingested fresh forage, containing carotenoids, thus raising the concentration of carotenoids in milk fat. Carotenoids found in milk also carry over into fluid whey during cheese production, where they may increase the color of spray-dried whey and require a bleaching process.

CAROTENOID MEASUREMENT IN FOODS

Carotenoid content can be difficult to measure because carotenoids isomerize rapidly due to their conjugated double bonds (de Oliveira and Rodriguez-Amaya, 2007). Isomerization can be promoted by acid, light, and heat (de Oliveira and Rodriguez-Amaya, 2007). As such, precautions must be taken to minimize these reactions during extraction and measurement. These precautions include, but are not limited to, completion of analysis within the shortest time possible, protection from light, avoiding high temperatures or contact with acid, and the use of high-purity solvents (de Oliveira and Rodriguez-Amaya, 2007). Once carotenoids are isolated, qualification and quantification are typically performed using the same methods. Before quantification, the carotenoid must be positively identified. This is done using chromatography—open column, thin layer, or HPLC (Bushway, 1985). The best of these 3 methods, HPLC, saves time and can distinguish between stereoisomers (Bushway, 1985). The carotenoid can be identified using retention times, co-chromatography with carotenoid standards, or UV-visible absorption spectra (de Oliveira and Rodriguez-Amaya, 2007). After the carotenoid is positively identified, it is then quantified by using an external standard curve (de Oliveira and Rodriguez-Amaya, 2007).
ANNATTO MEASUREMENT IN FOODS

Annatto can be found in a wide array of foods ranging from dairy products to other naturally colored products. A colorimetric method for measuring the amount of annatto in commercial bleached and unbleached dry whey powder by extracting the annatto using ammonia, ethanol, and phosphate solution was reported by Hammond et al. (1975). Lancaster and Lawrence (1995) extracted annatto from high-fat dairy products, with good repeatability, although methods were labor intensive. Annatto was confirmed and components were identified via HPLC (Lancaster and Lawrence, 1995). Scotter et al. (2002) extracted and analyzed annatto in margarine, ice cream, custard powder, breakfast cereals, cakes, fish, and jellies using various extraction methods tailored to each food matrix followed by HPLC coupled with spectral confirmation. Bareth et al. (2002) described a rapid, simple extraction and detection method specific for annatto in cheese and milk products using HPLC and a spectrophotometric method. Croissant et al. (2009) reported a successful method to extract and quantify norbixin (the primary annatto constituent in whey) from liquid whey, liquid retentate, and spray-dried whey protein concentrate. Analytical methods for annatto and analysis in food have been reviewed by Scotter (2009).

BLEACHING

Food is bleached not only to improve color, but in some cases to improve its quality such as improving gluten development in flour. The bleaching process can be categorized into 3 groups depending on the mechanism: oxidizing bleach, reducing bleach, and biochemical bleach (Balls et al., 1943; Bottomley et al., 1989; Roos et al., 2006).
Oxidizing Bleach

The portion of a molecule that emits color is called a chromophore and it is usually the most fragile part of the molecule. Destroying the chromophore will often render a colored molecule colorless. Oxidizing agents (bleach) destroy (oxidize) the double bond(s) within a chromophore. This reaction changes the molecule into a different substance in which the chromophore does not exist or a shorter chromophore exists. A shorter chromophore will absorb light of a shorter wavelength than visible light (UV light) and therefore will not appear colored (Winter et al., 2008). Many oxidizing bleaches are used in industry including chlorine dioxide, which is used for the bleaching of wood pulp, fats and oils, cellulose, flour, textiles, and beeswax. Hydrogen peroxide and benzoyl peroxide and bromates, are oxidizing bleaches used for whey and flour bleaching and as maturing agents. Another application is in the wood and pulp industry to produce chlorine-free paper for which the industry uses peracetic acid, ozone, hydrogen peroxide, and oxygen in bleaching sequences.

Reducing Bleach

A reducing bleach works by converting double bonds in a chromophore into single bonds. This eliminates the ability of the chromophore to absorb visible light. Sunlight releases high-energy photons, often in the violet or UV range, that can disrupt bonds in a chromophore, rendering the substance colorless. Many colored molecules are relatively fragile and are damaged by photons of UV light. Traditionally, exposure to sunlight was the way to bleach fabrics and make them white (Bloomfield, 2007). Reducing bleaches commonly used in foods include lemon juice (in combination with sunlight) and sulfur dioxide.
Biochemical Bleach

Enzymes such as lipoxygenase (EC 1.13.11.12; lipoxydase) can fall in the third category of bleaching agents: biochemical bleaches. Lipoxygenase was first discovered in 1928 by Bohn and Hass (1953) as a carotene-destroying enzyme (“carotene oxidase”) in soybeans, and later designated as lipoxygenase. Acceleration of the oxidation of xanthophylls to colorless products has long been recognized as a property of soybean lipoxygenase used in the bleaching of bread dough (Balls et al., 1943). A patent on biochemical bleaching of dairy products was introduced in the United States recently (Roos et al., 2006). Roos et al. (2006) disclosed the use of lipoxygenases to bleach dairy products, including whey. Only small amounts of lipoxygenases have a whitening effect on dairy products such as milk, cheese, butter oil, cream, and whey products. The mechanism of bleaching by lipoxygenase is based on the oxidative transition of double bonds in carotenoids by radicals produced in the reaction of lipoxygenase and linole(n)ic acid. Soy lipoxygenase has been used for bleaching purposes in wheat and maize flour; however, it has not been previously applied to bleach whey. Because a more specific enzymatic reaction is occurring rather than a general oxidation treatment, as is the case with oxidizing bleaches, enzymatic bleaching may reduce off-flavors. Recently (November 2009), an external enzymatic (biochemical) bleach was launched for bleaching of fluid whey. MaxiBright (DSM Food Specialties, Delft, the Netherlands) is a fungal peroxidase that can purportedly be used in place of an oxidizing bleach to bleach fluid whey. The enzyme is specific for carotenoids (Zorn et al., 2003), thus yielding whiter whey without the use of traditional bleaching agents, and possibly reducing unwanted side effects of traditional bleaching. Like all peroxidases, MaxiBright requires
activation with 0.5 to 1 mM hydrogen peroxide, but the hydrogen peroxide is consumed and catalase addition is not required. There are currently no available published studies on the use of MaxiBright with whey or in comparison to traditional oxidizing bleaches. Food and Drug Administration's “generally regarded as safe” (GRAS) status has been filed for MaxiBright, and approval is anticipated in September 2010.

**BLEACHING WHEY**

Annatto is used by the dairy industry to color cheese. The specific amount of annatto that partitions into cheese and whey has not, to our knowledge, been directly studied. However, approximately 20% of the annatto added to cheese milk is estimated to pass into whey (Barnicoat, 1950). The color from annatto is highly unfavorable in dried whey products and thus a decolorizing process must be performed and has been in place in the dairy industry for more than 40 yr (McDonough et al., 1968). This process involves bleaching of liquid whey or liquid retentate with hydrogen or benzoyl peroxide. Hydrogen peroxide and benzoyl peroxide are the only bleaching agents legally allowed for treatment of whey in the United States and there are several restrictions to their use.

**Hydrogen Peroxide**

Hydrogen peroxide (H₂O₂) is a clear, colorless liquid with a slightly pungent odor. Hydrogen peroxide decomposes to oxygen and water during bleaching (Table 1). Residual hydrogen peroxide must be removed from whey and cheese milk physically or by the addition of catalase according to FDA regulations 184.1366 and 133.113 (US FDA, 2009l,e). Catalase converts hydrogen peroxide into oxygen and water (Table 1). Catalase use must not exceed
20 ppm and must be sufficient to remove any residual hydrogen peroxide (US FDA, 2009e). As hydrogen peroxide is a GRAS substance, the maximum treatment level for bleaching annatto-colored whey using hydrogen peroxide is 0.05% (<500 ppm) of the whey (US FDA, 2009l). There are no specific provisions in European Union regulations regarding the use of hydrogen peroxide as a bleaching agent for dairy products. When no national provisions on processing aids exist, their use is controlled by general safety provisions. Therefore, it can be understood that European Union regulations are in agreement with FDA regulations for the use of hydrogen peroxide as a bleaching agent for whey.

In addition to being used as a bleaching agent, hydrogen peroxide can be used as an antimicrobial agent in milk, intended for cheese making at 0.05% (wt/wt) level (US FDA, 2009l). Likewise, hydrogen peroxide can be used as an antimicrobial agent during the preparation of modified whey by electrodialysis methods at 0.04% (wt/wt) level (US FDA, 2009l). Hydrogen peroxide can be used as a microbial agent in cheese milk and a side effect will be bleaching milk. However, federal food and drug regulations do not support the use of hydrogen peroxide solely for the use of bleaching milk for cheese and related cheese products (US FDA, 2009l).

**Benzoyl Peroxide**

Benzoyl peroxide (C₁₄H₁₀O₄) is a colorless, crystalline solid permitted for use in removing color in whey products that are not used for infant formula. Like hydrogen peroxide, benzoyl peroxide (or a mixture of benzoyl peroxide with potassium alum, calcium sulfate, and magnesium carbonate) is also a GRAS substance and can be used to bleach dairy ingredients.
Unlike hydrogen peroxide, benzoyl peroxide has no limitation on usage rates in foods other than current GMP rules (US FDA, 2009k). Benzoyl peroxide is GRAS as a direct human food ingredient upon the following current GMP conditions of use: (1) the ingredient is used as a bleaching agent in food, (2) the ingredient is used in the following foods, milk used for production of Asiago fresh and Asiago soft cheese (US FDA, 2009b), Blue cheese (US FDA, 2009c), Caciocavallo Siciliano cheese (US FDA, 2009d), Gorgonzola cheese (US FDA, 2009f), Parmesan and Reggiano cheese (US FDA, 2009g), Provolone cheese (US FDA, 2009h), Romano cheese (US FDA, 2009i), and Swiss and Emmentaler cheese (US FDA, 2009j), annatto-colored whey, such that the final bleached product conforms to the descriptions and specifications for whey, concentrated whey, or dried whey, at levels not to exceed current GMP. In contrast to hydrogen peroxide, benzoyl peroxide may be used to bleach milk for certain cheese productions as listed above, whereas hydrogen peroxide may not be used to bleach milk for cheese make in any cheese, but hydrogen peroxide is permitted to be used at 0.05% in milk for antimicrobial purposes. The current GMP is as follows: the weight of the benzoyl peroxide is not more than 0.002% (20 ppm) of the weight of the milk being bleached, and the weight of the potassium alum, calcium sulfate, and magnesium carbonate, singly or combined, should not be more than 6 times the weight of the benzoyl peroxide used (US FDA, 2009b,c,d,f,g,h,i,j). Whey is considered a GRAS substance and thus has no standard of identity. This means that there is no federal regulation for usage rates of benzoyl peroxide in whey for bleaching but using benzoyl peroxide as a preservative is not acceptable. Should benzoyl peroxide be used at levels >1% (wt/wt) in liquid whey, the manufacturer would need to justify the usage rate. Most additives are used at a rate of 1 to
3% (wt/wt), but any usage rate over 1% (wt/wt) raises many red flags (N. Ratzlaff, USDA, Lisle, IL; personal communication).

There are concerns about benzoyl peroxide bleaching as it has only been recently approved by Codex Alimentarius, with the maximum level (100 mg/kg) for liquid whey and whey products (excluding infant applications) being adopted in 2007, and the maximum level (100 mg/kg) for dried whey and whey products (excluding infant applications) being adopted in 2005 (Codex, 2008). The European Commission (EC) allows both hydrogen peroxide and benzoyl peroxide to be used as whey bleaching agents (designated as processing aids, not food additives), but individual country provisions may be stricter (Bianca Herr, Leatherhead Food Institute, Leatherhead, UK; personal communication). Many Asian and European government regulators do not like the use of benzoyl peroxide and consider benzoic acid and other breakdown products of benzoyl peroxide harmful. There is a possibility that the carrier used for benzoyl peroxide may be considered an allergen. In addition, certain carriers may pose problems with membrane fouling or leave residues of insoluble carrier on equipment. Benzoyl peroxide reacts with oxidizable compounds and is converted into water-soluble benzoic acid following bleaching (Table 1). A petition was submitted by the US Dairy Export Council (USDEC) to the Taiwan Department of Health, Food Sanitation, and Safety for the use of benzoyl peroxide in whey powder and was approved on December 20, 1999 (Johnson, 2006). Benzoyl peroxide is not an approved bleaching agent for whey in China or Japan and neither country's regulations allow for residual benzoic acid in dried whey products.
Because of stricter regulations in China with their new food safety law (June 1, 2009), the Chinese government is now routinely testing for the presence of benzoic acid (Anonymous, 2009). The safety of benzoic acid and the derivative benzoates have been studied extensively (Sharratt et al., 1964; Nair, 2001; Qi et al., 2009). Benzoic acid is found naturally in milk at low concentrations, 0 to 1 mg/kg, and in fermented dairy products at higher concentrations, typically about 20 mg/kg but up to 50 mg/kg (Sieber et al., 1995). During the fermentation process, lactic acid bacteria convert hippuric acid, a naturally occurring component of milk, to benzoic acid (Sieber et al., 1995). Benzoic acid can also be added as a preservative to cheese rennet and thus be found in cheeses from that route as well as from lactic acid fermentations. Benzoic acid is found in cheeses, but at lower concentrations than in fermented dairy products (Sieber et al., 1995). Subsequently, benzoic acid occurs naturally in whey and whey protein powders. Adverse reactions to benzoic acid-related compounds are rare, and life-threatening reactions are extremely rare (JECFA, 2004). Chang et al. (1977) found that when benzoyl peroxide was added to Edam cheese whey, after it was heated to 69°C for 6 h and cooled, 91.7% of the benzoyl peroxide used was recovered as benzoic acid, and minor amounts of hydroxybenzoic acids, phenylbenzoate, phenol, and benzoyl peroxide were found.

**Lactoperoxidase**

When the lactoperoxidase system is used for antimicrobiological purposes, external addition of thiocyanate or peroxide is required. However, when bleaching whey with hydrogen peroxide, companies often take advantage of the naturally existing lactoperoxidase system in
milk in combination with externally added hydrogen peroxide to obtain maximum bleaching effectiveness of fluid whey. No additional enzyme or thiocyanate needs to be added for bleaching purposes (E. Bastian; unpublished data). Lactoperoxidase (LP; EC 1.11.1.7) is a natural enzyme found in milk and constitutes about 0.5% of the total whey proteins in bovine milk. Lactoperoxidase is the second most abundant enzyme in raw milk (Kussendrager and van Hooijdonk, 2000; Fox and Kelly, 2006). The LP system consists of 3 components: lactoperoxidase, thiocyanate (SCN\(^-\)), and hydrogen peroxide, and the system is only active in the presence of these 3 components (Seifu et al., 2005). The LP system is a potent bacteriocidal system that has been used to preserve raw milk without refrigeration. Depending on the concentrations of SCN\(^-\) and hydrogen peroxide used to activate the LP system, preservation can vary from 24 h at 35°C to 48 h at 20 to 22°C (Fweja et al., 2007). This system is 50 to 100 times more effective than hydrogen peroxide alone (Fox and Kelly, 2006). Lactoperoxidase is relatively heat stable with denaturation starting at about 70°C (Kussendrager and van Hooijdonk, 2000). Heat stability studies were conducted in milk, whey, permeate, and buffer, and the enzyme was reported to be more stable to heat in whey and milk possibly because of their higher calcium ion concentration (Kussendrager and van Hooijdonk, 2000). Below pH 5.3, LP is less heat stable (Kussendrager and van Hooijdonk, 2000) and thus the optimal pH is 5.5 to 6.3 (Bottomley et al., 1989), which includes the pH range of whey. As such, sufficient amounts of the active enzyme are present in pasteurized milk and whey.

This system can be applied to bleach fluid whey by the addition of low concentrations of hydrogen peroxide to activate they system. In 1989, a patent was issued for decolorizing
whey products using the LP system (Bottomley et al., 1989). Lactoperoxidase reacts in the presence of hydrogen peroxide to convert SCN\(^{-}\) to hypothiocyanite (OSCN\(^{-}\)). Hypothiocyanite is a strong oxidizing agent that reacts with carotenoids such as norbixin and oxidizes the double bonds, removing the conjugation and thus removing the color of the compound. It should be noted that no additional enzyme or thiocyanate needs to be added, nor does catalase need to be added because the added hydrogen peroxide is consumed. The time required can be variable but is generally 20 to 45 min at 40°C. The recommended time and temperature is 30 min at 40°C; 40°C is acceptable from a regulatory perspective as long as the whey is not held at that temperature for more than 2 h. The bleaching reaction occurs within 30 min (Bottomley et al., 1989).

Lactoperoxidase catalyzes the oxidation of annatto but is inactivated by high concentrations of hydrogen peroxide (Bottomley et al., 1989). As such, hydrogen peroxide concentrations should be kept at or near 10 ppm (Bottomley et al., 1989). Additionally, the inactivation effect depends on the concentration of hydrogen peroxide and on the length of time the enzyme is exposed. If the concentration of hydrogen peroxide present in the whey falls below the inactivating concentration before complete inactivation of the enzyme has occurred, decolorization will still occur but at a slower rate (Bottomley et al., 1989).

**ADVANTAGES AND DISADVANTAGES OF BLEACHING**

**Hydrogen Peroxide**

Higher temperatures are generally more effective than lower temperatures. Reaction temperatures above 74°C increased neither the rate nor the extent of color removal but only
caused protein denaturation (McDonough et al., 1968). The oxidized flavors that were apparent immediately after bleaching purportedly disappear after evaporation and drying (McDonough et al., 1968), although a recent study demonstrated that this is not the case (Croissant et al., 2009). In addition, hydrogen peroxide has little to no effect on the nutrients present (Teply et al., 1958). Teply et al. (1958) analyzed milks and subsequent cheese and whey when the milk was bleached using 5, 10, and 25 times the normal amount of hydrogen peroxide and found that a strong treatment may alter proteins and amino acids in milk but in general there was no effect on the composition or nutritional value of the milk, cheese, or whey. The disadvantages to using hydrogen peroxide are that it must be inactivated with catalase, it could cause oxidized flavors, it is corrosive to equipment (Gilliland, 1969), and it is less economical to use because it requires more peroxide for satisfactory bleaching (Chang et al., 1977). A current review of prices for hydrogen peroxide and benzoyl peroxide suggests that the previous statement may be dependent on the bleaching conditions and usage rate. Hydrogen peroxide is sold in 53-gal drums for $200/drum; the liquid is 34% solids. Benzoyl peroxide is sold as a powder and costs $4.62 per kg. Depending on the concentration, temperature, reaction time, and pH, bleaching with hydrogen peroxide may alter the functionality of total and individual whey proteins (Cooney and Morr, 1972; Munyua, 1975). Unfortunately, the 2 prominent, albeit dated, studies (Cooney and Morr, 1972; Munyua, 1975) on the functional properties of whey protein bleached with hydrogen peroxide dealt with concentrations above the legal limit. Because these studies used a level above the legal maximum, the effects documented in those studies may not be representative of the effects of hydrogen peroxide used at lower levels.
According to Munyua (1975), hydrogen peroxide concentrations greater than 0.1% in fluid whey or milk caused a 5 to 8% decrease in the nonpolar amino acids such as aspartic acid, threonine, glutamic acid, methionine, trypsin, phenylalanine, histidine, lysine, tryptophan (25% decrease), and arginine. In contrast, free sulfhydryl groups increased as the hydrogen peroxide concentration increased. Increasing exposure time increased the number of free sulfhydryl groups up to 24 h. The researchers postulated that hydrogen peroxide reacted first with readily oxidized amino acids such as methionine. Higher concentrations of hydrogen peroxide, increased temperatures, and longer holding times all increased the amount of whey protein denaturation.

