Abstract

SMITH, TUCKER J. Bleaching Efficacy of Ozone Gas in Liquid Whey and its Effects on Flavor of 80% Whey Protein Concentrate and Norbixin Partitioning in Full Fat and Fat Free Cheddar Cheese (Under the direction of Dr. MaryAnne Drake).

As demand for whey ingredients increases, it is important to study alternative cheese and whey treatments which decrease the need for harsh chemical bleaching agents during the liquid whey production process. Currently, the two approved bleaching agents in the US are hydrogen peroxide and benzoyl peroxide. Bleaching with these agents causes off flavors in products containing whey protein ingredients. Studying the difference in annatto partitioning during full fat and fat free cheese makes, along with the use of a novel bleaching agent, ozone, assists in the search for a method of producing the highest quality whey protein ingredients with both optimal flavor and color characteristics.

The objective of the first study was to determine the viability of ozone as an alternative bleaching agent for fluid whey. Flavor effects and bleaching efficacy of ozone gas on liquid whey and whey retentate were evaluated in bench top experiments prior to pilot scale manufacture of 80% whey protein concentrate. Bleaching of retentate with ozone was higher at 35°C compared to 50°C ($p<0.05$); temperature did not affect liquid whey bleaching with ozone ($p > 0.05$). In bench top studies, a 63 % decrease in norbixin content was observed in fluid whey after 45 min ozone exposure. In pilot scale manufacture, WPC80 from HP bleached whey had 27% norbixin destruction while that bleached with ozone had a 15% reduction. Ozone-treated WPC80 exhibited animal and flour/pasta flavors and HP

bleached WPC80 was characterized by cabbage and fatty flavors not present in unbleached WPC80. Higher levels ($p<0.05$) of nonanal and decanal were present in the ozone WPC80 while higher levels ($p<0.05$) of pentanal, DMDS, hexanal, heptanal, 2-pentylfuran, and octanal were present in the HP WPC80 compared to the control. These results suggest that ozone bleaching does not represent a promising alternative to approved bleaching agents in whey protein production although it could possibly remain feasible at or close to saturation levels.

The objective of the second study was to better understand norbixin characteristics in whey by evaluating the norbixin partitioning differences in cheese and whey in full fat and fat free Cheddar cheese manufacture. Full fat and fat free cheeses along with corresponding wheys were produced in quadruplicate. Norbixin extractions were performed on the colored milk, the unseparated/unpasteurized whey, and the pressed Cheddar cheese. An average of 10% of the norbixin added to the full fat cheese milk was recovered in the whey and 82% was recovered in the cheese. Similarly, 12% of the norbixin added to skim milk was recovered in the skim milk cheese whey and 76% was recovered in the fat free cheeses. Level of norbixin addition to cheese milk and milk fat content had no impact on norbixin recovery in cheese or whey ($p>0.05$). These results suggest that fat content has little impact on norbixin binding or entrapment during the cheese make process.

Bleaching Efficacy of Ozone Gas in Liquid Whey and its Effects on Flavor of 80% Whey Protein Concentrate and Norbixin Partitioning in Full Fat and Fat Free Cheddar Cheese

by
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DEDICATION

To my wife and my daughter for whom I am doing this, and whose smiles are the reason I come home.

BIOGRAPHY

Tucker James Smith was born in Corpus Christi, Texas on March 30th, 1984 to Brad and Lois Smith. He has three brothers and one sister; Shad, Scott, Sam, and Barbi. Tucker's informative years were spent growing up in Wilmington, NC and he graduated from John T. Hoggard high school in 2002. After graduation from high school, Tucker spent one semester at Brigham Young University – Idaho in Rexburg ID, before serving a full time mission for the Church of Jesus Christ of Latter Day Saints in the Samara, Russia mission. After returning from his two year mission, Tucker transferred to Brigham Young University – Provo, where, after realizing he did not wish to be a physics major, found food science. The food science department at BYU offered much more than lectures and classes and Tucker had the great opportunity to work closely with several professors, including Dr. Laura Jefferies, Dr. Michael Dunn, Dr. Lynn Ogden, and Dr. Frost Steele in several capacities. Opportunities included the chance to teach two semester long labs in the essentials of food science and food engineering, assist in a PhD project over a summer, work in the sensory lab for years, work in the product development lab on two commercial products, and lead a grand prize winning product development team in the Idaho Milk Producers Association product development competition. Tucker graduated with his Bachelors of Science in Food Science in 2009 and began his graduate studies under Dr. MaryAnne Drake in 2010 where opportunity continues in abundance.

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I first wish to acknowledge my parents, who have shaped me in ways I'm not even aware of, and who I can only hope to be like some day. Dad, you are an example to me of a great scientist, a loving husband, a strong, faithful priesthood holder, and a gentle father. You are whom I most wish to be. Mom, you are the strength, determination and intelligence that I wish I had. You are the foil to so many of my weaknesses, and help me reach higher and want more.

To my siblings, who have shared much of my path with me and whom I care for and love more than I ever thought I could.