Reaction temperature during bleaching can affect whey protein denaturation. Cooney and Morr (1972) demonstrated 4% protein denaturation when whey was treated with 1% (10,000 ppm) hydrogen peroxide for 24 h at 25°C, whereas 28% protein denaturation was achieved by treating whey for 6 h at 50°C. It should be noted that the hydrogen peroxide concentration range used in this study was above the legal limit for hydrogen peroxide usage. More studies are needed in this area to determine if these effects are observed at lower concentrations. Although the effect of pH was minor compared with other variables, the pH resulting in the greatest amount of protein denaturation depended on the specific whey protein. For instance, immunoglobulins and bovine serum albumin were more readily denatured at lower pH, whereas β-lactoglobulin denaturation was enhanced at pH closer to neutral. α-Lactalbumin exhibited much less denaturation than β-lactoglobulin under the same conditions (Law and Leaver, 2000).
Benzoyl Peroxide

The effectiveness of benzoyl peroxide for removing color in whey depends on the amount used, how it is applied, the whey components present, and the exposure time and temperature. McDonough et al. (1968) reported that both benzoyl peroxide and hydrogen peroxide were effective bleaching agents, but that benzoyl peroxide was more effective at all temperatures. The rate and extent of decolorization by hydrogen peroxide and benzoyl peroxide for annatto in Cheddar cheese whey increased as the temperature was increased from 32.2 to 63°C. However, no additional increase with either agent was seen at 74°C. The advantages to using benzoyl peroxide are that it is effective at lower usage levels than hydrogen peroxide, it does not require addition of catalase to remove residues, and it does not pit stainless steel, and therefore is less corrosive to equipment (Chang et al., 1977). As with reports of off-flavors from hydrogen peroxide, oxidized flavors in Cheddar cheese whey were strong immediately after treatment with benzoyl peroxide; however, off-flavors purportedly dissipated following evaporation and drying (McDonough et al., 1968), an effect not observed in a more recent study (Croissant et al., 2009).

Contrary to the findings of McDonough et al. (1968), our recent unpublished research has reported that hydrogen peroxide is more temperature-dependent than benzoyl peroxide. McDonough et al. (1968) recommended bleaching temperatures in the range of 55 to 65°C. Benzoyl peroxide reacts quickly to remove color and additional time will not increase color removal. At 30°C, benzoyl peroxide may never completely bleach whey (Roos et al., 2006).
Peroxide bleaching (hydrogen or benzoyl) may have an effect on flavor in whey proteins, although few studies have been conducted. Mortenson et al. (2008) studied the flavor of whey protein concentrates (WPC) and whey protein isolates. Contrary to expectations, they found that flavor of WPC34 (WPC with 34–36% protein) and whey protein isolate were not affected by instantizing, ion exchange, or bleaching. However, lack of strict experimental controls and other processing variables suggest confounding factors in this study. Other recent studies have unequivocally established sensory and volatile compound differences associated with whey sourced from different cheeses, agglomeration and instantization (Carunchia Whetstine et al., 2005; Gallardo-Escamilla et al., 2005; Drake et al., 2009; Wright et al., 2009). Kuramoto and Jezeski (1954) studied the bleaching effects of benzoyl peroxide in cream (30% fat) for blue cheese manufacture at temperatures ranging from 52 to 85°C for periods of up to 4 h with concentrations of benzoyl peroxide of 4.5, 9, and 18 mg/kg. They found that flavor problems, such as tallow or oxidized flavors, were more apparent with increasing temperature, contact time, and benzoyl peroxide concentration. McDonough et al. (1968) reported that oxidized flavors present in fluid whey bleached with benzoyl peroxide at 20 and 10 mg/kg at 52°C and 63°C for 1.5 h or hydrogen peroxide at 500 and 300 mg/kg at 52°C and 63°C for 1.5 h were not detected in the dried whey powder. However, Croissant et al. (2009) conducted a controlled study with hot bleaching of liquid whey, with either hydrogen peroxide at 250 or 500 mg/kg at 60°C for 90 min or benzoyl peroxide at 10 or 20 mg/kg at 60°C for 90 min, and then manufactured WPC from those wheys. They demonstrated sensory effects and volatile compound changes in WPC from hydrogen peroxide or benzoyl peroxide bleached whey compared with unbleached whey.
Whey with higher total solids, such as condensed whey, needs greater amounts of peroxide to remove color (JECFA, 2004). The most effective conditions are 60°C for 15 min at pH 6 to 7 (El-Samragy, 2004). Longer holding times are required if lower temperatures are used (McDonough et al., 1968; El-Samragy, 2004). Once whey has been dried, the annatto becomes highly resistant to bleaching. It is important to note that much of the above discussion regarding the use of hydrogen peroxide and benzoyl peroxide for bleaching whey originates from old literature or unpublished data and has not recently been thoroughly evaluated.

**REGULATORY CONCERNS**

Regulatory concerns focus on the use of either hydrogen or benzoyl peroxide for preservation of whey rather than bleaching. That is, these agents are approved for bleaching, not for maintenance of membrane flux during processing or for microbial control. The use of peroxide for preservation of whey during any process other than electrodialysis is prohibited. When bleaching is applied, the agent, concentration, time, and temperature vary widely within the industry depending on the existing facility and its specific process regimen. Regulatory agencies typically use the point of peroxide addition in the process to determine if the purpose of peroxide is bleaching or preservation (USDA/AMS/Dairy Division, 2008). Bleaching whey is usually applied at 1 of 2 possible steps during the whey production process. Peroxide can be added to fluid whey after pasteurization, before or after fat separation, as it is pumped into a storage tank, or when whey retentate is in the hot well of the evaporator. Bleaching is also conducted under a wide range of temperatures from 5 to
70°C. The USDA cites 2 specific cases where use of peroxide would be assumed to be for preservation purposes (Hammond et al., 1975; USDA/AMS/Dairy Division, 2008). The first case is addition of peroxide before the separator or any point in the process before preheating for the evaporator. Fluid whey can legally be bleached following pasteurization if the preheated whey goes into a storage tank for bleaching followed by fat separation. In this situation, the plant typically alternates between 2 tanks. Alternating between 2 tanks for bleaching must be completed within 4 h for microbiological reasons. In addition, legal bleaching may also be carried out if the bleach is added to the hot well before condensing. The second situation generating concern is addition of peroxide before holding the whey for more than 2 h at temperatures between 7 and 63°C (USDA/AMS/Dairy Division, 2008). Legally, whey can be bleached at any temperature so long as the whey is not held between 7 and 63°C for more than 2 h. To determine the legality of bleaching during ultrafiltration (UF) of whey, the point of application at which the agent is added and how long the whey is being held before UF is considered. If the bleaching agent is immediately added and flow begins, this is well within legal limits, regardless of the temperature at which flow occurs. In addition, should the bleaching agent be added to a balance tank before UF, the feed is considered fast enough that these bleaching conditions are considered acceptable. It should be noted that UF units have maximum temperatures at which the membranes can operate and this should be considered when deciding bleaching conditions. Hot bleaching temperatures during UF may not lend themselves to long membrane life in the case of spiral wound membranes. In contrast, should the bleaching agent be added to a silo tank before UF and held for more than 2 h between 7 and 63°C, this would be considered illegal because the bleaching agent would
now be considered a preservation agent (N. Ratzlaff, USDA, Lisle, IL; personal communication).

CONCLUSIONS

Bleaching can create a more desirable color in whey proteins but it may also alter functionality and flavor. The majority of published literature dealing with bleaching of whey is quite dated (>25 yr). Since these studies have been conducted, milk quality, cheese making practices, and whey protein processing have all greatly evolved, emphasizing a need to scientifically evaluate bleaching and its effect on whey protein. Only two agents, hydrogen peroxide and benzoyl peroxide, are currently approved for bleaching. Of these two, the latter is viewed negatively in many countries and some regulations prevent its use. Both bleaching agents can negatively affect whey protein flavor. More precise application of currently approved bleach agents (e.g., minimum concentrations, optimal time/temperature exposure) or development of bleaching alternatives may facilitate enhanced whey protein flavor.

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Table 1. Summary of hydrogen peroxide and benzoyl peroxide for whey bleaching.

<table>
<thead>
<tr>
<th>Bleaching Agent</th>
<th>Hydrogen Peroxide</th>
<th>Benzoyle Peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image1" alt="Structure" /> (USDA, 2008)</td>
<td><img src="image2" alt="Structure" /> (National Center for Biotechnology Information, 2008)</td>
</tr>
<tr>
<td>Breakdown</td>
<td>$2H_2 \rightarrow O_2 + 2H_2O$</td>
<td><img src="image3" alt="Breakdown" /> (Smith, 2004)</td>
</tr>
</tbody>
</table>
| Pros            | • little to no effect on the nutrients present  
                 • more acceptable for usage in other countries | • effective at lower usage levels than hydrogen peroxide  
                 • does not require a catalase addition to remove residues  
                 • does not pit stainless steel therefore is less corrosive to equipment  
                 • effective across a wide range of temperatures |
| Cons            | • must be inactivated with catalase  
                 • could possibly cause oxidized flavors  
                 • corrosive to equipment  
                 • less economical to use since it requires much more peroxide for satisfactory bleaching | • possible formation of oxidized flavors  
                 • possibility that the carrier used may be considered an allergen  
                 • concerns from other countries because it has just been recently approved by CODEX |
CHAPTER 2:

ALTERNATIVE BLEACHING METHODS FOR CHEDDAR WHEY

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ABSTRACT

Residual annatto colorant (norbixin) in fluid Cheddar whey is bleached. The two approved chemical bleaching agents for whey, hydrogen peroxide (HP) and benzoyl peroxide (BP), negatively impact flavor of dried whey protein. The objective of this study was to evaluate alternative methods for bleaching liquid whey: ultraviolet radiation (UV) and acid-activated bentonite (BT). Colored Cheddar cheese whey was manufactured followed by pasteurization and fat separation. Liquid whey was subjected to one of four treatments: control (CT) (no bleaching; 50°C, 1 h), HP (250 mg/kg; 50°C, 1 h), UV (1 min exposure; 50°C) or BT (0.5% w/w; 50°C, 1 h). The treated whey was then ultrafiltered, diafiltered, and spray dried to 80 percent whey protein concentrate (WPC80). The entire experiment was replicated three times. Color (norbixin extraction and measurement), descriptive sensory and instrumental volatile analyses were conducted on WPC80. Norbixin destruction was 29, 53 and 79 for HP, UV and BT treatments, respectively. WPC80 from bleached whey, regardless of bleaching agent, had lower sweet aromatic and cooked/milky flavors compared to unbleached CT (p<0.05). The HP and BT WPC80 had higher fatty flavor compared to the CT WPC80 (p<0.05), and the UV WPC80 had distinct mushroom/burnt and animal flavors. Volatile compound results were consistent with sensory results and confirmed higher relative abundances of volatile aldehydes in UV and HP WPC80 compared to CT and BT WPC80. Based on bleaching efficacy and flavor, BT may be an alternative to chemical bleaching of fluid whey.

Keywords: Whey protein concentrate, bleaching, alternatives
PRACTICAL APPLICATION

The two approved chemical bleaching agents for whey, hydrogen peroxide (HP) and benzoyl peroxide (BP), negatively impact flavor of dried whey protein, and restrictions on these agents are increasing. This study evaluated two alternatives to chemical bleaching of fluid whey: UV radiation and bentonite.
INTRODUCTION

The U.S. is the largest cheese producer in the world, at 4,570,000 metric tons in 2009 (USDEC 2010) and therefore is naturally the largest whey producer worldwide. Liquid whey is frequently further processed into whey protein concentrates (WPC) or whey protein isolate (WPI). WPC is obtained by removing sufficient non-protein constituents from whey so that the finished product contains a protein level of 34 – 80 %. WPI has a higher protein concentration, ≥90 % protein. The functional and nutritional properties of whey protein are valued by food manufacturers and in recent years its applications have expanded tremendously (Smithers 2008).

Prior to being processed into powder, colored cheese whey is bleached. In the U.S., a natural colorant called annatto is added to Cheddar cheese milk. Some of the colorant remains in the whey which negatively impacts the color of dried whey protein. Hydrogen peroxide (HP) and benzoyl peroxide (BP) are the two chemical bleaching agents that are approved for bleaching whey in the U.S (Kang and others 2010). These bleaching agents cause off flavors that carry through to the final spray dried product. Croissant and others (2009) demonstrated that both BP and HP impacted the flavor of liquid whey and subsequent spray dried WPC. Subsequent studies have confirmed the role of bleaching in off flavors in WPC34 and WPC80 (Listiyani and others 2011; Jervis and others 2012). Alternatives to chemical bleaching agents may be a viable and attractive alternative for whey bleaching and were the focus of the current study. The two alternative bleaching agents evaluated were acid activated bentonite and UV radiation.
Bentonite has a high surface area and tends to absorb molecules into its interlayer sites (Christidis and Kosiari 2003). When acid activated, a procedure during which bentonite is dispersed in a strong acid at high temperatures and subsequently rinsed with deionized water to remove the acid, the absorbent properties are enhanced (Odom, 1984; Rupert and others 1987; Boyd and Jaynes 1994). In the food industry, acid activated bentonite is mainly used for the purification, decolorization and stabilization of vegetable oils (Christidis and Kosiari 2003). In addition to color removal during bleaching, bentonite removes undesirable products such as hydroperoxides formed by oxidation, unsaturated fatty acids and glycerides.

Ultraviolet (UV) light has a wavelength between 10 and 400 nm which falls between the visible light and x-ray spectrum. UV light has a damaging effect on the stability of annatto colorants (Petersen and others 1999). Previous studies have measured the effect of light (100 W bulb, 1380 or 40 W bulb, 430 lux) on the color stability of annatto in fluid systems (Najar and others 1988; Pesek and Warthesen 1988) and colored Cheddar cheese (Hong and others, 1995 a, b) with light exposure resulting in the greatest degree of color destabilization in fluid systems (Najar and others 1988).

MATERIALS AND METHODS

Experimental design

Two experiments were performed. Experiment I was conducted on liquid Cheddar whey using each bleaching agent at varying temperatures and/or varying exposure times. The purpose of experiment I was to select the appropriate treatments for subsequent WPC80 production (experiment II). For experiment II, unbleached fluid whey and whey bleached
with 250 ppm HP were used as controls. Unbleached colored whey served as a negative control while the HP bleached whey served as a current industrially relevant point of reference.

**Liquid whey production**

Liquid Cheddar whey was manufactured from whole vat pasteurized bovine milk with the addition of double strength annatto food color (3% norbixin wt/vol, Danisco, St. Louis, MO) at a rate of 0.033mL/Kg milk as described by Listiyani and others (2011). The whey was drained at pH 6.40 and was immediately pasteurized at 63°C for 30 min. The hot whey was immediately processed with a hot bowl cream separator (Model FJ 125 EAR, Clair, Althofen, Austria) to reduce the fat content. Total percent solids and percent fat content of fluid milk and whey were analyzed using the SmartSystem 5 moisture-solids analyzer with Smart Trac rapid fat analysis (CEM, Matthews, NC).

**Bleaching treatments for liquid whey (Experiment 1)**

Following pasteurization and fat separation, whey was subjected to different treatments. For the control (CT), the liquid whey was heated at 60°C for 1 h. For HP treatment, 250 mg HP/kg (30% wt/vol; Nelson Jameson Inc., Marshfield, WI) was added to the liquid whey and held for 1 h at 60°C with gentle agitation followed by addition of 20 ppm catalase (Food Pro CAT, Danisco) to remove any residual HP.

For ultraviolet radiation (UV), the apparatus and methods of Mahmoud and Ghaly (2004) were adopted with minor changes. Liquid whey was circulated through a stainless steel UV apparatus at 60°C. The flow of the whey was fixed so that the liquid whey was exposed to UV light for 1 min. The UV apparatus was composed of a stainless steel feed
tank, a peristaltic pump, and an overflow tank. A 380 mm arc length low-pressure mercury lamp enclosed in a 21 mm diameter (o.d.) quartz tube was used (Aqua Treatment Service, Inc., Mechanicsburg, PA). The UV reactor had a 0.201 L working volume and the stainless steel chamber was 475 mm long with an i.d. of 33 mm. An inlet port was located at one end of the reactor and was connected to a peristaltic pump, which pumped the cheese whey from the feed tank. The outlet port was located at the other end of the reactor and was connected to the overflow tank.

For BT treatment, first, acid activated bentonite was made by heating bentonite (Sigma-Aldrich, St. Louis, MO; 20g) with 2 N sulfuric acid (VWR International, West Chester, PA) at 90°C for 4 h followed by rinsing with deionized water until it reached pH 4 (Srasra and others 1989). The acid activated bentonite powder was dispersed in liquid whey and recirculated. Following the respective 15 min, 30 min, or 1 h treatment time, the liquid whey was centrifuged for 10 min at 8000 x g to remove the bentonite. After each treatment, color and volatile analyses of the liquid wheys were conducted. The experiments were replicated in triplicate. Based on the results, one treatment for each alternative bleaching method was selected for manufacture of WPC80.

**Bleaching treatments for WPC80 (Experiment 2)**

Following pasteurization and fat separation, whey was subjected to one of five different treatments selected from experiment I. The temperature of all treatments was decreased to 50°C to maintain consistency with industrial UF practices. For ultraviolet (UV) radiation, the liquid whey flow was regulated by the peristaltic pump to allow 1 min
exposure. For acid activated bentonite treatment, 0.5% (w/v) bentonite was mixed with the liquid whey for 1 h and removed using the method described previously.

**WPC80 production**

Following bleaching for heat treatment (control), whey was concentrated using a UF system (Model Pellicon 2, Millipore Inc., Billerica, MA). The system and procedure has been described by Listiyani and others (2011). The whey was concentrated using an ultrafiltration (UF) system equipped with four polyethersulfone cartridge membrane filters (Model P2V010V05, nominal separation cut off: 10 kDa, surface area: 0.5 m²). A variable speed peristaltic pump (Model 77410-10) equipped with pump heads (Model 77601-00) with silicone tubing (Model 96440-73) was used to circulate the product. DF was initiated after UF had reduced the volume of whey to approximately 50% original weight. The total volume of deionized water added was approximately 65% of the original volume of the whey, and was added in three parts over the course of the UF process. The total time of the UF and DF was approximately 3.5 h.

After DF, the retentate was spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark). A peristaltic pump (model 9154K43, McMaster-Carr) was used to feed the spray dryer at a rate of 1kg/h. Retentate feed temperature was 22°C. The spray dryer inlet temperature was 190°C and the outlet temperature was 80°C. Powders were sealed in Mylar bags (TF-4000, Impak Corp., Central City, SD) and stored at -80°C. Production of the liquid whey and the five spray-dried WPC80 treatments was completed in 1 d. This experiment was replicated three times.
Composition Analyses

Total solids of WPC were determined by air oven drying (AOAC, 2000; method number 990.20; 33.2.44). Fat was quantified by ether extraction (AOAC, 2000; method number 989.05; 33.2.26). Protein was determined using the Kjeldahl method (AOAC, 2000; method number 991.20; 33.2.11). Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) was determined by the North Carolina State University analytical services laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy. All samples were measured in duplicate.

Color analysis: reflectance measurement and annatto extraction

Reflectance measurement

The Hunter CIE Lab L* (lightness), a* (red-green), and b* (yellow-blue) values for the fresh liquid whey, WPC80, and 10% solids (w/v) rehydrated WPC80 were determined in triplicate using a Minolta Chroma meter (CR-410, Ramsey, NJ) as described by Listiyani and others (2011).

Annatto Extraction

Annatto extraction and quantitation methods were modified from Croissant and others (2009). Ten grams of Cheddar liquid whey or 1 g WPC80 powder in 2 mL HPLC grade water (EMD Chemicals) was weighed into a 50 mL centrifuge tube (Nalgene, Rochester, NY). To this, 6 mL ethanol (EMD Chemicals) was added and vortexed with centrifugation at 16,500 x g for 10 min at 4°C (Model RC5B, Thermo Scientific, Waltham, MA). The supernatant was removed to a separate centrifuge tube. To the remaining solids, 3 mL of
chloroform (EMD Chemicals) was added, the sample was vortexed and centrifuged again at 16,500 x g for 10 min. The bottom chloroform layer containing the annatto was collected and the volume was recorded. The collected sample was further purified using a strata-NH₂ SPE column (500mg/3ml, Phenomenex) and the absorbance were measured at 458nm with a UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Durham, NC) and quantified using an external calibration curve (Listiyani and others 2011). All extraction procedures and measurements were performed with premium full spectrum F885 flat sheet filters (Ergomart, Dallas, TX) covering all lights to minimize norbixin isomerization and degradation (Mercadante 2008).

**Descriptive Sensory Analysis**

Sensory testing was conducted in compliance with the North Carolina State University Institutional Review Board for Human Subjects guidelines. Descriptive sensory analysis was performed on 10% solids (w/v) rehydrated WPC80 using an established lexicon for dried dairy ingredients (Drake and others 2003, 2009; Wright and others 2009). Eight trained sensory panelists (7 females, 1 male, ages 23-45 y) with more than 100 h of experience each with the Spectrum™ descriptive analysis method and sensory analysis of dried dairy ingredients (Meilgaard and others 1999) participated. Rehydrated (30ml) samples were dispensed into lidded 59 ml cups (Solo Cup Company, Champaign, IL) with 3-digit codes. Each product replication was evaluated in duplicate by each panelist at room temperature in a randomized design. Data was collected using paper ballots or by computerized data entry using Compusense five, release 4.8 (Compusense Inc., Guelph, Ontario, Canada).
**Gas Chromatography/Mass Spectrometry**

Volatile compounds in liquid whey and WPC80 were extracted by solid phase microextraction (SPME). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using the method of Listiyani and others (2011). Liquid samples were tested as-is on the day of manufacture, and spray dried powders were rehydrated at 10% solids (w/v). In order to minimize light oxidation, samples were prepared with overhead lights off. All samples contained 10% (w/v) sodium chloride (Fisher Scientific) using HPLC grade water (EMD Chemicals). Five grams of each sample were transferred to SPME vials (Supelco). Each SPME vial was spiked with a 10 ul of 8.1 mg/kg internal standard 2-methyl-3-heptanone (Sigma-Aldrich, St. Louis, MO) dissolved in methanol (EMD Chemicals). Each vial was sealed airtight with a teflon –sided silicon septa and a steel screw cap (MicroLiter Analytical Supplies Inc., Sawanee, FL).

Samples were injected using a combiPal auto sampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890 N 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 31mm 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. A Zb-5ms column (Zb-5ms 30m length x 0.25 mm inner diameter x 0.25um film thickness; Phenomenex) was used for
all analysis at a constant flow rate of 1ml/min in SIM mode. 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 National Institute of Standards and Technology (2002) (Gaithersburg, MD) mass spectral database, retention indices (RI) based on an alkane series (Sigma Aldrich) and comparison of spectra of authentic standards injected under identical conditions. Concentration of compounds were determined using single point external standard calibration curves, where the area of the mass chromatogram was calibrated with a known amount of analyte injected on the GC-MS under the same conditions.

Statistical analysis

Proximate analyses, sensory, and instrumental results were analyzed using XLSTAT statistical software (version 2009. 1.02, Addinsoft, New York, NY). One-way analysis of variance (ANOVA) was conducted to determine the effect of bleaching on color, chemical composition, and sensory and instrumental volatile analyses. Tukey’s least significant difference with a confidence interval of 95% was conducted as a post-hoc test. Principle component analysis using the correlation matrix was also conducted to determine how different bleaching methods affected the sensory and instrumental measurements.

RESULTS AND DISCUSSION

Composition analyses

No significant differences (p>0.05) were observed for the fat (5.75%±0.19%), moisture (2.82%±1.55) or protein (81.7%±0.92%) content of bleached and unbleached WPC80. The magnesium, iron, aluminum and silicon content for BT WPC80 were higher
(p<0.05) than CT WPC80 or other WP80, which was probably due to residual bentonite that was not removed by pilot scale procedures (Table 1).

**Color analysis: reflectance measurement and annatto extraction**

**Experiment I: Liquid Whey**

The HP treatment of liquid whey decreased norbixin content by 47% and decreased yellowness (b* value) by 17% which is consistent with previous published results (Listiyani and others 2011). BT treatment of liquid whey at 60°C was more effective than 4°C for color removal (p<0.05, results not shown). Based on norbixin concentration, 60°C for 30 min or 1 h resulted in 49 and 62% norbixin decrease, respectively. BT exposure for 1 hr was selected for WPC80 production. Fluid whey exposed to UV light for as little as 0.5 min resulted in 74% norbixin destruction (results not shown). A 1 min exposure to UV was selected for experiment 2.

**Experiment II: WPC 80**

HP, BT and UV bleached WPC 80 displayed a 29, 79 and 53 % norbixin destruction, respectively (Figure 1). Based on norbixin recovery, BT and UV were more effective (p<0.05) than traditional HP (250ppm) bleaching. HP was the least effective of all treatments. For bleaching, HP and UV chemically destroy the structure of norbixin, while BT physically removes or sequesters norbixin from the whey and was the most effective of the agents evaluated.

**Volatile compounds and Flavor analyses**

In order to remove norbixin in whey, bleaching agents are employed. In the case of chemical bleaching agents, rapid loss of color occurs when free radicals are formed which
possess enough energy to break less stable double bonds, such as those in norbixin. Norbixin itself may quench free radicals prior to lipid oxidation (Campbell and others 2011) and was the only carotenoid that inhibited the oxidative deterioration of oil stored at 60°C (Kiokias and Gordon 2003). However, the same free radicals that destroy norbixin and bleach whey also promote lipid oxidation. Lipid oxidation products are the major source of off-flavors (cardboard, fatty, cabbage flavors) in dried whey proteins (Wright and others 2009; Whitson and others 2010). Consistent with previous studies (Croissant and others 2009; Listiyani and others 2011; Jervis and others 2012), HP bleaching significantly decreased sweet aromatic and cooked/milky flavors and increased cardboard and fatty flavors in WPC80 (Table 2). Consistent with oxidative reactions, UV also increased cardboard and fatty flavors and decreased sweet aromatic flavor in WPC80 and contributed unique atypical mushroom, animal and pasta flavors. BT treatment had the least impact on sensory properties of WPC80. No change in cardboard flavor relative to the control was observed in BT WPC80. BT WPC80 had decreased sweet aromatic and cooked/milky flavors and a slight but distinct fatty flavor.

A total of 24 volatile compounds were identified and quantified (Table 3). Selected compounds have previously shown relevance to whey off-flavors (Croissant and others 2009; Liaw and others 2010; Whitson and others 2010; Listiyani and others 2011). Consistent with sensory results, volatile lipid oxidation products were generally higher in UV and HP WPC80 compared to BT and CT WPC80 (P<0.05). The ability of HP to bleach is connected to its oxidative nature. Unfortunately, its predisposition to oxidize also contributes to lipid oxidation off flavors in WPC34 and WPC80. Hydrogen peroxide bleached WPC80 was
higher in many lipid oxidation products, including pentanal, hexanal, heptanal, E-2-heptanal, 1-octen-3-one, 1-octen-3-ol, octanal, E-2-octenal, and (E)-2-nonenal when compared to the CT WPC80. HP treated WPC80 was also higher in DMDS (Table 3).

High energy photons of the UV range disrupt the bonds in the chromophore of the annatto, resulting in colorless or decreased color in whey (Chen and others 2008). When whey was exposed to UV light, some compounds that were not found in the WPC80 from CT and HP treated whey were observed: p-cresol and guiacol (Table 3). These compounds may be formed from radical-induced oxidation of aromatic amino acids. WPC80 from UV treated whey was also higher (p<0.05) in pentanal, hexanal, heptanal, 1-octen-3-one, e-2-octenal and e-2-nonenal compared to CT WPC80. Given the off flavors production in WPC80, UV radiation is not a viable alternative bleaching agent for whey.

Natural bentonites are effective adsorbents after acid activation (Christidis and Kosiari 2003). Acid activated bentonite has an ability to adsorb phospholipids, trace metals, and organic compounds such as carotenoids (Christidis and Kosiari 2003). BT also removes hydroperoxides formed by oxidation, unsaturated fatty acids and glycerides. Unlike HP or UV, bentonite is not an oxidizing agent and should not initiate or promote lipid oxidation and this result was observed. Volatile liquid oxidation products were generally lower in BT WPC80 compared to other bleached WPC80 and the sensory profile of BT WPC80 was most similar to unbleached WPC80. Given that restrictions on chemical bleaching agents continue to increase, future studies should investigate optimization of BT and its effect on protein functionality.
CONCLUSION

This was the first study, to our knowledge, to investigate and compare alternative bleaching methods for annatto destruction in colored whey. Based on bleaching efficacy and flavor, acid activated bentonite was the most promising alternative bleaching treatment.

ACKNOWLEDGMENTS

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Determination of the sensory attributes of dried milk powders and dairy

Drake MA, Miracle RE, Wright JM. 2009. Sensory properties of dairy proteins. p 429-


Table 1. Mineral analyses of unbleached and bleached WPC80.

<table>
<thead>
<tr>
<th>Category</th>
<th>S (%)</th>
<th>P (%)</th>
<th>K (%)</th>
<th>Ca (%)</th>
<th>Mg (%)</th>
<th>Fe (ppm)</th>
<th>Na (ppm)</th>
<th>Al (ppm)</th>
<th>Si (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.479&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.562&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.828&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.072&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1709&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.72&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HP</td>
<td>1.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.489&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.578&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.830&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.069&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1796&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>UV</td>
<td>1.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.483&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.589&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.839&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.071&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1784&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BT</td>
<td>1.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.413&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.511&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.752&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;a&lt;/sup&gt;</td>
<td>339&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1500&lt;sup&gt;a&lt;/sup&gt;</td>
<td>730&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1925&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>CT = control; HP = hydrogen peroxide; BT = acid activated bentonite; UV = ultraviolet radiation.

<sup>a-c</sup> Samples within the same column with different letters are significantly different (p<0.05).
Table 2. Sensory properties of bleached and unbleached Cheddar WPC80.

<table>
<thead>
<tr>
<th>Category</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
</tr>
<tr>
<td>Aroma intensity</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sweet aromatic</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cooked/milky</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mushroom</td>
<td>ND</td>
</tr>
<tr>
<td>Cardboard</td>
<td>1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fatty</td>
<td>ND</td>
</tr>
<tr>
<td>Burnt/animal</td>
<td>ND</td>
</tr>
<tr>
<td>Flour/pasta</td>
<td>ND</td>
</tr>
<tr>
<td>Astringency</td>
<td>2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Means in the same row with different letters are significantly different (p<0.05). Intensities were scored using a 0–15 point universal scale, with 0 being no intensity and 15 the highest intensity. Most dried dairy ingredient flavors fall between 0 and 4 on this scale (Whitson and others 2010; Wright and others 2009).

ND = Not detected; CT = control; HP = hydrogen peroxide; BT = acid activated bentonite; UV = ultraviolet radiation.
Table 3. Concentration in parts per billion (ppb) of selected compounds in WPC80 by SPME-GC-MS.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>CT</th>
<th>HP</th>
<th>BT</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-heptanone</td>
<td>0.045&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.158&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.070&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Butanal</td>
<td>22.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>DMS</td>
<td>0.293&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.258&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.396&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.173&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Diacetyl</td>
<td>0.750&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.697&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.750&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.717&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>Isobutyraldehyde</td>
<td>0.824&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.924&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>3-methylbutanal</td>
<td>0.067&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.066&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.551&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>2-methylbutanal</td>
<td>0.120&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.110&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.209&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.112&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>Pentanal</td>
<td>0.147&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.933&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.427&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.339&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>DMDS</td>
<td>0.038&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.504&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.045&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>Hexanal</td>
<td>11.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>Heptanal</td>
<td>0.492&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.922&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.692&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.112&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>E-2-heptanal</td>
<td>0.021&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.086&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.031&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.107&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>13</td>
<td>DMTS</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.028&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>1-octen-3-one</td>
<td>0.012&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.056&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.170&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>1-octen-3-ol</td>
<td>0.882&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>2-pentyfuran</td>
<td>0.087&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.314&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.156&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.217&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>Octanal</td>
<td>0.230&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.034&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.311&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.838&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>E-2-octenal</td>
<td>0.170&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.863&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.268&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.915&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>19</td>
<td>Guaiacol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.278&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>20</td>
<td>Nonanal</td>
<td>0.553&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.571&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>21</td>
<td>(E)-2-nononal</td>
<td>0.030&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.138&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.040&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.179&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>22</td>
<td>Decanal</td>
<td>0.017&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.013&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>23</td>
<td>β-ionone</td>
<td>0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.006&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

Mean concentration of selected compounds in WPC80 by SPME GC-MS using SIM mode quantified using single point response factors.

<sup>a</sup>-<sup>d</sup> Means in a row followed by different letters are statistically different (p<0.05).

ND = not detected; CT = control; HP = hydrogen peroxide; BT = acid activated bentonite; UV = ultraviolet radiation.
Figure 1. Norbixin destruction (%) of bleached WPC80. Percent norbixin destruction relative to a control unbleached WPC80 manufactured from colored Cheddar whey. HP=hydrogen peroxide; BT=acid activated bentonite; UV = ultraviolet radiation.
CHAPTER 3.

THE IMPACT OF CHEESE MILK HEAT TREATMENT ON CHEMICAL AND ENZYMATIC BLEACHING OF FLUID WHEY

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ABSTRACT

Chemical bleaching is a commonly applied unit operation in whey protein processing that negatively influences whey protein flavor. Recent studies have shown that native milk lactoperoxidase (LP) is active in cheese whey and can be used to bleach annatto colored fluid whey. A low concentration (10-50 mg/kg) of hydrogen peroxide (H₂O₂) is required for LP bleaching activity. When excess concentrations of H₂O₂ (> 100 mg/kg) are applied, LP is inactivated and H₂O₂ chemical bleaching occurs. Fluid milk can be subjected to a wide range of heat treatment prior to cheese manufacture and heat treatment may influence subsequent whey bleaching efficacy. The objective of this study was to investigate the impact of milk pasteurization temperature on the bleaching efficacy of fluid whey. Milk was not pasteurized, pasteurized at 63°C for 30 min (low heat) or 78°C for 28 sec (high heat) and colored Cheddar wheys were produced from each milks. The whey from raw milk was either unpasteurized (URW) or pasteurized at 63°C for 30 min (RW). Wheys from low heat and high heat treated milk were pasteurized at 63°C for 30 min (LW and HW). Hydrogen peroxide was then added at 250 mg/kg (chemical bleaching) or 25 mg/kg (LP bleaching) at 35 or 50°C and aliquots were collected after 0, 3, 5, 7, 10, 15, 20, and 60 min. Unbleached colored wheys were used as controls. Norbixin was extracted to determine bleaching efficacy. LW had a higher bleaching efficacy (p<0.05) from 25 mg/kg H₂O₂ (90% norbixin destruction) than 250 mg/kg H₂O₂ (20% norbixin destruction) at 35°C and 50°C, consistent with LP activity. The norbixin content of unbleached HW was 18% lower than that of unbleached URW (p<0.05), and the protein content was decreased by 27% for HW compared to LW. Percent norbixin destruction in whey from HW was lower than LW in all treatments and there were no
differences (p>0.05) between 25 and 250 mg/kg H₂O₂ (22% norbixin destruction).

Regardless of milk pasteurization temperature and bleaching agent, greater bleaching was observed with increased time. These results demonstrate the influence of milk heat treatment on bleaching efficacy and also suggest differences in norbixin association with whey components with different milk heat treatment.
INTRODUCTION

Heat treatment is an important process step in manufacturing dairy products. Heating in the range 70 – 140°C is commonly used for milk or milk products in order to reduce bacterial counts, extend shelf life, and change rheological properties (Jelen and Rattray 1995). Milk for cheese manufacture is generally pasteurized by high temperature short time (HTST) pasteurization (72°C x 15 sec) to destroy non-pathogenic and pathogenic microorganisms in raw milk (Rynne and others 2004). Pasteurization temperatures above 72°C are not recommended for cheese milk because of their adverse effects on curd formation (Guinee and others 1997; Singh and Waungana 2001) and syneresis (Pearse and others 1985; Pearse and MacKinlay 1989). However, extensive studies have shown improvement in cheese yield when pasteurization temperature was increased (El-Koussy and others 1977; Marshall 1986; Green 1990a; Green 1990b; Guinee and others 1998; Lebeuf and others 1998; Kelly 1999). This increase in cheese yield is due to increased moisture content (Maubois and Mocquot 1975) due to inclusion of whey proteins in the cheese matrix (Singh and Waughana 2001).

Bovine milk contains 3-3.5 % protein and consists of two major protein groups: casein and whey protein, which are differentiated by their solubility at pH 4.6 (Fox and McSweeney 1998). In bovine milk, 76-86% of total milk protein is casein, and the remaining 14-24% is whey protein (Park and others 1998, Swaisgood 2003). Several studies have demonstrated whey protein denaturation when milk was heated at temperatures above 70°C. The main whey protein, ß-lactoglobulin (ß-lg) which makes up about 48-58% of the whey protein, is heat labile (Dannenberg and Kessler 1988a, 1988b; Rüegg and others 1977). At 65°C, denaturation begins and is almost complete when whey proteins are heated at 90°C for
5 min. Up to 80% of β-lg could be complexed to casein micelles after a heat treatment of 90°C for 20 min (Smits and van Brouwershaven 1980). The complex between β-lg and κ-casein involves disulfide bonds (Jang and Swaisgood 1990), and is affected by temperature and pH (Singh and Fox 1987; Dannenberg and Kessler 1988b). Singh and Fox (1987) reported that complex formation with β-lg prevented the dissociation of micellar κ-casein on heating and stabilized the micelles in the pH range 6.5 to 6.7. The other primary whey protein, α-lactalbumin (α-lac) makes up about 13-19% of the whey protein and is more heat stable than β-lg. For this reason, α-lac is often chosen as an indicator of heat treatment (Sun 2012). Maximum α-lac association with casein is about 55% at temperatures below 90°C, and 40% at temperatures of 95-130°C (Oldfield and others 1998). Lactoperoxidase (LP) is another component in whey, which makes up about 0.03% of the whey protein. LP is inactivated when exposed to 78°C for 15 sec (de Wit and van Hooijdonk, 1996).

To produce yellow colored Cheddar cheese, a natural colorant called annatto which contains the carotenoid pigment norbixin is added. An average of 10% of the annatto added to cheese milk is recovered in the cheese whey (Smith, 2012). The removal of annatto from whey can be done either physically or chemically. For physical removal, an adsorbent such as bentonite may be added (Kang and others 2012). Bentonite forms a complex with the annatto colorant, and the complex is filtered to be separated from the rest of the whey. The commonly used bleaching method in colored Cheddar whey, however, is chemical bleaching (Kang and others 2010). Chemical bleaching interrupts the conjugated double bonds within the annatto colorant structure, which are responsible for the visible color. Hydrogen peroxide at concentrations of up to 500mg/kg has been the popular chemical bleaching agent for
colored whey; however, its use results in off-flavors in dried whey ingredients (Croissant and others 2009; Jervis and others 2012). Very recently, Campbell and others (2012) reported the use of the endogenous milk enzyme lactoperoxidase (LP) as an alternative enzymatic whey bleaching agent when a low concentration of hydrogen peroxide of 10-20 mg/kg was supplied. Although some literature regarding the thermal stability of LP in whey and milk can be found (Barrett and others 1999; Boscolo and others 2007), very little is known about the impact of milk heat treatment on bleaching of annatto colored whey. In this context, the effect of milk or whey heat treatment on LP activity and its impact on the bleaching of annatto colored Cheddar whey was the focus of this study.

MATERIALS AND METHODS

Whey manufacture and bleaching treatments
Whole raw bovine milk (114 kg; fat: 3.56±0.3%, protein: 3.2±0.02%) was obtained from the North Carolina State University Dairy Education Unit (Raleigh, NC, USA). The standard plate count (SPC) for raw milk was <10^3 CFU/ml, and coliform count (CC) was <20 CFU/ml. After heat treatment the SPC was < 50 CFU/ml, and CC were not detected. Three batches of Cheddar cheeses were made using different heat treated milks from the same lot of raw milk (Table 1). For cheese manufacture, milk was heated to temperature (31°C) and was inoculated with a freeze-dried mesophilic starter culture (50 DCU/454 kg, Choozit MA 11, Danisco, New Century, NJ). Calcium chloride solution (50% w/v, Dairy Connection Inc., Madison, Wis., U.S.A.) was added at the rate of 0.39 mL/kg of milk. The milk was agitated and allowed to ripen for 60 min. Then, the milk was coagulated with double strength
recombinant rennet (Dairy Connection Inc.) for 30 min at a rate of 0.09 mL/kg of milk
diluted 80 times in deionized water. The coagulum was cut and the curd and whey were
allowed to rest for 5 min followed by gentle stirring for 10 min without added heat. The
temperature was increased gradually (39°C) over 30 min while pH and titratable acidity were
closely monitored. The curd was continuously stirred until the target whey drain pH of 6.40
was attained. As the whey was drained, a sieve was used to remove fines and the whey was
either unpasteurized or immediately pasteurized at 63°C for 30 min (Table 1). Cheese trials
were performed in triplicate (Figure 1). Either 25mg H₂O₂/kg (enzymatic bleaching) or 250
mg H₂O₂/kg (chemical bleaching) was used at two different temperatures (35°C and 50°C)
for bleaching purposes. Samples were collected at appropriate time points (Figure 1) for
corresponding analyses.

**Determination of LP**

Raw milk, low heat treated milk, high heat treated milk, unpasteurized raw whey
(URW), pasteurized raw whey (RW), low heat treated milk whey (LW), and high heat treated
milk whey (HW) were subjected to LP measurement. The activity of LP was determined
using the International Dairy Federation method (Pruitt and Kamau, 1994) with 2,2′azino-
bis-(3-ethyl-benxothiazoline-6-sulphonate) (ABTS) as a co-substrate with hydrogen peroxide
(Hernandez and others 1990; Marks and others 2001; Trujillo and others 2007). The LP
catalyses the oxidation of ABTS in the presence of hydrogen peroxide forming ABTS⁺,
which emits a green-blue hue soluble compound with UV absorption at 412 nm.

In a disposable plastic cuvette (VWR International LLC), 2.0 mL of ABTS stock
solution (1 mM, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) and 0.1mL of the milk or
whey was placed. To start the reaction, 1.0 mL of hydrogen peroxide solution (0.3 mM, Nelson Jameson Inc.) was added and rapidly mixed thoroughly and the absorbance (412 nm) was recorded immediately every 5 sec until the reaction reached plateau (less than 2 min). Concentrations of LP were determined using the least squares regression of the absorbance data and Beer's law using the equation below (Pruitt and Kamau 1994). One enzyme activity unit (U) is defined as the amount of enzyme that catalyses the oxidation of 1 μmol/min of ABTS (Hernandez and others 1990). A standard curve was created using bovine lactoperoxidase standard (Sigma-Aldrich; stock solution concentration was 115U/ml). The units (U) were calculated using the extinction coefficient (Δε) 32.4 x 10^{-3} /μM·cm of the oxidized ABTS at 412 nm (Shindler and others 1976).

\[
[E]_{\text{milk/whey}} = \frac{(R - R_0)(V_{\text{sample}} + V_{\text{assay}})}{mV_{\text{sample}}}
\]

\[R = \frac{\Delta A}{\Delta t}/\Delta \varepsilon\]
\[R_0 = -3 \mu M \text{ product/min}\]
\[m = 320 \frac{(\mu M \text{ product/min})}{(\mu g/ml)}\]
\[V_{\text{sample}} \text{ (volume)} = 0.100 \text{ ml}\]
\[V_{\text{assay}} \text{ (volume)} = 3.0 \text{ ml}\]

(Pruitt and Kamau 1994)

**Composition Analyses**

Total solids of liquid whey were determined by performing air oven drying (AOAC, 2000; method number 990.20; 33.2.44). Fat was quantified by ether extraction (AOAC, 2000; method number 989.05; 33.2.26). Protein was determined using a Sprint Protein Analyzer (CEM, Matthews, NC). All samples were measured in duplicate.
**Norbixin measurement**

Residual norbixin was extracted from liquid wheys using a method from Van Scheppingen and others (2012) with minor changes. Two-hundred microliters of liquid whey was dissolved in acetonitrile (99.9% w/v, ACROS, Fair Lawn, NJ) and vortexed for 30 sec and centrifuged at 14000 x g for 5 min in a Microfuge 18 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). Fifty microliters of the supernatant was injected in duplicate onto an HPLC column (Waters 2707 Auto sampler, Waters, Milford, MA) (Phenomenex Kinetex 2.6μ 100x4.6mm, 40°C). The auto sampler was set at 4C throughout the injections. An isocratic mobile phase (70% v/v acetonitrile (99.9% w/v, ACROS, Fair Lawn, NJ), 29.9% v/v HPLC grade water (Honeywell, Burdick & Jackson, Muskegon, MI) and 0.1% v/v formic acid (98-100% w/v Sigma-Aldrich, St. Louis, MO)) was used at a flow rate of 1mL/min (Waters 1525 Binary Pump, Waters, Milford, MA). The injector temperature was at 4°C and a photodiode array detector (Waters 2998) at 460 nm was used. The norbixin recovery of bleached samples were measured and calculated as percentage norbixin destruction compared to liquid unbleached control samples. A norbixin standard (45% w/w, Chr. Hansen, Milwaukee, WI) was used to positively identify the compound.

**Gas Chromatography/Mass Spectrometry**

Volatile compounds in liquid and milk whey were extracted by solid phase microextraction (SPME). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using a modified method of Wright et al. (2006). Liquid samples were tested the day of manufacture. In order to minimize light oxidation, samples were prepared with overhead lights off. All samples contained 10% (w/v)
sodium chloride (Fisher Scientific, Pittsburg, PA) using HPLC grade water. Five grams of each sample were transferred to SPME vials (Supelco, Bellefonte, PA). Each SPME vial was spiked with a 10 ul of internal standard (2-methyl-3-heptanone (Sigma-Aldrich, St. Louis, MO) in methanol (EMD Chemicals Inc., Gibbstown, NJ), stock concentration = 8.1 ppm). Each vial was sealed airtight with a teflon — sided silicon septa and a steel screw cap (Microliter Analytical, Sawanee, Fl., U.S.A.).

Samples were injected using a CombiPAL auto sampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890 N 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 31mm 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 3min 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 30m length x 0.25 mm inner diameter x 0.25µm film thickness; Phenomenex) was used for all analysis at a constant flow rate of 1ml/min in single ionization mode (SIM). 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 National Institute of Standards and Technology (2002) mass spectral database and authentic standards injected under identical conditions. The relative abundance of compounds was determined by comparing the area to the known concentration of the recovered standards. These analyses were conducted in triplicate.
**SDS-PAGE**

Unpasteurized raw whey (URW), pasteurized raw whey (RW), low heat treated milk whey (LW), and high heat treated milk whey (HW) were subjected to SDS-PAGE. A NuPAGE Bis-Tris electrophoresis system (Invitrogen, Carlsbad, CA) was used to identify the protein composition under reduced conditions. The purpose of conducting SDS-PAGE was to observe protein differences in the milks and wheys due to heat treatment. SDS-PAGE gels were conducted using two different approaches. First, the protein concentration of milks and liquid wheys were standardized to the same protein concentration prior to loading on to the gel. When the % protein was standardized, the effects of heat treatment on protein content of whey would not be evident. In the second approach, the wheys were not standardized to identical % protein, but were only diluted in order to prevent overloading of each lane on the gel. One ml aliquot of whey was diluted with 1 ml of DI water. Each standardized or diluted milk and whey sample (65ul) was dissolved in NuPAGE LDS sample buffer (25ul) with NuPAGE reducing agent (10ul). Final protein concentrations of samples were 2.0mg/ml for the milk and whey standardized samples. For the unstandardized samples the protein concentrations were 2.3, 2.0, 2.0, 1.3 mg/ml for URW (unpasteurized raw whey), RW (pasteurized raw whey), LW (low heat treated milk whey), and HW (high heat treated milk whey), respectively. Samples were then heated at 70°C for 10 min and 10uL aliquot of each sample was loaded per well. Proteins were separated on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) using NuPAGE MES SDS running buffer (Invitrogen) at a constant 200 volts for 45 min. After the electrophoresis was complete, the gel was removed from the cast and soaked in 50% methanol (EMD Chemicals Inc., Gibbstown, NJ) and 10% acetic acid.
(99.5%; J.T. Baker Chemicals, Phillipsburg, NJ) solution for 10 min. Then the gel was
transferred and stained with colloidal blue staining solution (Invitrogen). After 3 h, the gel
was destained in DI water overnight. Novex sharp protein standard (Invitrogen) was used to
identify components in wheys. A gel-dry drying solution (Invitrogen) was used to preserve
the gel. Each experimental replication was evaluated by SDS PAGE in duplicate.

**Statistical analysis**

Proximate analyses and instrumental results were analyzed using XLSTAT statistical
software (version 2009.1.02, Addinsoft, New York, NY). One-way analysis of variance
(ANOVA) was conducted in order to determine the effect of bleaching on color, chemical
composition, and instrumental analyses. Tukey’s least significant difference with a
confidence interval of 95% was conducted as a post-hoc test. Principle component analysis
using the correlation matrix was also conducted to determine how different bleaching
conditions affected the sensory and instrumental measurements.

**RESULTS AND DISCUSSION**

No significant differences were observed among the raw and heated milks for total
solids and moisture (p>0.05, results not shown). Compositional differences among the heat
treated wheys were documented (Table 2). The HW had lower (p<0.05) protein concentration
due to the loss of protein from high heat treatment. At temperatures above 70°C the
polypeptide chains of whey (β-lg) unfold and the exposed thiol groups react with casein
forming a complex that is retained in the cheese curd (Dalgleish 1990; Jang and Swaigsood
1990). The denatured whey proteins remain in the cheese, decreasing the protein
concentration in liquid whey. URW had a higher (p<0.05) fat content compared to the other liquid wheys (Table 3) due to the absence of a fat separation step.

Thermal inactivation of LP in milk and subsequent whey was observed under high heat treatment, as expected (Table 3). LP activity in milk ranged from 0-11.5 U/ml in this study which falls in the range of LP activity from other studies (Turner and others 2007; Katekan and others 2008; Fonteh and others 2002). Past studies have shown that the LP activity in milk can vary greatly ranging from 0.05 - 16.9 U/ml (Turner and others 2007; Katekan and others 2008; Fonteh and others 2002). Such variation in LP activity in milk is affected by factors including the individual animals, species, feed and stage of lactation (Fonteh and others 2002). The low heat treated milk had an 8.70% decrease in LP concentration compared to raw milk, while no LP was detected in high heat treated milk. When milk is heated above 76°C for at least 15 sec, LP activity decreased more than 95% (Barrett and others 1999). According to the European Commission Council Directive 92/46/EEC, milk heated to greater than 85°C for at least 5 sec has LP activity below 0.02 U/mL. Therefore, the result of no LP activity was expected.

LP activity increased in whey when compared to milk (Table 3). The isoelectric point of LP is 9.2-9.9; therefore, during cheese manufacture, LP is retained in whey and gets concentrated since the curds are removed. Another possible reason for the LP activity to be lower in milk is due to the interference of casein in milk towards LP activity (Fonteh and others 2005). Fonteh and others (2005) demonstrated that casein inhibited LP activity by approximately 50%. The reason for this is unclear, however, a possible explanation for casein (most abundant protein fraction in milk) to have a negative impact on LP activity is some sort
of interaction or entrapment of the enzyme molecules within the three-dimensional network of the casein micelles, thus making a barrier between LP and substrates. The LP activity in whey was highest for URW, which did not undergo any heat treatment. The two liquid wheys, RW and LW, had the same level of LP, 27.6% lower LP than that of URW. In other words, when milk was heated at 63°C for 30 min no change (p>0.05) in LP activity was detected. However, when the whey was heat treated at 63°C for 30 min there was a decrease (p<0.05) in LP activity.

The temperature during LP bleaching (25 mg/kg H$_2$O$_2$) did not impact the bleaching efficacy of whey (P>0.05) (Figures 2, 3). After 10 min LP bleaching (25 mg/kg H$_2$O$_2$), >95% norbixin reduction was achieved for all wheys except for the HW at 35 or 50°C. Due to the high heat treatment of milk, the LP was destroyed in HW and was not available as a bleaching agent in liquid whey. Less than 10% norbixin was eliminated in HW after 1 h of bleaching. These results are consistent with Campbell and others (2012) who reported >99% bleaching in fluid whey at either 35 or 50°C for 1 h utilizing the LP system.

A distinctive feature of LP is its ability to exist in various oxidation states. The five known enzyme intermediates for LP are ferric peroxidase (the native enzyme), Compound I, Compound II, Compound III, and ferrous peroxidase (Pruitt and Kamau 1991). The peroxidative reactions are complex and follow diverse pathways depending on the H$_2$O$_2$ concentration and whether electron donors are present (de Wit and van Hooydonk 1996). Enzymatic bleaching takes place when LP is active and when 25 mg/kg H$_2$O$_2$ is used to activate the LP to initiate the bleaching process (>90 % norbixin destruction). Low concentration of H$_2$O$_2$ (25 mg/kg) is optimal to activate the LP-catalyzed H$_2$O$_2$ system to
follow the peroxidatic cycle of ferric LP $\rightarrow$ compound I $\rightarrow$compound II $\rightarrow$ ferric LP conversion (Kohler and Jenzer 1989). This cycle regenerates the LP so it remains active. In the presence of high concentrations of H$_2$O$_2$, the reaction is very fast in the first few minutes. When H$_2$O$_2$ concentration is high (250 mg/kg), Compound III is the predominantly detected state of the enzyme. Compound III is associated with irreversible LP inactivation by cleavage of the heme moiety and liberation of iron in the LP (Seifu and others 2005, Jervis and Drake 2012).

When 250 mg/kg H$_2$O$_2$ bleaching was utilized (Figures 4, 5), at 35°C, the milk/whey heat treatment displayed no effect on bleaching of norbixin, and a gradual increase up to 20% norbixin destruction was observed after 1 h of bleaching. However, for H$_2$O$_2$ bleaching at 50°C, bleaching occurred at a faster rate, reaching 10% norbixin reduction within the first 10 min. At 35°C, H$_2$O$_2$ bleaching required more than 30 min to reach the same norbixin reduction. Listiyani and others (2012) investigated the impact of temperature on bleaching of liquid Cheddar whey and demonstrated that bleaching at 68°C (30 min) (27.5% norbixin destruction) with H$_2$O$_2$ at either concentration (250 or 500 mg/kg) was more effective in reducing norbixin content in whey compared to 4°C (16 h) (10.5% norbixin destruction). Our results are in alignment with Listiyani and others (2012) in that the norbixin destruction values fell between 27.5 and 10.5%.

The results from 250 mg/kg H$_2$O$_2$ bleaching showed approximately 20% bleaching in fluid whey at 35 and 50°C for 1 h which are also in agreement with previous studies. Listiyani and others (2011) reported 43% norbixin reduction when 500 mg/kg H$_2$O$_2$ was used to bleach liquid whey at 60°C. Li and others (2012) also reported 44% norbixin reduction.
when 250 mg/kg H₂O₂ was used at 60°C for 1 h to bleach liquid whey. Campbell and others (In press) reported 50.8% norbixin destruction when serum protein was bleached using 500 mg/kg H₂O₂ at 68°C for 1 h. Campbell and others (2012) also reported 32 and 47% norbixin destruction in fluid whey using 250mg/kg H₂O₂ at 35 and 50°C for 1 h, respectively. Similarly, Jervis and others (2012) reported 44.3% norbixin destruction in liquid whey bleached using 500 mg/kg H₂O₂ at 68°C for 1 h. The results from this study confirm that the bleaching temperature is more important than the H₂O₂ concentration when H₂O₂ chemical bleaching occurs.

Norbixin destruction in whey due to heat treatment was distinct. When the % norbixin in unbleached URW and unbleached HW were compared, a 16.4±1.7 % norbixin decrease in HW was observed compared to URW (data not included). This norbixin decrease in HW coincides with a decrease in protein in HW compared to URW. When milk is heated above 70°C, whey protein denatures and forms a complex with casein micelles and is retained in the cheese curd (Fox and McSweeney 1998). This is the most likely reason why the protein concentration was lower in HW compared to the other wheys (0.6% (HW) versus 0.84% average of URW, RW, LW), an average of a 28% decrease with high heat treatment of milk (Table 2). This result was confirmed in unstandardized SDS-PAGE (Figure 6). The unstandardized SDS-PAGE gel bands of HW show a decrease in β-lg and a lack of LP, high molecular weight serum proteins (SP1-3), and casein proteolysis products (CN1,2) (Figure 6) compared to the gel bands of URW. Govindarajan and Morris (1973) suggested the possibility of norbixin binding with β-lg. The high heat treatment encourages β-lg association with casein and if norbixin is associated with β-lg, norbixin will be retained in the casein
matrix with β-lg and therefore there will be less norbixin in the HW. Another explanation for the loss of norbixin in the unbleached HW is due to more water being trapped in the cheese matrix leading to less residual norbixin in the whey. Both are possible explanations, however, based on the SDS-PAGE results and the β-lg decrease in HW, the former speculation is the more likely reason for observed loss of norbixin in the unbleached (control) HW. However it is important to note that this observation occurred following heat treatment of milk in excess of what would occur in typical cheese manufacture conditions. So this process does not represent what happens with norbixin under typical cheese manufacture.

In normal milk the most abundant proteins in the whey are β-lg, α-la and bovine serum albumin (BSA) (Hogarth and others 2004). In the present study, protein bands of 4 different heat treated wheys were evaluated using SDS-PAGE (Figure 6). Changes in whey composition, including the level of protein constituents of β-lg, α-la, and LP are shown. In the unstandardized HW gels, the β-lg band was thinner than the other samples. Knowing that β-lg composes about half of whey protein, it makes sense that the decrease in β-lg impacts the overall protein concentration in HW (Table 2). For both standardized and unstandardized gels, HW lacked an LP band which also supports the LP concentration results (Table 3).

Selected volatile compounds were previously documented to be associated with off flavors in dried whey ingredients (Croissant and others 2009; Whitson and others 2011; Listiyani and others 2011, Campbell and others 2012). A total of 12 volatiles were identified in fluid milk (Table 4). Pentanal, heptanal, hexanal, 2-pentylfuran, 1-hexen-3-one, nonanal, and DMTS were detected at different levels due to different heat treatments. Overall, milk volatile compounds (Table 4) were detected at lower concentrations than that of whey (Table
5). These differences are due to milk not going through the cheese manufacture procedure of adding rennet, additional heat treatment and culture addition. Thus, lower volatile compound concentrations were expected. Campbell and others (2011) recorded volatile compounds of milk to fall in similar range as those found in this study.

Volatile compound differences were also evident among wheys. Volatiles identified in liquid wheys included diacetyl, nonanal, heptanal, hexanal, phenylacetaldehyde, 2-pentylfuran, octanal, pentanal, z-4-heptenal, e-2-octenal, and 1-hexen-3-one (Table 5, 6). All of these volatile compounds have previously been identified in liquid whey (Carunchia-Whetstine and others 2003; Karagul-Yuceer and others 2003; Tomaino and others 2004; Gallardo-Escamilla and others 2005). Diacetyl concentration was highest for URW prior to bleaching. DMS, DMTS and were detected at low concentrations (<0.010 ppb) in wheys however, different H$_2$O$_2$ or LP bleaching treatments did not have any impact on these volatile compounds. In general, unbleached wheys (controls) had lower concentrations of aldehydes compared to wheys that were bleached which is consistent with previous studies (Listiyani and others 2012; Campbell and others 2012). As bleaching time progressed, concentrations of hexanal, heptanal, octanal, nonanal, and 2-pentylfuran increased in wheys (Table 6). Except for the HW, the liquid Cheddar wheys (URW, RW, LW) bleached by chemical H$_2$O$_2$ bleaching (250 mg/kg H$_2$O$_2$) were characterized by lower relative abundances of lipid oxidation products (aldehydes), than liquid wheys bleached using the LP system (25 mg/kg H$_2$O$_2$) (Table 5, 6; Figure 7). These results are consistent with a previous study conducted by Campbell and others (2012) who reported higher lipid oxidation compounds in LP bleached whey compared to H$_2$O$_2$ bleached whey. HW was different from the other wheys. Nonanal,
heptanal, hexanal, phenylaetaldehyde, 2-pentylfuran, pentanal, z-4 heptenal, e-2-octenal and 1-hexen 3-one were all higher (p<0.05) in HW compared to other wheys. This result demonstrates that the high heat treatment of milk contributed to lipid oxidation prior to bleaching. The abundance of several ketones, aldehydes and sulfur compounds have been reported to increase during thermal processing of milk (Lloyd and others 2009, Hall and others 1985). Suloff (2002) also demonstrated an increase in hexanal, heptanal, octanal, nonanal, and decanal in UHT milk (142°C, 4.6sec) compared to pasteurized milk (75°C, 13 sec).

CONCLUSIONS

Abundant literature was already published regarding LP in milk and its usage as an antimicrobial agent. It is well documented that LP activity is highly variable due to many uncontrollable factors. The effect of bleaching temperature (35°C vs. 50°C) of H$_2$O$_2$ (250mg/kg H$_2$O$_2$) and LP (25mg/kg H$_2$O$_2$) on liquid whey was minor compared to the effect of LP bleaching due to various milk heat treatments. The overall bleaching effect of LP was higher than that of H$_2$O$_2$. Hydrogen peroxide bleaching of colored whey was less effective (20% destruction) at reducing the norbixin content in whey; while LP bleaching resulted in decreasing the norbixin content by 90%. These results demonstrated that LP was a more effective bleaching agent compared with H$_2$O$_2$ in bleaching colored whey at the conditions evaluated. Overall, the results from this study suggest that bleaching with LP is more effective than bleaching with H$_2$O$_2$ in fluid whey. It is also critical to note that LP bleaching is H$_2$O$_2$ concentration dependent. Milk heat treatment impacts enzymatic bleaching efficacy.
of whey. In this study, chemical bleaching took place in two different occasions. First, when LP was thermally inactivated, chemical bleaching occurred and resulted in 20 % norbixin destruction regardless of the concentration of H₂O₂, time or temperature. Second, even though LP was active in liquid whey, if it was treated with 250 mg/kg H₂O₂, the high concentration of H₂O₂ inactivated the LP and chemical bleaching occurred. Additionally, the differences in norbixin concentration of the different heat treated unbleached liquid wheys suggest differences in norbixin association with whey proteins following heat treatment. The results from this study demonstrated the influence of milk heat treatment on bleaching efficacy and also suggested differences in norbixin association with whey components with different milk heat treatments.
REFERENCES


<table>
<thead>
<tr>
<th>Milk</th>
<th>Milk heat treatment</th>
<th>Whey heat treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heat treatment</td>
<td>Raw milk was used as is</td>
<td>No heat treatment (URW)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey was heated at 63°C for 30 min (RW)</td>
</tr>
<tr>
<td>Low heat treatment</td>
<td>Raw milk was heated at 63°C for 30 min</td>
<td>Whey was heated at 63°C for 30 min (LW)</td>
</tr>
<tr>
<td>High heat treatment</td>
<td>Raw milk was heated at 78°C for 28 sec</td>
<td>Whey was heated at 63°C for 30 min (HW)</td>
</tr>
</tbody>
</table>
Table 2. Proximate analysis of bleached wheys.

<table>
<thead>
<tr>
<th></th>
<th>URW</th>
<th>RW</th>
<th>LW</th>
<th>HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solids</td>
<td>6.9 ± 0.34a</td>
<td>6.1 ± 0.34a</td>
<td>6.2 ± 0.18a</td>
<td>5.6 ± 0.45a</td>
</tr>
<tr>
<td>Protein</td>
<td>0.89 ± 0.05a</td>
<td>0.80 ± 0.02a</td>
<td>0.82 ± 0.03a</td>
<td>0.60 ± 0.04b</td>
</tr>
<tr>
<td>Fat</td>
<td>0.36 ± 0.03a</td>
<td>0.06 ± 0.05b</td>
<td>0.04 ± 0.03b</td>
<td>0.03 ± 0.02b</td>
</tr>
</tbody>
</table>

a-b: Means in a row with different letters are different (p<0.05)

URW: Milk – raw; whey – unpasteurized
RW: Milk – raw; whey – pasteurized at 63°C for 30 min
LW: Milk – pasteurized at 63°C for 30 min; whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; whey – pasteurized at 63°C for 30 min
Table 3. LP activity in milks and wheys.

<table>
<thead>
<tr>
<th>Milk</th>
<th>LP activity (Unit/ml)</th>
<th>Whey</th>
<th>LP activity (Unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>11.5a</td>
<td>URW</td>
<td>54.9a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RW</td>
<td>40.2b</td>
</tr>
<tr>
<td>Low heat treated</td>
<td>10.5a</td>
<td>LW</td>
<td>39.3b</td>
</tr>
<tr>
<td>treated milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High heat</td>
<td>ND</td>
<td>HW</td>
<td>ND</td>
</tr>
<tr>
<td>treated milk</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a-b: Means in a column with different letters are different (p<0.05)
LP: Lactoperoxidase
URW: Milk – raw; whey – unpasteurized
RW: Milk – raw; whey – pasteurized at 63°C for 30 min
LW: Milk – pasteurized at 63°C for 30 min; whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; whey – pasteurized at 63°C for 30 min
ND: not detected
Table 4. Volatile compound relative abundance of milks (ppb).

<table>
<thead>
<tr>
<th>Category</th>
<th>Raw milk</th>
<th>Low heat treated milk</th>
<th>High heat treated milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonanal</td>
<td>0.007</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Octanal</td>
<td>0.181a</td>
<td>0.219a</td>
<td>0.159a</td>
</tr>
<tr>
<td>Pentanal</td>
<td>0.014a</td>
<td>0.007b</td>
<td>0.009ab</td>
</tr>
<tr>
<td>z-4-heptenal</td>
<td>0.004a</td>
<td>0.006a</td>
<td>0.005a</td>
</tr>
<tr>
<td>DMS</td>
<td>0.125a</td>
<td>0.092a</td>
<td>0.106a</td>
</tr>
<tr>
<td>Heptanal</td>
<td>0.002</td>
<td>0.002</td>
<td>ND</td>
</tr>
<tr>
<td>e,e-2,4-nonadienal</td>
<td>0.002a</td>
<td>0.001a</td>
<td>0.001a</td>
</tr>
<tr>
<td>2-pentylfuran</td>
<td>0.005a</td>
<td>0.004ab</td>
<td>0.003b</td>
</tr>
<tr>
<td>Hexanal</td>
<td>0.049a</td>
<td>0.036b</td>
<td>0.019c</td>
</tr>
<tr>
<td>DMTS</td>
<td>0.008a</td>
<td>0.006ab</td>
<td>0.004b</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>0.003a</td>
<td>0.006a</td>
<td>0.005a</td>
</tr>
<tr>
<td>1-hexen-3-one</td>
<td>0.001</td>
<td>0.001</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a-c Means in the same row not sharing a common letter are different (p<0.05).
ND = not detected

Low heat treated milk: pasteurized at 63°C for 30 min
High heat treated milk: pasteurized at 78°C for 28 sec
DMS: dimethylsulfide
DMTS: dimethyltrisulfide
Table 5. Relative abundance (μg/kg) of selected volatile compounds for LP (25 mg H$_2$O$_2$/kg)/ H$_2$O$_2$ bleached (250 mg H$_2$O$_2$/kg) wheys at 35/50°C for 1 h.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Temp.</th>
<th>Trt.</th>
<th>URW</th>
<th>RW</th>
<th>LW</th>
<th>HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetyl</td>
<td>35°C</td>
<td>LP</td>
<td>0.038a</td>
<td>0.022ab</td>
<td>0.023ab</td>
<td>0.021ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.034ab</td>
<td>0.021ab</td>
<td>0.022ab</td>
<td>0.018b</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>LP</td>
<td>0.028a</td>
<td>0.021a</td>
<td>0.025a</td>
<td>0.017a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.024a</td>
<td>0.018a</td>
<td>0.018a</td>
<td>0.014a</td>
</tr>
<tr>
<td>Nonanal</td>
<td>35°C</td>
<td>LP</td>
<td>0.034b</td>
<td>0.205a</td>
<td>0.104ab</td>
<td>0.121ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.013b</td>
<td>0.045b</td>
<td>0.024b</td>
<td>0.087ab</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>LP</td>
<td>0.023b</td>
<td>0.154a</td>
<td>0.113a</td>
<td>0.090a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.011b</td>
<td>0.070a</td>
<td>0.050a</td>
<td>0.079a</td>
</tr>
<tr>
<td>Heptanal</td>
<td>35°C</td>
<td>LP</td>
<td>1.890ab</td>
<td>2.493a</td>
<td>1.452ab</td>
<td>0.971bc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.078c</td>
<td>0.981bc</td>
<td>0.484bc</td>
<td>0.895bc</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>LP</td>
<td>0.973a</td>
<td>2.005a</td>
<td>1.554a</td>
<td>0.967a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.994b</td>
<td>1.281a</td>
<td>0.824a</td>
<td>0.860a</td>
</tr>
<tr>
<td>Phenylacetaldelyde</td>
<td>35°C</td>
<td>LP</td>
<td>0.007b</td>
<td>0.056a</td>
<td>0.022b</td>
<td>0.015b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.001a</td>
<td>0.015a</td>
<td>0.006a</td>
<td>0.012af</td>
</tr>
<tr>
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<td>50°C</td>
<td>LP</td>
<td>0.002b</td>
<td>0.033a</td>
<td>0.021ab</td>
<td>0.010ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.001b</td>
<td>0.015ab</td>
<td>0.009ab</td>
<td>0.009ab</td>
</tr>
<tr>
<td>2-pentylfuran</td>
<td>35°C</td>
<td>LP</td>
<td>0.044ab</td>
<td>0.163a</td>
<td>0.093ab</td>
<td>0.072ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.004b</td>
<td>0.057ab</td>
<td>0.026b</td>
<td>0.062ab</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>LP</td>
<td>0.038a</td>
<td>0.265a</td>
<td>0.203a</td>
<td>0.136a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.008a</td>
<td>0.099a</td>
<td>0.061a</td>
<td>0.116a</td>
</tr>
<tr>
<td>Octanal</td>
<td>35°C</td>
<td>LP</td>
<td>0.075a</td>
<td>0.077a</td>
<td>0.062a</td>
<td>0.038b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.078a</td>
<td>0.071a</td>
<td>0.078a</td>
<td>0.036b</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>LP</td>
<td>0.060a</td>
<td>0.090a</td>
<td>0.058a</td>
<td>0.031a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.048a</td>
<td>0.029a</td>
<td>0.022a</td>
<td>0.027a</td>
</tr>
</tbody>
</table>
Table 5. Continued

<table>
<thead>
<tr>
<th></th>
<th>35°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pentanal</strong></td>
<td>0.047a</td>
<td>0.057a</td>
</tr>
<tr>
<td></td>
<td>0.003a</td>
<td>0.018a</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.036a</td>
<td>0.037a</td>
</tr>
<tr>
<td><strong>LW</strong></td>
<td>0.027a</td>
<td>0.057a</td>
</tr>
<tr>
<td><strong>LP</strong></td>
<td>0.042a</td>
<td>0.021a</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.034a</td>
<td>0.021a</td>
</tr>
<tr>
<td><strong>z-4-heptenal</strong></td>
<td>0.003a</td>
<td>0.034a</td>
</tr>
<tr>
<td></td>
<td>0.007a</td>
<td>0.018a</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.007a</td>
<td>0.037a</td>
</tr>
<tr>
<td><strong>LW</strong></td>
<td>0.004a</td>
<td>0.021a</td>
</tr>
<tr>
<td><strong>LP</strong></td>
<td>0.002b</td>
<td>0.025a</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
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<td>0.037a</td>
</tr>
<tr>
<td><strong>z-4-heptenal</strong></td>
<td>0.002b</td>
<td>0.018a</td>
</tr>
<tr>
<td></td>
<td>0.007a</td>
<td>0.034a</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.004a</td>
<td>0.011a</td>
</tr>
<tr>
<td><strong>e-2-octenal</strong></td>
<td>0.009ab</td>
<td>0.003ab</td>
</tr>
<tr>
<td></td>
<td>0.003a</td>
<td>0.008ab</td>
</tr>
<tr>
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<td>ND</td>
</tr>
<tr>
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<td>0.003ab</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.003a</td>
<td>0.004a</td>
</tr>
<tr>
<td><strong>LP</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.006a</td>
<td>0.002a</td>
</tr>
<tr>
<td><strong>LP</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>0.006a</td>
<td>0.002a</td>
</tr>
</tbody>
</table>

a-c Samples with different letters are significantly different from each other within each row (P<0.05)

URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
LP: 25mg H₂O₂/kg used for bleaching
H₂O₂: 250mg H₂O₂/kg used for bleaching
Table 6. Relative abundance (μg/kg) of selected volatile compounds in LP bleached (25mg H$_2$O$_2$/kg) wheys.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment</th>
<th>URW</th>
<th>RW</th>
<th>LW</th>
<th>HW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diacetyl</strong></td>
<td>Control</td>
<td>0.032a</td>
<td>0.019a</td>
<td>0.023a</td>
<td>0.020a</td>
</tr>
<tr>
<td></td>
<td>35°C 5min</td>
<td>0.042a</td>
<td>0.019b</td>
<td>0.020b</td>
<td>0.017b</td>
</tr>
<tr>
<td></td>
<td>10min</td>
<td>0.033a</td>
<td>0.020a</td>
<td>0.020a</td>
<td>0.018a</td>
</tr>
<tr>
<td></td>
<td>20min</td>
<td>0.033a</td>
<td>0.021a</td>
<td>0.025a</td>
<td>0.019a</td>
</tr>
<tr>
<td></td>
<td>60min</td>
<td>0.038a</td>
<td>0.022ab</td>
<td>0.023ab</td>
<td>0.021ab</td>
</tr>
<tr>
<td></td>
<td>50°C 5min</td>
<td>0.027a</td>
<td>0.023a</td>
<td>0.023a</td>
<td>0.014a</td>
</tr>
<tr>
<td></td>
<td>10min</td>
<td>0.017a</td>
<td>0.019a</td>
<td>0.023a</td>
<td>0.015a</td>
</tr>
<tr>
<td></td>
<td>20min</td>
<td>0.026a</td>
<td>0.017a</td>
<td>0.025a</td>
<td>0.016a</td>
</tr>
<tr>
<td></td>
<td>60min</td>
<td>0.028a</td>
<td>0.021a</td>
<td>0.025a</td>
<td>0.017a</td>
</tr>
<tr>
<td><strong>Nonanal</strong></td>
<td>Control</td>
<td>0.009b</td>
<td>0.023b</td>
<td>0.023b</td>
<td>0.095a</td>
</tr>
<tr>
<td></td>
<td>35°C 5min</td>
<td>0.012b</td>
<td>0.054ab</td>
<td>0.043b</td>
<td>0.112a</td>
</tr>
<tr>
<td></td>
<td>10min</td>
<td>0.013b</td>
<td>0.088a</td>
<td>0.043ab</td>
<td>0.106a</td>
</tr>
<tr>
<td></td>
<td>20min</td>
<td>0.025a</td>
<td>0.139a</td>
<td>0.133a</td>
<td>0.116a</td>
</tr>
<tr>
<td></td>
<td>60min</td>
<td>0.034b</td>
<td>0.205a</td>
<td>0.104ab</td>
<td>0.121ab</td>
</tr>
<tr>
<td></td>
<td>50°C 5min</td>
<td>0.023a</td>
<td>0.050a</td>
<td>0.064a</td>
<td>0.089a</td>
</tr>
<tr>
<td></td>
<td>10min</td>
<td>0.023a</td>
<td>0.082a</td>
<td>0.070a</td>
<td>0.090a</td>
</tr>
<tr>
<td></td>
<td>20min</td>
<td>0.025a</td>
<td>0.086a</td>
<td>0.094a</td>
<td>0.095a</td>
</tr>
<tr>
<td></td>
<td>60min</td>
<td>0.023a</td>
<td>0.154a</td>
<td>0.113a</td>
<td>0.090a</td>
</tr>
<tr>
<td><strong>Heptanal</strong></td>
<td>Control</td>
<td>0.002b</td>
<td>0.011b</td>
<td>0.007b</td>
<td>0.041a</td>
</tr>
<tr>
<td></td>
<td>35°C 5min</td>
<td>0.005b</td>
<td>0.021b</td>
<td>0.016b</td>
<td>0.049a</td>
</tr>
<tr>
<td></td>
<td>10min</td>
<td>0.007a</td>
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a-c Samples with different letters are significantly different from each other within each row (P<0.05)
URW: Milk – raw; whey – unpasteurized
RW: Milk – raw; whey – pasteurized at 63°C for 30 min
LW: Milk – pasteurized at 63°C for 30 min; whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; whey – pasteurized at 63°C for 30 min
LP: 25mg H₂O₂/kg used for bleaching
H₂O₂: 250mg H₂O₂ /kg used for bleaching
Figure 1. Experimental design for liquid whey bleach treatments and measurements.

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URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
LP: 25mg H₂O₂/kg used for bleaching
H₂O₂: 250mg H₂O₂/kg used for bleaching
Figure 2. Norbixin % elimination after bleaching fluid whey with LP (25 mg H$_2$O$_2$/kg) at 35°C for 1 h.

URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
LP: 25mg H$_2$O$_2$/kg used for bleaching
Figure 3. Norbixin % elimination after bleaching fluid whey with LP (25 mg H₂O₂/kg) at 50°C for 1 h.

URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
LP: 25mg H₂O₂/kg used for bleaching
Figure 4. Norbixin % elimination after bleaching fluid whey with H$_2$O$_2$ (250 mg H$_2$O$_2$/kg) at 35°C for 1 h.

URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
H$_2$O$_2$: 250mg H$_2$O$_2$/kg used for bleaching
Figure 5. Norbixin % elimination after bleaching fluid whey with H₂O₂ (250 mg H₂O₂/kg) at 50°C for 1 h.

URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
H₂O₂: 250mg H₂O₂/kg used for bleaching
Figure 6. Sodium dodecyl sulfate-PAGE of proteins in milk and liquid wheys. The loading of the samples were 10 μL and samples were run in duplicate.

URW: Milk – raw; whey – unpasteurized
RW: Milk – raw; whey – pasteurized at 63°C for 30 min
LW: Milk – pasteurized at 63°C for 30 min; whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; whey – pasteurized at 63°C for 30 min
STD: standard

Bands are identified on the gel: LP = lactoperoxidase; BSA = bovine serum albumin; SP1, SP2, SP3 = high molecular weight serum proteins, CN1 = αs-CN (combination of αs1 and αs2-CN), CN2 = casein proteolysis products, β-lg (β-lactoglobulin), α-la (α-lactalbumin), β-CN (β-casein) and κ-CN (κ-casein) CN3 = proteolysis products of casein.
Figure 7. PCA biplot of selected volatile compounds in fluid whey after bleaching with LP (25 mg H2O2/kg) or chemical bleaching with H2O2 (250 mg H2O2/kg) at 35 or 50°C for 1 h.

URW: Milk – Raw; Whey – unpasteurized
RW: Milk – Raw; Whey – pasteurized at 63°C for 30 min
LW: Milk – Pasteurized at 63°C for 30 min; Whey – pasteurized at 63°C for 30 min
HW: Milk – pasteurized at 78°C for 28; Whey – pasteurized at 63°C for 30 min
LP: 25mg H2O2/kg used for bleaching
H2O2: 250mg H2O2/kg used for bleaching
Invited review: Annatto usage and bleaching in dairy foods

E. J. Kang,* R. E. Campbell,* E. Bastian,† and M. A. Drake**†
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ABSTRACT

Annatto is a yellow/orange colorant that is widely used in the food industry, particularly in the dairy industry. Annatto, consisting of the carotenoids bixin and norbixin, is most commonly added to produce orange cheese, such as Cheddar, to achieve a consistent color over seasonal changes. This colorant is not all retained in the cheese, and thus a percentage remains in the whey, which is highly undesirable. As a result, whey is often bleached. Hydrogen peroxide and benzoyl peroxide are the 2 bleaching agents currently approved for bleaching whey in the United States. Recent studies have highlighted the negative effect of bleaching on whey flavor while concurrently there is a dearth of current studies on bleaching conditions and efficacy. Recent international mandates have placed additional concern on the use of benzoyl peroxide as a bleaching agent. This review discusses the advantages, disadvantages, regulatory concerns, flavor implications, and optimal usage conditions of 2 widely used bleaching agents, hydrogen peroxide and benzoyl peroxide, as well as a few alternative methods including lipoxygenase, peroxidase, and lactoperoxidase systems.

Key words: annatto, bleach, flavor, whey

INTRODUCTION

Annatto is a yellow/orange carotenoid that is widely used in the food industry. Annatto comes from seeds of the tropical tree fruit *Bixa orellana*, which was named after Francisco de Orellana, a scientist and explorer of the upper Amazon (Giuliano et al., 2003). Clusters of the fruit, which is capsule shaped and covered in burrs, grow on the tree and there are about 10 to 50 small seeds inside the fruit (Ames and Hofmann, 2001). The seeds are covered in a bright red pulp and this pulp contains the annatto pigment (Giuliano et al., 2003). Latin America produces about 60% of the world’s annatto, followed by Africa (27%) and Asia (12%) (Giuliano et al., 2000). Prices for annatto seeds depend on production and are proportional to the content of bixin, the major pigment (Giuliano et al., 2003). The United States and Europe are the 2 largest importers of annatto seeds, but the Japanese market has grown rapidly since the introduction of the colorant in 1965 (Ames and Hofmann, 2001).

Annatto as a colorant is not a new concept. The Aztecs used annatto extract as a dye for textiles, body dye (such as in lipsticks), and as a food colorant in the drink cacahuate (Giuliano et al., 2003). In addition to being used to impart color in various items, annatto has been used as a spice. Native Americans commonly used ground bixa seeds in their cacao beverages to impart a slight red color and to add a musky flavor comparable to that of paprika or saffron (Mikszteck et al., 1981; Norton, 2006). Today, annatto is still used as a spice, especially in Latin American dishes such as *cochinita pibil*, a pork dish with ground bixa seeds and bitter orange juice (Gerlach and Gerlach, 2002). It is also used in sausages, fish, margarine, snacks, dressings, sauces, and confections, but usage varies from country to country due to different food customs and regulations (Ames and Hofmann, 2001; Scotter, 2000). The major application of annatto in the United States is within the dairy industry, where it is used to color cheeses and other dairy products (Emerton, 2008).

Extraction of Annatto

Three commercial processes are used to extract the carotenoid pigment from dried annatto seeds: direct extraction into oil, direct extraction into aqueous alkali, or indirect extraction with solvents (Preston and Rickard, 1980). Extraction with oil or solvent yields a colorant that is mainly bixin (Mortenson et al., 2008). Extraction with aqueous alkali (the form used in the dairy industry, e.g., for cheese color) saponifies bixin’s methyl group on bixin, yielding norbixin as the principal colorant (Giuliano et al., 2003; Mortenson et al., 2008). Both bixin and norbixin naturally occur in the cis form but can be converted to the trans form by light and heat (McKeown 1963, 1965). The trans forms of both
bixin and norbixin are more red than their cis forms (Preston and Richard, 1980; Scotter et al., 1994).

**Volatile Compounds**

Although annatto has been used in foods as a spice (flavoring), there is little information about how volatile compounds of annatto contribute to flavor. Galindo-Cuspinera et al. (2002) evaluated volatile profiles of oil and water-soluble annatto extracts and reported that \( \beta \)-humulene was the major volatile present. \( \beta \)-Humulene is described as having a woody, spicy aroma and a slightly bitter taste (Galindo-Cuspinera et al., 2002). Other important volatiles found in annatto extracts that might contribute to flavor were \( p \)-xylene, toluene, \( \alpha \)- and \( \beta \)-pinene, \( \gamma \)-elemene, and spathulenol. Annatto extraction uses heat (Scotter et al., 2002), which means that different commercial products may experience varying degrees of thermal degradation. Further studies also suggest that heat treatment of annatto in foods may result in additional flavor-contributing volatile compounds. Scotter (1995, 1998, 2000, 2001) studied the thermal degradation products of both bixin and norbixin and found that heat released the aromatic compounds \( m \)-xylene and toluene. As mentioned previously, annatto is a major colorant used in the dairy industry, often in cheese and in processed cheese that involves heat treatment. Volatiles from thermal degradation of annatto in actual dairy products (e.g., cheese) have yet to be studied. Studies conducted by Scotter et al. (2002) and Galindo-Cuspinera et al. (2002) indicate that there are numerous odorants in annatto, such as \( \beta \)-humulene, \( p \)-xylene, toluene, \( \alpha \)- and \( \beta \)-pinene, \( \gamma \)-elemene, and spathulenol, with potential to influence food aroma; however, more studies are needed in this area.

**Discoloration – Oxidation**

Annatto has been used in dairy products since the 1800s to standardize the color of cheese, which varies due to seasonal feed variations in the milk. Chr. Hansen Inc., a major, modern-day supplier of annatto to the dairy industry, opened their first factory in 1874 (Kristin Schneider, Chr. Hansen Inc., Milwaukee, WI; personal communication). Over the years, an orange color has become expected in many cheeses, requiring addition of annatto to cheesemilk. According to 21 CFR 73.30 (US FDA, 2006), annatto extract may be used for coloring foods as long as good manufacturing practices (GMP) are followed. Therefore, in the United States, there is no “maximum level” of usage for annatto; however, Scotter et al. (2002) reported that the maximum level of annatto addition in commercial Red Leicester cheese in the United Kingdom was 50 mg of norbixin/kg of cheese and the estimated range of annatto in Red Leicester was 23.7 to 37.5 mg of norbixin/kg of cheese. For ripened orange, yellow, and broken white cheese, and flavored processed cheese in the United Kingdom, the maximum level of annatto addition is 15 mg/kg. The estimated range of annatto is 0.2 to 9.6 mg/kg (n = 16) and 0.2 to 21.4 mg/kg (n = 8) for ripened orange, yellow, and broken white cheese, and for flavored processed cheese, respectively. Currently in the United States, no study has been conducted to determine the amounts of annatto typically found in cheese. In the United States, annatto is approved as a color additive in foods at GMP, meaning that the “Annatto extract may be safely used for coloring foods generally, in amounts consistent with good manufacturing practice...” (Hulagan et al., 1995; Giuliano et al., 2005; US FDA, 2006a). Although annatto is used to standardize the color of cheese, some studies have reported the usage of annatto leading to pinking or coloration of cheeses (Hood and White, 1929; Moir, 1933; Morgan, 1938; Bannister, 1937; 1950; Govindaraj and Morris, 1973).

Bixin and norbixin have highly conjugated structures making these compounds susceptible to both oxidation and reduction. Oxidation is important to the fluid whey industry because oxidation leads to color loss, which is the primary goal of bleaching and will be discussed later in this review. Also important, a study suggested that norbixin might be able to bind with proteins such as \( \beta \)-casein or \( \beta \)-lactoglobulin and form a stable complex that can help prevent oxidation and color loss (Govindaraj and Morris, 1973). This reaction has not been proven; it may be desirable in some products such as cheese but reduce color removal during bleaching of whey.

**CAROTENOIDS IN MILK**

Carotenoids are lipophilic molecules that are found in milk fat. The diet of the cow can influence the color of cheese (Caropino, et al., 2004). A wide variety of carotenoids and their degradation products are present in the forage that cows eat, but only a small number of different carotenoids can be identified in milk. This is because carotenoid transfer from diet to milk is low and carotenoids can be broken down to colorless compounds in the gastrointestinal tract (Noniere et al., 2006). Some of these colorless compounds may be transferred to the milk and have effects on the sensory profile even though they do not impart any color to the final product. Carotenoids identified in milk include lutein, violaxanthin, anthaxanthin, zeaxanthin, neoxanthin, all-trans \( \beta \)-carotene, \( \alpha \)-carotene, and 13-cis \( \beta \)-carotene. Among these, lutein and \( \beta \)-carotene are the most quantitatively
important (Noziere et al., 2006) with β-carotene comprising about 90% of total carotenoids present in cow's milk (Hulshof et al., 2005).

Carotenoids can influence the sensory properties of milk either indirectly through their antioxidant properties by stabilizing oxidizable compounds or directly through their yellowing properties. β-Carotene can be converted to vitamin A, but incomplete conversion leaves a yellow color in the milk (Patton et al., 1980).

The main components in milk are riboflavin (vitamin B2), a green compound present in the aqueous phase, and β-carotene in the lipid phase (Noziere et al., 2006).

Riboflavin and carotenoid contents vary widely in milk and are influenced by diet, breed, and season (Schröder, 2003; Noziere et al., 2006). Croissant et al. (2007) reported that milk from pasture-fed Holstein and Jersey cows exhibited more yellow color than that from their similar counterparts fed a conventional, TMR diet. These findings were expected because pasture-fed cows ingested fresh forage, containing carotenoids, thus raising the concentration of carotenoids in milk fat. Carotenoids found in milk also carry over into fluid whey during cheese production, where they may increase the color of spray-dried whey and require a bleaching process.

**Carotenoid Measurement in Foods**

Carotenoid content can be difficult to measure because carotenoids isomerize rapidly due to their conjugated double bonds (de Oliveira and Rodriguez-Amaya, 2007). Isomerization can be promoted by acid, light, and heat (de Oliveira and Rodriguez-Amaya, 2007). As such, precautions must be taken to minimize these reactions during extraction and measurement. These precautions include, but are not limited to, completion of analysis within the shortest time possible, protection from light, avoiding high temperatures or contact with acid, and the use of high-purity solvents (de Oliveira and Rodriguez-Amaya, 2007). Once carotenoids are isolated, quantification and quantification are typically performed using the same methods.

Before quantification, the carotenoid must be positively identified. This is done using chromatography—open column, thin layer, or HPLC (Bushway, 1985). The best of these 3 methods, HPLC, saves time and can distinguish between stereoisomers (Bushway, 1985).

The carotenoid can be identified using retention times, co-chromatography with carotenoid standards, or UV-visible absorption spectra (de Oliveira and Rodriguez-Amaya, 2007). After the carotenoid is positively identified, it is then quantified by using an external standard curve (de Oliveira and Rodriguez-Amaya, 2007).

**Annatto Measurement in Foods**

Annatto can be found in a wide array of foods ranging from dairy products to other naturally colored products. A colorimetric method for measuring the amount of annatto in commercial bleached and unbleached dry whey powder by extracting the annatto using ammonia, ethanol, and phosphate solution was reported by Hammond et al. (1973). Lancaster and Lawrence (1985) extracted annatto from high-fat dairy products, with good repeatability, although methods were labor intensive. Annatto was confirmed and components were identified via HPLC (Lancaster and Lawrence, 1995).

Scotter et al. (2002) extracted and analyzed annatto in marigold, ice cream, custard powder, breakfast cereals, cakes, fish, and jellies using various extraction methods tailored to each food matrix followed by HPLC coupled with spectral confirmation. Bareth et al. (2002) described a rapid, simple extraction and detection method specific for annatto in cheese and milk products using HPLC and a spectrophotometric method. Very recently, Croissant et al. (2009) reported a successful method to extract and quantify aecubin (the primary annatto constituent in whey) from liquid whey, liquid retentate, and spray-dried whey protein concentrate. Analytical methods for annatto and analysis in food have been reviewed by Scotter (2009).

**BLEACHING**

Food is bleached not only to improve color, but in some cases to improve its quality such as improving gluten development in flour. The bleaching process can be categorized into 3 groups depending on the mechanism: oxidizing bleach, reducing bleach, and biochemical bleach (Bals et al., 1942; Bottomley et al., 1989; Ross et al., 2000).

**Oxidizing Bleach**

The portion of a molecule that emits color is called a chromophore and it is usually the most fragile part of the molecule. Destroying the chromophore will often render a colored molecule colorless. Oxidizing agents (bleach) destroy (oxidize) the double bond(s) within a chromophore. This reaction changes the molecule into a different substance in which the chromophore does not exist or a shorter chromophore exists. A shorter chromophore will absorb light of a shorter wavelength than visible light (UV light) and therefore will not appear colored (Winter et al., 2008). Many oxidizing bleaches are used in industry including chlorine dioxide, which is used for the bleaching of wood pulp, fats and oils.

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cellulose, flour, textiles, and beeswax. Organic peroxides, such as hydrogen peroxide and benzoyl peroxide and bromates, are oxidizing bleaches used for whey and flour bleaching and as maturing agents. Another application is in the wood and pulp industry to produce chlorine-free paper for which the industry uses persacetic acid, ozone, hydrogen peroxide, and oxygen in bleaching sequences.

Reducing Bleach

A reducing bleach works by converting double bonds in a chromophore into single bonds. This eliminates the ability of the chromophore to absorb visible light. Sunlight releases high-energy photons, often in the violet or UV range, that can disrupt bonds in a chromophore, rendering the substance colorless. Many colored molecules are relatively fragile and are damaged by photons of UV light. Traditionally, exposure to sunlight was the way to bleach fabrics and make them white (Bloomefield, 2007). Reducing bleaches commonly used in foods include lemon juice (in combination with sunlight) and sulfur dioxide.

Biochemical Bleach

Enzymes such as lipoygenase (EC 1.13.11.12; lipoydase) can fall in the third category of bleaching agents: biochemical bleaches. Lipoygenase was first discovered in 1928 by Bohn and Hase (1933) as a carotene-destroying enzyme (“carotene oxidase”) in soybeans, and later designated as lipoygenase. Acceleration of the oxidation of xanthophylls to colorless products has long been recognized as a property of soybean lipoygenase used in the bleaching of bread dough (Balls et al., 1945). A patent on biochemical bleaching of dairy products was introduced in the United States recently (Roos et al., 2006). Roos et al. (2005) disclosed the use of lipoygenases to bleach dairy products, including whey. Only small amounts of lipoygenases have a whitening effect on dairy products such as milk, cheese, butter oil, cream, and whey products. The mechanism of bleaching by lipoygenases is based on the oxidative transition of double bonds in carotenoids by radicals produced in the reaction of lipoygenase and linole(n)ic acid. Soy lipoygenase has been used for bleaching purposes in wheat and maize flour; however, it has not been previously applied to bleach whey. Because a more specific enzymatic reaction is occurring rather than a general oxidation treatment, as is the case with oxidizing bleaches, enzymatic bleaching may reduce off-flavors. Recently (November 2009), an external enzymatic (biochemical) bleach was launched for bleaching of fluid whey: MaxiBright (DSM Food Specialties, Delft, the Netherlands) is a fungal peroxidase that can purportedly be used in place of an oxidizing bleach to bleach fluid whey. The enzyme is specific for carotenoids (Zorn et al., 2003), thus yielding whiter whey without the use of traditional bleaching agents, and possibly reducing unwanted side effects of traditional bleaching. Like all peroxidases, MaxiBright requires activation with 0.5 to 1 m/l hydrogen peroxide, but the hydrogen peroxide is consumed and catalase addition is not required. There are currently no available published studies on the use of MaxiBright with whey or in comparison to traditional oxidizing bleaches. Food and Drug Administration’s “generally regarded as safe” (GRAS) status has been filed for MaxiBright, and approval is anticipated in September 2010.

BLEACHING WHEY

Annatto is used by the dairy industry to color cheeses. The specific amount of annatto that partitions into cheese and whey has not, to our knowledge, been directly studied. However, approximately 20% of the annatto added to cheese milk is estimated to pass into whey (Barnes, 1950). The color from annatto is highly unfavorable in dried whey products and thus a decolorizing process must be performed and has been in place in the dairy industry for more than 40 yr (McDonough et al., 1968). This process involves bleaching of liquid whey or liquid retentate with hydrogen or benzoyl peroxide. Hydrogen peroxide and benzoyl peroxide are the only bleaching agents legally allowed for treatment of whey in the United States and there are several restrictions to their use.

Hydrogen Peroxide

Hydrogen peroxide (H2O2) is a clear, colorless liquid with a slightly pungent odor. Hydrogen peroxide decomposes to oxygen and water during bleaching (Table 1). Residual hydrogen peroxide must be removed from whey and cheese milk physically or by the addition of catalase according to FDA regulations 184.1366 and 133.133 (US FDA, 2009a,c). Catalase converts hydrogen peroxide into oxygen and water (Table 1). Catalase use must not exceed 20 ppm and must be sufficient to remove any residual hydrogen peroxide (US FDA, 2009a). As hydrogen peroxide is a GRAS substance, the maximum treatment level for bleaching annato-colored whey using hydrogen peroxide is 0.05% (100 ppm) of the whey (US FDA, 2009a). There are no specific provisions in European Union regulations regarding the use of hydrogen peroxide as a bleaching agent for dairy products. When no national provisions on processing aids exist, their use is controlled by general safety pro
Table 1. Summary of hydrogen peroxide and benzoyl peroxide for whey bleaching

<table>
<thead>
<tr>
<th>Item</th>
<th>Hydrogen peroxide</th>
<th>Benzoyl peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Breakdown</td>
<td>$\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O}$ (hydrogen peroxide $\rightarrow$ oxygen + water)</td>
<td><img src="image" alt="Breakdown" /></td>
</tr>
<tr>
<td>(Smith, 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pros</td>
<td>• little to no effect on the nutrients present</td>
<td>• effective at lower usage levels than hydrogen peroxide</td>
</tr>
<tr>
<td></td>
<td>• more acceptable for usage in other countries</td>
<td>• does not require a catalase addition to remove odors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• does not pit stainless steel; therefore, is less corrosive to equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• effective across a wide range of temperatures</td>
</tr>
<tr>
<td>Cons</td>
<td>• must be inactivated with catalases</td>
<td>• possible formation of oxidized flavors</td>
</tr>
<tr>
<td></td>
<td>• could possibly cause oxidized flavors</td>
<td>• possibility that the carrier used may be considered an allergen</td>
</tr>
<tr>
<td></td>
<td>• corrosive to equipment</td>
<td>• concerns from other countries because it has just been recently approved by Codex</td>
</tr>
<tr>
<td></td>
<td>• less economical to use because it requires much more peroxide for satisfactory bleaching</td>
<td></td>
</tr>
</tbody>
</table>

Therefore, it can be understood that European Union regulations are in agreement with FDA regulations for the use of hydrogen peroxide as a bleaching agent for whey.

In addition to being used as a bleaching agent, hydrogen peroxide can be used as an antimicrobial agent in milk intended for cheese making at 0.05% (wt/wt) level (US FDA, 2009b). Likewise, hydrogen peroxide can be used as an antimicrobial agent during the preparation of modified whey by electrodialysis methods at 0.04% (wt/wt) level (US FDA, 2009b). Hydrogen peroxide can be used as an antimicrobial agent in cheese milk and a side effect will be bleaching milk. However, federal food and drug regulations do not support the use of hydrogen peroxide solely for the use of bleaching milk for cheese and related cheese products (US FDA, 2009b).

**Benzoyl Peroxide**

Benzoyl peroxide ($\text{C}_9\text{H}_8\text{O}_4$) is a colorless, crystalline solid permitted for use in removing color in whey products that are not used for infant formulas. Like hydrogen peroxide, benzoyl peroxide (or a mixture of benzoyl peroxide with potassium alum, calcium sulfate, and magnesium carbonate) is also a GRAS substance and can be used to bleach dairy ingredients. Unlike hydrogen peroxide, benzoyl peroxide has no limitation on usage rates in foods other than current GMP rules (US FDA, 2009b). Benzoyl peroxide is GRAS as a direct human food ingredient upon the following current GMP conditions of use: (1) the ingredient is used as a bleaching agent in food, (2) the ingredient is used in the following foods, milk used for production of Asiago fresh and Asiago soft cheese (US FDA, 2009b), Blue cheese (US FDA, 2009b), Caciocavallo Siciliano cheese (US FDA, 2009d), Gorgonzola cheese (US FDA, 2009f), Parmesan and Reggiano cheese (US FDA, 2009g), Provolone cheese (US FDA, 2009h), Romano cheese (US FDA, 2009f), and Swiss and Emmemtal cheese (US FDA, 2008b).
benzoyl peroxide as a preservative is not acceptable. Should benzoyl peroxide be used at levels >1% (wt/wt) in liquid whey, the manufacturer would need to justify the usage rate. Most additives are used at a rate of 1 to 3% (wt/wt), but any usage rate over 1% (wt/wt) raises many red flags (N. Ratslaff, USDA, Lisle, IL; personal communication).

There are concerns about benzoyl peroxide bleaching as it has only been recently approved by Codex Alimentarius, with the maximum level (100 mg/kg) for liquid whey and whey products (excluding infant applications) being adopted in 2007, and the maximum level (100 mg/kg) for dried whey and whey products (excluding infant applications) being adopted in 2005 (Codex, 2008). The European Commission (EC) allows both hydrogen peroxide and benzoyl peroxide to be used as whey bleaching agents (designated as processing aids, not food additives), but individual country provisions may be stricter (Bianca Herr, Leatherhead Food Institute, Leatherhead, UK; personal communication). Many Asian and European government regulators do not like the use of benzoyl peroxide and consider benzoic acid and other breakdown products of benzoyl peroxide harmful. There is a possibility that the carrier used for benzoyl peroxide may be considered an allergen. In addition, certain carriers may pose problems with membrane fouling or leave residues of insoluble carrier on equipment. Benzoyl peroxide reacts with oxidizable compounds and is converted into water-soluble benzoic acid following bleaching (Table 1). A petition was submitted by the US Dairy Export Council (US-DEC) to the Taiwan Department of Health, Food Sanitation, and Safety for the use of benzoyl peroxide in whey powder and was approved on December 20, 1999 (Johnson, 2006). Benzoyl peroxide is not an approved bleaching agent for whey in China or Japan and neither country’s regulations allow for residual benzoic acid in dried whey products.

Because of stricter regulations in China with their new food safety law (June 1, 2009), the Chinese government is now routinely testing for the presence of benzoic acid (Anonymous, 2009). The safety of benzoic acid and the derivative benzesturol have been studied extensively (Sharratt et al., 1984; Nair, 2001; Qi et al., 2009). Benzoic acid is found naturally in milk at low concentrations, 0 to 1 mg/kg, and in fermented dairy products at higher concentrations, typically about 20 mg/kg but up to 50 mg/kg (Sieber et al., 1995). During the fermentation process, lactic acid bacteria convert hippuric acid, a naturally occurring component of milk, to benzoic acid (Sieber et al., 1995). Benzoic acid can also be added as a preservative to cheese rennet and thus be found in cheeses from that route as well as from lactic acid fermentations. Benzoic acid is found in cheeses, but at lower concentrations than in fermented dairy products (Sieber et al., 1995). Subsequently, benzoic acid occurs naturally in whey and whey protein powders. Adverse reactions to benzoic acid-related compounds are rare, and life-threatening reactions are extremely rare (JECPA, 2004). Chang et al (1977) found that when benzoyl peroxide was added to Edam cheese whey, after it was heated to 69°C for 6 h and cooled, 91.7% of the benzoyl peroxide used was recovered as benzoic acid, and minor amounts of hydroxybenzoic acids, phenylbenzoate, phenol, and benzoyl peroxide were found.

**Lactoperoxidase**

When the lactoperoxidase system is used for antimicrobial purposes, external addition of thiocyanate or peroxide is required. However, when bleaching whey with hydrogen peroxide, companies often take advantage of the naturally existing lactoperoxidase system in milk in combination with externally added hydrogen peroxide to obtain maximum bleaching effectiveness of fluid whey. No additional enzyme or thiocyanate needs to be added for bleaching purposes (Z. Bastian; unpublished data). Lactoperoxidase (LP; EC 1.11.1.7) is a natural enzyme found in milk and constitutes about 0.5% of the total whey proteins in bovine milk. Lactoperoxidase is the second most abundant enzyme in raw milk (Kussendrager and van Hooijdonk, 2000; Fox and Kelly, 2006). The LP system consists of 3 components: lactoperoxidase, thiocyanate (SCN⁻), and hydrogen peroxide, and the system is only active in the presence of these 3 components (Seifi et al., 2005). The LP system is a potent bactericidal system that has been used to preserve raw milk without refrigeration. Depending on the concentrations of SCN⁻ and hydrogen peroxide used to activate the LP system, preservation can vary from 24 h at 35°C to 48 h at 20 to 22°C (Fweja et al., 2007). This system is 50 to 100 times more effective than hydrogen peroxide alone (Fox and Kelly, 2006). Lactoperoxidase is relatively heat stable with denaturation starting at about 70°C (Kussendrager and van Hooijdonk, 2000). Heat-stability studies were conducted in milk, whey, permeate, and buffer, and the enzyme was reported to be more stable to heat in whey and milk possibly because of their higher calcium ion concentration (Kussendrager and van Hooijdonk, 2000). Below pH 5.3, LP is less heat stable (Kussendrager and van Hooijdonk, 2000) and thus the optimal pH is 5.5 to 6.3 (Bottomley et al., 1989), which includes the pH range of whey. As such, sufficient amounts of the active enzyme are present in pasteurized milk and whey.

This system can be applied to bleach fluid whey by the addition of low concentrations of hydrogen peroxide.
to activate the system. In 1969, a patent was issued for decolorizing whey products using the LP system (Bottomley et al., 1989). Lactoperoxidase reacts in the presence of hydrogen peroxide to convert SCN⁻ to hypoiodite (OSCN⁻). Hypoiodite is a strong oxidizing agent that reacts with carotenes such as norbixin and oxidizes the double bonds, removing the conjugation and thus removing the color of the compound. It should be noted that an additional enzyme or thiocyanate needs to be added, nor does catalase need to be added because the added hydrogen peroxide is consumed. The time required can be variable but is generally 20 to 45 min at 40°C. The recommended time and temperature is 30 min at 40°C; 40°C is acceptable from a regulatory perspective as long as the whey is not held at that temperature for more than 2 h. The bleaching reaction occurs within 30 min (Bottomley et al., 1989).

Lactoperoxidase catalyzes the oxidation of annatto but is inactivated by high concentrations of hydrogen peroxide (Bottomley et al., 1989). As such, hydrogen peroxide concentrations should be kept at or near 10 ppm (Bottomley et al., 1989). Additionally, the inactivation effect depends on the concentration of hydrogen peroxide and on the length of time the enzyme is exposed. If the concentration of hydrogen peroxide present in the whey falls below the inactivating concentration before complete inactivation of the enzyme has occurred, decolorization will still occur but at a slower rate (Bottomley et al., 1989).

ADVANTAGES AND DISADVANTAGES OF BLEACHING

Hydrogen Peroxide

Higher temperatures are generally more effective than lower temperatures. Reaction temperatures above 74°C increased neither the rate nor the extent of color removal but only caused protein denaturation (McDonough et al., 1968). The oxidized flavors that were apparent immediately after bleaching purportedly disappear after evaporation and drying (McDonough et al., 1968), although a recent study demonstrated that this is not the case (Croissant et al., 2009). In addition, hydrogen peroxide has little to no effect on the nutrients present (Teply et al., 1958). Teply et al. (1958) analyzed milks and subsequent cheese and whey when the milk was bleached using 5, 10, and 20 times the normal amount of hydrogen peroxide and found that a strong treatment may alter proteins and amino acids in milk but in general there was no effect on the composition or nutritional value of the milk, cheese, or whey. The disadvantages to using hydrogen peroxide are that it must be inactivated with catalase, it could cause oxidized flavors, it is corrosive to equipment (Gilliland, 1969), and it is less economical to use because it requires more peroxide for satisfactory bleaching (Chang et al., 1977). A current review of prices for hydrogen peroxide and benzoyl peroxide suggests that the previous statement may be dependent on the bleaching conditions and usage rate. Hydrogen peroxide is sold in 53-gal drums for $200/drum; the liquid is 34% solids. Benzoyl peroxide is sold as a powder and costs $4.62 per kg.

Depending on the concentration, temperature, reaction time, and pH, bleaching with hydrogen peroxide may alter the functionality of total and individual whey proteins (Cooney and Morr, 1972; Munyua, 1975). Unfortunately, the 2 prominent, albeit dated, studies (Cooney and Morr, 1972; Munyua, 1975) on the functional properties of whey protein bleached with hydrogen peroxide dealt with concentrations above the legal limit. Because these studies used a level above the legal maximum, the effects documented in those studies may not be representative of the effects of hydrogen peroxide used at lower levels. According to Munyua (1975), hydrogen peroxide concentrations greater than 0.1% in fluid whey or milk caused a 5 to 8% decrease in the nonpolar amino acids such as aspartic acid, threonine, glutamic acid, methionine, tryptophan, phenylalanine, histidine, lysine, tryptophan (25% decrease), and arginine. In contrast, free sulfhydryl groups increased as the hydrogen peroxide concentration increased. Increasing exposure time increased the number of free sulfhydryl groups up to 24 h. The researchers postulated that hydrogen peroxide reacted first with readily oxidized amino acids such as methionine. Higher concentrations of hydrogen peroxide, increased temperatures, and longer holding times all increased the amount of whey protein denaturation.

Reaction temperature during bleaching can affect whey protein denaturation. Cooney and Morr (1972) demonstrated 4% protein denaturation when whey was treated with 1% (10,000 ppm) hydrogen peroxide for 24 h at 25°C, whereas 28% protein denaturation was achieved by treating whey for 6 h at 50°C. It should be noted that the hydrogen peroxide concentration range used in this study was above the legal limit for hydrogen peroxide usage. More studies are needed in this area to determine if these effects are observed at lower concentrations. Although the effect of pH was minor compared with other variables, the pH resulting in the greatest amount of protein denaturation depended on the specific whey protein. For instance, immunoglobulins and bovine serum albumin were more readily denatured at lower pH, whereas β-lactoglobulin
denaturation was enhanced at pH closer to neutral. α-Lactalbumin exhibited much less denaturation than β-lactoglobulin under the same conditions (Law and Leaver, 2000).

**Benzoyl Peroxide**

The effectiveness of benzoyl peroxide for removing color in whey depends on the amount used, how it is applied, the whey components present, and the exposure time and temperature. McDonough et al. (1968) reported that both benzoyl peroxide and hydrogen peroxide were effective bleaching agents, but that benzoyl peroxide was more effective at all temperatures. The rate and extent of decolorization by hydrogen peroxide and benzoyl peroxide for annatto in Cheddar cheese whey increased as the temperature was increased from 32.2 to 63°C. However, no additional increase with either agent was seen at 74°C. The advantages to using benzoyl peroxide are that it is effective at lower usage levels than hydrogen peroxide, it does not require addition of catalase to remove residues, and it does not pit stainless steel, and therefore is less corrosive to equipment (Chang et al., 1977). As with reports of off-flavors from hydrogen peroxide, oxidized flavors in Cheddar cheese whey were strong immediately after treatment with benzoyl peroxide; however, off-flavors purportedly dissipated following evaporation and drying (McDonough et al., 1968), an effect not observed in a more recent study (Croissant et al., 2009).

Contrary to the findings of McDonough et al. (1968), our recent unpublished research has reported that hydrogen peroxide is more temperature-dependent than benzoyl peroxide. McDonough et al. (1968) recommended bleaching temperatures in the range of 55 to 65°C. Benzoyl peroxide reacts quickly to remove color and additional time will not increase color removal. At 30°C, benzoyl peroxide may never completely bleach whey (Roos et al., 2006).

Peroxide bleaching (hydrogen or benzoyl) may have an effect on flavor in whey proteins, although few studies have been conducted. Mortenson et al. (2008) studied the flavor of whey protein concentrates (WPC) and whey protein isolates. Contrary to expectations, they found that flavor of WPC34 (WPC with 34–36% protein) and whey protein isolate were not affected by instantizing, ion exchange, or bleaching. However, lack of strict experimental controls and other processing variables suggest confounding factors in this study. Other recent studies have unequivocally established sensory and volatile compound differences associated with whey sourced from different cheesemakes, agglomeration and instantization (Carunchia Whetstine et al., 2005; Gallardo-Escamilla et al., 2005; Drake et al., 2009; Wright et al., 2009). Kuramoto and Jezeski (1954) studied the bleaching effects of benzoyl peroxide in cream (30% fat) for blue cheese manufacture at temperatures ranging from 52 to 85°C for periods of up to 4 h with concentrations of benzoyl peroxide of 4.5, 9, and 18 mg/kg. They found that flavor problems, such as tallow or oxidized flavors, were more apparent with increasing temperature, contact time, and benzoyl peroxide concentration. McDonough et al. (1968) reported that oxidized flavors present in fluid whey bleached with benzoyl peroxide at 20 and 10 mg/kg at 52°C and 63°C for 1.5 h or hydrogen peroxide at 500 and 300 mg/kg at 52°C and 63°C for 1.5 h were not detected in the dried whey powder. However, Croissant et al. (2009) conducted a controlled study with hot bleaching of liquid whey, with either hydrogen peroxide at 250 or 500 mg/kg at 60°C for 90 min or benzoyl peroxide at 10 or 20 mg/kg at 60°C for 90 min, and then manufactured WPC from those wheys. They demonstrated sensory effects and volatile compound changes in WPC from hydrogen peroxide or benzoyl peroxide bleached whey compared with unbleached whey.

Whey with higher total solids, such as condensed whey, needs greater amounts of peroxide to remove color (JECA, 2004). The most effective conditions are 60°C for 15 min at pH 6 to 7 (El-Samragy, 2004). Longer holding times are required if lower temperatures are used (McDonough et al., 1968; El-Samragy, 2004). Once whey has been dried, the annatto becomes highly resistant to bleaching. It is important to note that much of the above discussion regarding the use of hydrogen peroxide and benzoyl peroxide for bleaching whey originates from old literature or unpublished data and has not recently been thoroughly evaluated.

**REGULATORY CONCERNS**

Regulatory concerns focus on the use of either hydrogen or benzoyl peroxide for preservation of whey rather than bleaching. That is, these agents are approved for bleaching, but for maintenance of membrane flux during processing or for microbial control. The use of peroxide for preservation of whey during any process other than electrodialysis is prohibited. When bleaching is applied, the agent, concentration, time, and temperature vary widely within the industry depending on the existing facility and its specific process regimen. Regulatory agencies typically use the point of peroxide addition in the process to determine if the purpose of peroxide is bleaching or preservation (USDA/AMS/Dairy Division, 2008). Bleaching whey is usually applied at 1 of 2 possible steps during the whey production process. Fer-
coide can be added to fluid whey after pasteurization, before or after fat separation, as it is pumped into a storage tank, or when whey retentate is in the hot well of the evaporator. Bleaching is also conducted under a wide range of temperatures from 5 to 70°C. The USDA cites 2 specific cases where use of peroxide would be assumed to be for preservation purposes (Hammond et al., 1975; USDA/AMS/Dairy Division, 2008). The first case is addition of peroxide before the separator or any point in the process before preheating for the evaporator. Fluid whey can legally be bleached following pasteurization if the pasteurized whey goes into a storage tank for bleaching followed by fat separation. In this situation, the plant typically alternates between 2 tanks. Alternating between 2 tanks for bleaching must be completed within 4 h for microbiological reasons. In addition, legal bleaching may also be carried out if the bleach is added to the hot well before condensing. The second situation generating concern is addition of peroxide before holding the whey for more than 2 h at temperatures between 7 and 63°C (USDA/AMS/Dairy Division, 2008).

Legally, whey can be bleached at any temperature for more than 2 h at temperatures between 7 and 63°C (USDA/AMS/Dairy Division, 2008). To determine the legality of bleaching during ultrafiltration (UF) of whey, the point of application at which the agent is added and how long the whey is being held before UF is considered. If the bleaching agent is immediately added and flow begins, this is well within legal limits, regardless of the temperature at which flow occurs. In addition, should the bleaching agent be added to a balance tank before UF, the feed is considered fast enough that these bleaching conditions are considered acceptable. It should be noted that UF units have maximum temperatures at which the membranes can operate and this should be considered when deciding bleaching conditions. Hot bleaching temperatures during UF may not lead themselves to long membrane life in the case of spiral wound membranes. In contrast, should the bleaching agent be added to a sloop tank before UF and held for more than 2 h at between 7 and 63°C, this would be considered illegal because the bleaching agent would now be considered a preservation agent (N. Ratzlaff, USDA, Lisle, IL; personal communication).

CONCLUSIONS

Bleaching can create a more desirable color in whey proteins but it may also alter functionality and flavor. The majority of published literature dealing with bleaching of whey is quite dated (>25 yr). Since these studies have been conducted, milk quality, cheese making practices, and whey protein processing have all greatly evolved, emphasizing a need to scientifically evaluate bleaching and its effects on whey protein. Only two agents, hydrogen peroxide and benzoyl peroxide, are currently approved for bleaching. Of these two, the latter is viewed negatively in many countries and some regulations prevent its use. Both bleaching agents can negatively affect whey protein flavor. More precise application of currently approved bleach agents (e.g., minimum concentrations, optimal time/temperature exposure) or development of bleaching alternatives may facilitate enhanced whey protein flavor.

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Appendix B. Publication 2

Alternative Bleaching Methods for Cheddar Cheese Whey

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Abstract: Residual anatoxins (notoaxin) in fluid Cheddar cheese whey can be bleached. The 2 approved chemical bleaching agents for whey: hydrogen peroxide (HP) and benzoyl peroxide (BP), negatively impact the flavor of dried whey protein. The objective of this study was to evaluate alternative methods for bleaching liquid whey: ultraviolet radiation (UV), acid-activated bentonite (BT), and ozone (O3). Colored Cheddar cheese whey was manufactured followed by pasteurization and fat separation. Liquid whey was subjected to one of 5 treatments: control (CT) (no bleaching; 50 °C, 1 h), HP (250 mg/kg, 50 °C, 1 h), UV (1 min exposure; 50 °C), BT (0.5% w/v; 50 °C, 1 h), or O3 (2.25h, 50 °C, 1 h). The treated whey was then ultrafiltered, diafiltered, and spray-dried to 80% whey protein concentrate (WPC80).

The entire experiment was replicated 3 times. Color (notoaxin extraction and measurement), descriptive sensory, and instrumental volatile analyses were conducted on WPC80. Notoxins elimination was 28%, 79%, 39%, and 15% for HP, BT, UV, and O3 treatments, respectively. WPC80 from bleached whey, regardless of bleaching agent, had lower sweet aromatic and cooked/milky flavors compared to unbleached CT (P < 0.05). The HP and BT WPC80 had higher fatty flavor compared to the CT WPC80 (P < 0.05), and the UV and O3 WPC80 had distinct mushroom and animal flavors. Volatile compound results were consistent with sensory results and confirmed higher relative abundances of volatile aldehydes in UV, HP, and O3 WPC80 compared to CT and BT WPC80. Based on bleaching efficacy and flavor, BT may be an alternative to chemical bleaching of fluid whey.

Keywords: alternatives, bleaching, whey protein concentrate

Practical Application: The 2 approved chemical bleaching agents for whey: hydrogen peroxide (HP) and benzoyl peroxide (BP), negatively impact flavor of dried whey protein, and restrictions on these agents are increasing. This study evaluated 3 alternatives to chemical bleaching of fluid whey: UV radiation, ozone, and bentonite.

Introduction

The United States is the largest cheese producer in the world, at 457000 metric tons in 2009 (USDEC 2010), and therefore is also the largest whey producer. Liquid whey is frequently further processed into whey protein concentrate (WPC) or whey protein isolate (WPI). WPC is obtained by removing sufficient non-protein constituents from whey so that the finished product contains a protein level of 34% to 80%. WPI has a higher protein concentration (≥90%). The functional and nutritional properties of whey protein are valued by food manufacturers and in recent years its applications have expanded (Strubers 2008).

Prior to bring processed into powder, the anatoxins (yellow) colored cheese whey is bleached. In the United States, a natural colorant, anatoxin, is added to Cheddar cheese milk. Some of the colorant remains in the whey and negatively impacts the color of dried whey protein. Hydrogen peroxide (HP) and benzoyl peroxide (BP) are the 2 chemical bleaching agents that are approved for bleaching whey in the United States (Kang and others 2010). These bleaching agents cause off-flavors that carry through to the final spray-dried product. Cressman and others (2009) have demonstrated that both BP and HP impact the flavor of liquid whey and the subsequent spray-dried WPC. Other studies have confirmed the role of bleaching on off-flavors in WPC34 and WPC80 (Jethwa and others 2011; Jethwa and others 2012). Other bleaching methods may be a viable and attractive alternative for whey bleaching and were the focus of this study. The 3 alternative bleaching methods evaluated were acid-activated bentonite (BT), ultraviolet (UV) radiation, and ozone (O3).

BT has a high surface area and tends to absorb molecules into its interlayer sites (Christidis and Kostari 2003). When acid-activated, a procedure during which BT is dispensed in a strong acid at high temperature and subsequently rinsed with distilled water to remove the acid, the adsorbent properties are enhanced (Gidom 1944; Rupert and others 1987; Boyd and others 1994). In the food industry, acid-activated BT is mainly used for the purification, decolorization, and stabilization of vegetable oils (Christidis and Kostari 2003). In addition to color removal during bleaching, BT also removes undesirable products such as hydroperoxides formed by oxidation, unsaturated fatty acids, and glycolipids.

UV light has a wavelength between 10 and 400 nm, which falls between the visible light and x-ray spectrum. UV light has a damaging effect on the stability of anatoxin colorants (Kostari and others 1999). Previous studies have measured the effect of visible light on the color stability of anatoxin in fluid systems (Najar and others 1988; Pesek and Warthesen 1988) and Cheddar cheese...
Cheddar whey bleaching...

(Hung and others 1995a, 1995b) with light exposure resulting in the greatest degree of color desulfuration in fluid systems (Njau and others 1988).

Commonly used as a strong oxidizing agent, ozone is a triatomic oxygen molecule with an oxidizing potential of 2.07 eV, higher than that of HP (1.77 eV) and the perhydroxy radical (1.70 eV) (Perinçek and others 2007). Ozone received generally recognized as safe (GRAS) status for use in bottled water in 1982 and GRAS status for use as a disinfectant or sanitizer for foods when used in accordance with good manufacturing practices in 1997 (USDA 1997). Ozone has also been used in various products as a bleaching agent. Ozone naturally decomposes to O₂ and an oxygen radical. The oxygen radical is a strong oxidizer, which readily oxidizes the conjugated double bonds of chromophores. Ozone has been used to reduce yellow color in concentrated gelatin solutions and cotton fabrics (Cataldo 2007). Ozone requires a low quantity of water and requires no harmful carrier chemicals (Perinçek and others 2007). The objective of this study was to evaluate the impact of these 3 alternative bleaching methods on the color and flavor of WPC80. Unbleached WPC80 and WPC80 from HP-bleached whey were used as controls.

Materials and Methods

Experimental design

Two experiments were performed. Experiment 1 was conducted on liquid Cheddar whey using each bleaching agent at varying temperatures and/or varying exposure times. The purpose of experiment 1 was to select the appropriate treatments for subsequent WPC80 production (experiment 2). For experiment 2, unbleached fluid whey and whey bleached with 250 ppm HP were used as controls. Unbleached colored whey served as a negative control, while the HP-bleached whey served as a current industrially relevant point of reference.

Liquid whey production

Liquid Cheddar whey was manufactured from whole vat-pasteurized bovine milk with the addition of double-strength annatto food color (3% Annatto extract w/v), Danisco, St. Louis, Mo., U.S.A. at a rate of 0.035 ml/kg milk as described by Listyanyi and others (2011). The whey was drained at pH 6.40 and pasteurized at 63 °C for 30 min. The hot whey was immediately processed with a hot bowl cream separator (Model FF 125 EAR, Clar, Aithofen, Austria) to reduce the fat content. Total percent solids and percent fat content of fluid milk and whey were analyzed using the SmartSystems 5 moisture/bulksolid analyzer with SmartTrac rapid fat analysis (CEM, Matthews, N.C., U.S.A.).

Bleaching treatments for liquid whey (experiment 1)

Following pasteurization and fat separation, whey was subjected to different treatments. For the control (CT), the liquid whey was heated at 60 °C for 1 h. For HP treatment, 250 mg HP/kg (0.005 w/w; Nelson & Jameson Inc., Manfield, Wn., U.S.A.) was added to the liquid whey and held for 1 h at 60 °C with gentle agitation followed by addition of 20 ppm catalase (Food Pro CAT, Danisco) to remove any residual HP.

For UV radiation, the apparatus and methods of Mahmoud and Ghaly (2004) were adopted with minor changes. Liquid whey was circulated through a stainless steel UV apparatus at 60 °C. The flow of the whey was fixed so that the liquid whey was exposed to UV light for 1 min. The UV apparatus was composed of a stainless steel feed tank, a peristaltic pump, and an overflow tank. A 380-mm arc length low-pressure mercury lamp enclosed in a 21-mm-dia (f.d.) quartz tube was used (Aqua Treatment Service, Inc., Mechanicsburg, Pa., U.S.A.). The UV reactor had a 0.201-L working volume and the stainless steel chamber was 475 mm long with an i.d. of 33 mm. An inlet port was located at one end of the reactor and was connected to a peristaltic pump, which pumped the cheese whey from the feed tank. The outlet port was located at the other end of the reactor and was connected to the overflow tank.

For BT treatment, first, acid-activated BT was made by heating 20 g BT (Sigma-Aldrich, St. Louis, Mo., U.S.A.) with 2 N sulfuric acid (VWR Int., West Chester, Pa., U.S.A.) at 50 °C for 4 h followed by rinsing with deionized water until it reached pH 4 (Sears and others 1989). The acid-activated BT powder was dispersed in liquid whey (0.5% w/v) and recirculated through a peristaltic pump (model 9154K-G3; McMaster-Carr, Atlanta, Ga., U.S.A.) to prevent the BT from settling. Following the respective 15 min, 30 min, or 1 h treatment times, the liquid whey was centrifuged for 10 min at 8000 x g to remove the BT. For Oz, 2 samples (700 ml), in 2-L Pyrex beakers, were placed in a 35 °C or 60 °C water bath. Upon reaching 35 °C or 60 °C, respectively, an Oz generator (Model OZX-300; Enaly M&F Ltd., Shanghai, China) was activated, pumping approximately 200 mg/h Oz into the whey through an 80-micron diffusion stone. Oz was pumped through each sample for 15, 30, and 45 min with a 60-ml sample of whey taken after each time point. After each treatment, color and volatile analyses of the liquid whey were conducted. The experiments were replicated in triplicate. Based on the results, one treatment for each alternative bleaching method was selected for manufacture of WPC80.

Bleaching treatments for WPC80 (experiment 2)

Following pasteurization and fat separation, whey was subjected to one of 3 different treatments selected from experiment 1. The temperature of all treatments was decreased to 50 °C to maintain consistency with industrial ultrafiltration (UF) practices. For UV radiation, the liquid whey flow was regulated by the peristaltic pump to allow 1 min of exposure. For acid-activated BT treatment, 0.3% (w/v) BT was mixed with the liquid whey for 1 h and removed using the method described earlier. Oz (2.2 g/l) was continuously bubbled through 10-micron diffusion stones into 95 L liquid whey at 1 h for using 3 Oz generators (3 model OZX-3001, 1 model 300BT-12, Enaly M&F Ltd.). Rate of Oz production was increased due to the increased volume of whey, but kept low enough to ensure safety during processing.

WPC80 production

Following bleaching for heat treatment (control), whey was concentrated using a UF system (Model Pellicon 2; Millipore Inc., Billericia, Mass., U.S.A.). The system and procedure has been described by Listyanyi and others (2011). The whey was concentrated using a UF system equipped with 4 polyethersulfone cartridge membrane filters (Model P2V910V05, nominal separation cut-off 10 kDa, surface area: 0.5 m²). A variable speed peristaltic pump (Model 77410-10) equipped with pump heads (Model 77401-00) with silicone tubing (Model 96440-73) was used to circulate the product. Di-filtration (DF) was initiated after UF had reduced the volume of whey to approximately 50% original weight. The total volume of deionized water added was approximately 65% of the original volume of the whey, and was added in 3 parts over the course of the UF process. The filtration process continued until the protein concentration reached 80% (on
Cheddar whey briefing…

a dry basis). The total time of the UF and DF was approximately 3.5 h. After DF, the retentate was spray-dried (Model Lab 1, Asea Inc., Soeborg, Denmark). A peristaltic pump (model 9144654; MoMaster-Cart) was used to feed the spray dryer at a rate of 1 kg/h. Retentate feed temperature was 22 °C. The spray dryer inlet temperature was 190 °C and the outlet temperature was 80 °C. Powders were stored in Nylar bags (TTE-4000 Inpaip Corp., Central City, SDak., U.S.A.) and stored at -80 °C. Production of the liquid whey and the 5 spray-dried WPC80 treatments were completed in 1 d. This experiment was replicated 3 times.

Composition analysis
Total solids of WPC were determined by air oven drying (AOAC 2000; method number 990.20; 33.2-4.4). Fat was quantified by ether extraction (AOAC 2000; method number 997.05; 33.2-26). Protein was determined using the Kjeldahl method (AOAC 2000; method number 985.19; 33.2-11). Mineral analyses (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) were done by the North Carolina State University Analytical Service Laboratory (Raleigh, N.C., U.S.A.) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy. All samples were measured in duplicate.

Color analysis: reflectance measurement and annatto extraction
Reflectance measurements. The Hunter CIE Lab L* (lightness), a* (red-green), and b* (yellow-blue) values for the fresh liquid whey, WPC80, and 10% solids (w/w) rehydrated WPC80 were determined in triplicate using a Minolta Chroma meter (CR-410; Ramsey, N.J., U.S.A.) as described by Listiyni and others (2011).

Anatto extraction. Annatto (bixin) extraction and quantification methods were modified from Cincinato and others (2009). Cheddar liquid whey (10 g) or WPC80 (1 g) powder in 2 mL HPLC-grade water (EMD Chemicals, Gibbstown, N.J., U.S.A.) was weighed into a 50-mL centrifuge tube (Nalgene, Rochester, NY., U.S.A.). To this, 6 mL ethanolic EMD Chemicals) was added and vortexed with centrifugation at 16500 × g for 10 min at 4 °C (Model R-3CSB; Thermo Scientific, Waltham, Mass., U.S.A.). The supernatant was removed to a separate centrifuge tube. To the remaining solids, 3 mL chloroform (EMD Chemicals) was added, the sample was vortexed and centrifuged again at 16500 × g for 10 min. The bottom chloroform layer containing the annatto was collected and the volume was recorded. The collected sample was further purified using a silica-NH2 SPE column (500 mg/5 mL; Phenomenex, Torrance, Calif., U.S.A.) and the absorbance was measured at 458 nm with a UV-visible spectrophotometer (UV-1700 Pharmaspec; Shimadzu, Durham, N.C., U.S.A.) and quantified using an external calibration curve (Listiyni and others 2011). All extraction procedures and measurements were performed with quadruplicate full-spectrum F685 flat-sheet filters (Engomart, Dallas, Tex., U.S.A.) covering all lights to minimize norbixin isomerization and degradation (Mercalante 2008).

Descriptive sensory analysis
Sensory testing was conducted in compliance with the North Carolina State Univ. Institutional Review Board for Human Subjects guidelines. Descriptive sensory analysis was performed on 10% solids (w/w) rehydrated WPC80 using an exhaust flash lexico for dried dairy ingredients (Drake and others 2003, 2009; Wright and others 2009). Eight trained panellists (7 females, 1 male, ages 23 to 45 y) with more than 100 h of experience each with the Spectrum TMD descriptive analysis method and sensory analysis of dried dairy ingredients (Malgaard and others 1999) participated. Rehydrated (30 mL) samples were dispensed into lidded 59-mL cups (Solo Cup Co., Champaign, Ill., U.S.A.) with 3-digit codes. Each product replication was evaluated in duplicate by each panelist at room temperature in a randomized design. Data were collected using paper ballots or by computerized data entry using Compusense five, release 4.8 (Compusense Inc., Guelph, Ontario, Canada).

Gas chromatography/mass spectrometry
Volatile compounds in liquid whey and WPC80 were extracted by solid-phase microextraction (SPME). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using the method of Listiyni and others (2011). Liquid samples were tested as-is on the day of manufacture, and spray-dried powders were rehydrated at 10% solids (w/w). In order to minimize light oxidation, samples were prepaid with white overhead lights off. All samples contained 10% (w/w) sodium chloride (Fisher Scientific) using HPLC grade water (EMD Chemicals). Each sample (5 g) was transferred to a SPEM vial (Supelco, Bellefonte, Pa., U.S.A.). Each SPEM vial was spiked with 10 mL of an 8.1 mg/kg internal standard 2-methyl-3-heptanone (Sigma-Aldrich) dissolved in methanol (EMD Chemicals). Each vial was sealed airtight with a teflon-saided silicon septum and a steel screw cap (MicroLab Analytical Supplies Inc., Sarasota, Fla., U.S.A.). Samples were injected using a CombiSLB auto sampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890 N GC with 5973 inlet MSD Agilent Technologies Inc., Santa Clara, Calif., U.S.A.). Samples were maintained at 6 °C prior to fiber exposure. Sample were equalized at 40 °C for 25 min before 30 min of fiber exposure of a 1-cm DVB/CAR/PDMS fiber (Supelco) at 31 °C with 4 psi 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. A ZB-5ms column (30 cm × 0.25 mm film thickness; Phenomenex) was used for all analyses at a constant flow rate of 1 mL/min in SIM mode. Purge time was set at 1 min. The M5 transfer line was maintained at 250 °C with the Quad at 230 °C and source at 250 °C.

Compounds were identified using the NIST, Inst. of Standards and Technology (2002) (Gaithersburg, Md., U.S.A.) mass spectral database, retention indices (RI) based on an alkane series (Sigma Aldrich), and comparison of spectra of authentic standards injected under identical conditions. Concentrations of compounds were determined using single-point external standard validation curves, where the area of the mass chromatogram was calibrated with a known amount of analyte injected on the GC-MS under the same conditions.

Statistical analysis
Proximate analyses, sensory, and instrumental results were analyzed using XLSTAT statistical software (version 2009. 1.02; Addinsoft, New York, N.Y., U.S.A.). One-way analysis of variance (ANOVA) was conducted to determine the effect of each factor on color, chemical composition, and sensory and instrumental volatility analyses. Tukey’s least significant difference calculation with a confidence interval of 95% was conducted as a post hoc test.
Results and Discussion

Composition analyses

No significant differences (P > 0.05) were observed for the fat (5.75 ± 1.19%), moisture (2.82 ± 1.55%), or protein (81.7 ± 0.92%) contents of bleached and unbleached WPC80. The magnesium, iron, aluminum, and silicon contents for BT WPC80 were higher (P < 0.05) than for CT WPC80 or other WPC80, which was probably due to residual BT that was not removed by pilot-scale procedures (Table 1).

Color analysis: reflectance measurement and annatto extraction

Experiment 1: liquid whey. The HP treatment of liquid whey decreased norbixin content by 47% and decreased yellowness (b* value) by 17%, which is consistent with previous published results (Listyymi and others 2011). BT treatment of liquid whey at 60 °C was more effective than 4 °C for color removal (P < 0.05, results not shown). Based on norbixin concentration, 60 °C for 30 min or 1 h resulted in 49% and 62% norbixin decrease, respectively. BT exposure for 1 h was selected for WPC80 production. Fluid whey exposed to UV light for as little as 0.5 min resulted in 74% norbixin elimination (results not shown). A 1-min exposure to UV was selected for experiment 2. Ozone saturation levels decreased norbixin content in liquid whey by 49% at 25 °C and 63% at 40 °C after 45 min. Therefore, 45 min exposure at 40 °C was selected for WPC80 production. Due to safety reasons, ozone levels were decreased for pilot-scale manufacture.

Experiment 2: WPC80, HP, BT, UV, and Ozone bleached WPC80 displayed a 28%, 79%, 39%, and 15% norbixin elimination, respectively (Figure 1). Based on norbixin recovery, BT and UV were more effective (P < 0.05) than traditional HP (250 ppm) bleaching. Ozone was the least effective of all treatments. For bleaching, HP, UV and Ozone chemically destroy the structure of norbixin, while BT physically removes or sequesters norbixin from the whey, and it was the most effective of the agents evaluated.

Volatile compounds and flavor analyses

In order to remove norbixin in whey, bleaching agents are employed. In the case of chemical bleaching agents, rapid loss of color occurs when free radicals are formed which possess enough energy to break less stable double bonds, such as those in norbixin. Norbixin itself may quench free radicals prior to lipid oxidation (Campbell and others 2011), and it was the only carotenoid that inhibited the oxidative degradation of oil stored at 60 °C (Kroksas and Gordan 2003). However, the same free radicals that destroy norbixin and bleach whey also promote lipid oxidation. Lipid oxidation products are the major source of off-flavors (cardboard, fatty, cabbage flavors) in dried whey (Wright and others 2009; Whiten and others 2010). Consistent with previous studies (Croissant and others 2009; Listyymi and others 2011; Jervis and others 2012), HP-bleaching significantly decreased sweet aromatic and cooked/musty flavors and increased cardboard and fatty flavors in WPC80 (Table 2). Consistent with oxidative reactions, UV and Ozone also increased cardboard and fatty flavors and decreased sweet aromatic flavor in WPC80 and contributed unique styphlic mushroom, animal, and peanut flavors. BT treatment had the least impact on sensory properties of WPC80. No change in cardboard flavor relative to the control was observed in BT WPC80. BT WPC80 had decreased sweet aromatic and cooked/musty flavors and a slight but distinct fatty flavor.

A total of 34 volatile compounds were identified and quantified (Table 3). Selected compounds have previously shown relevance to whey off-flavors (Croissant and others 2009; Liaw and others)

| Table 1—Mineral analyses of unbleached and bleached WPC80. |
|-----------------|-----------------|-----------------|-----------------|
| Category        | Ca (%)          | Mg (%)          | Fe (ppm)        |
| CT              | 0.29*           | 0.73*           | 0.26*           |
| HP              | 0.73*           | 1.21*           | 0.75*           |
| UV              | 0.98*           | 1.74*           | 0.93*           |
| BT              | 1.07*           | 1.51*           | 0.98*           |
| Ozone           | 1.03*           | 1.46*           | 2.04*           |

Figure 1—Norbixin elimination (%) of bleached WPC80. Percent norbixin elimination relative to a control (unbleached WPC80) manufactured from colored Cheddar whey. HP = hydrogen peroxide; BT = acid-activated bentonite; UV = ultraviolet radiation; Ozone = ozone.
Cheddar whey bleaching…

2010; Wharton and others 2010; Lin and others 2011). Consistent with sensory results, volatile lipid oxidation products were generally higher in UV, OZ, and HP WPC80 compared to BT and CT WPC80 (Table 3). The ability of HP to bleach is connected to its oxidative nature. Unfortunately, its predisposition to oxidize also contributes to lipid oxidation off-flavors in WPC34 and WPC80. HP-bleached WPC80 was higher in many lipid oxidation products, including pentanal, hexanal, heptanal, E-2-heptanal, 1-octen-3-ol, 1-octen-3-yl, octanal, E-2-octanal, and E(2)-nonenal when compared to the CT WPC80. HP-treated WPC80 was also higher in DMDS (Table 3).

High-energy photons of the UV range disrupt the bonds in the chromophore of the amine, resulting in colorless or decreased color in whey (Chen and others 2008). When whey was exposed to UV light, some compounds that were not found in the WPC80 from CT and HP-treated whey were observed: p-cresol and guaiacol (Table 3). These compounds may be formed from radical-induced oxidation of aromatic amino acids. WPC80 from UV-treated whey was also higher (P < 0.05) in pentanal, hexanal, heptanal, 1-octen-3-ol, e2-octanal, and e2-nonanal compared to CT WPC80. Given the off-flavor production in WPC80, UV radiation is not a viable alternative bleaching agent for whey. OZ also had an effect on flavor and volatile profiles of WPC80. OZ, like UV light, is a strong oxidizing agent with a oxidation potential much higher than that of HP (Iglesiass 2002). As such, one would expect a significant increase in lipid oxidation products compared to the CT WPC80. Hexanal, E-2-heptanal, E(2)-nonenal nonanal and decanal were higher (P < 0.05) in comparison in OZ WPC80 compared to CT WPC80. Given that a relatively low level of OZ was used to bleach whey for WPC80 manufacture and that miniscule boronix elimination occurred, it is likely the degree of off-flavor formation caused by an effective amount of OZ needed to bleach at equivalent levels to HP would be unacceptable in WPC80.

Natural BTs are effective absorbents after acid activation (Chris-tidis and Kosiari 2003). Acid-activated BT has an ability to adsorb phospholipids, rancid metals, and organic compounds such as carotenoids (Christidis and Kosiari 2003). BT also removes compounds, like deproteins formed by oxidation, unreacted fatty acids, and glycerides. Unlike HR UV, or OZ, BT is not an oxidizing agent and should not initiate or promote lipid oxidation and this result was observed. Volatile liquid oxidation products were generally lower in BT WPC80 compared to other bleached WPC80, and the sensory profile of BT WPC80 was most similar to unbleached WPC80. Given that restrictions on chemical bleaching agents continue to increase, future studies should investigate optimization of BT and its effect on protein functionality.

Conclusion

This was the 1st study, to our knowledge, to investigate and compare alternative bleaching methods for amine elimination in colored whey. Based on bleaching efficacy and flavor, acid-activated BT was the most promising alternative bleaching treatment.

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References