To my professors at BYU who provided me with everything a curious mind could wish for. I never could have imagined how meaningful my relationship with you could be. This is especially to Dr. Laura Jefferies who believed in me, certainly more than I did, and without whom I wouldn't be half the food scientist that I am today.

To my lab mates here in the MAD lab. It is a crazy and frustrating ride at times, but is bearable because I'm sharing the ride with you all.

To Dr. MaryAnne Drake who works harder on our individual projects than we do. It is your sacrifice that makes this lab function.

Lastly, to my wonderful, beautiful wife, and delightful, adorable daughter. I can't say how much you mean to me. You are my purpose, my determination. You are my reason to become more than I am. This work is a symbol of the choice I made to completely dedicate myself to you. It was a choice well made.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Whey is the natural byproduct of the cheese make process. Whey, along with cheese, was initially discovered about 3000 years ago when the stomachs of cows began to be used as holding vessels for milk (Smithers, 2008). An enzyme which naturally appears in the stomachs of cows, chymosin, cleaves the k-casein from the casein micelle, reducing steric stabilization and causing the casein to coagulate and form what we now know as curds and whey (Fox and McSweeney, 1998). Whey consists mostly of lactose (44-52g/L, 4.4-5.2% w/w), proteins (6-10g/L, 0.6-1% w/w), minerals (2.5-7.2g/L, 0.3-0.7% w/w), and water. Composition does depend on whether sweet (pH <5.6) or acid (pH >5.1) whey is being produced and differences in the cheese make process. (Farkye, 2004; Tunick, 2008). The protein fraction of whey consists mainly of beta-lactoglobulin, alpha-lactalbumin, serum albumin, immunoglobulin, and protease-peptone (Jayaprakasha and Brueckner, 1999). About 80% of the total whey proteins are made up of Beta-lactoglobulin and alpha-lactalbumin (Schmidt et al., 1984).

In the modern era, whey has largely been considered a waste product of the cheese make process. Whey was considered a pollutant and was difficult and expensive to dispose of. Environmental concerns began to rise as cheese production in the US increased. Previously, whey was disposed of by selling it as animal feed, spraying it over fields or simply dumping whey into the sewer system, rivers, lakes, or the ocean. (Smithers, 2008) By 2010, total US cheese production, excluding cottage cheese, was 10.4 billion pounds, with Wisconsin leading cheese production with 2.6 billion pounds and California in second,

producing 2.06 billion pounds. Idaho, New York and Minnesota were other top cheese-producing states. Internationally, only the European Union-27 countries produced more cheese than the United States in 2010. A production of 10.4 billion pounds is a 42% increase in cheese production since 1998 (USDA 2011). As the unavoidable byproduct of cheese making, whey production increases have followed cheese production increases. Needless to say, an economical usage of whey and other byproducts of the cheese making process is absolutely vital to the dairy industry.

Relatively recently, whey protein has grown in popularity as a food ingredient. Whey proteins have valuable functional properties such as gelation, thermal stability, foam formation, or emulsification (Foegeding et al., 2002). As a natural byproduct of cheese, whey enjoys a natural, wholesome image (Russell et al., 2006). Functional foods, like whey ingredients, which have a well-established and specific health benefit, have been estimated to reach well over $100 billion by 2012 (Smithers, 2008). Consumption of whey protein provides many health benefits, including exercise recovery, weight management, cardiovascular health, anti-cancer effects, anti-infection activity, wound repair, and infant nutrition (Smithers 2008).

Due to its various functional properties, whey ingredients are used in many different food systems. In some fish products, up to 20% of meat proteins may be replaced with whey proteins to be used as a binding agent (Jayaprakasha and Bruenckner, 1999). They have also been used to improve cheese and yogurt yields and in cakes, as a replacement for egg whites (Jayaprakasha and Bruenckner, 1999). Many foods utilize the gelling functional property of

whey protein. Research has been done to induce gelation at "cold" (ambient to refrigeration) temperatures in order to use whey for its functional properties in food systems too sensitive to heat. To achieve this cold gelation the whey proteins must be heat denatured and aggregated without forming a gel and then added to a solvent which will cause the whey proteins to gel at cold temperatures (Foegeding et al., 2002).

Another important property of whey protein is its ability to form emulsions. This allows whey ingredients to be used in foods such as minced meats, cakes, coffee whiteners, salad dressings, and frozen desserts (Jayaprakasha and Brueckner, 1999). Whey proteins are soluble at acidic pH and can continue to provide emulsion stability in heated foods (Huffman 1996). Such properties are important since many food products have low pH and/or are heated at some point during processing. Using whey proteins in conjunction with other functional ingredients as thickening agents or emulsifiers can improve their functionality (Demetriades et al., 1997).

In addition to the gelation and emulsion properties of whey proteins, foaming is an additional functional property which is important to the food industry. Frozen desserts, cakes, nougat confections, meringues, and frothed drinks are just some of the popular foods which use some sort of foam (Foegeding et al., 2002, 2006). The foaming stability and foamability of whey proteins depends on the protein type, denaturation, fat and carbohydrate concentrations, foam formation method, whey protein concentration, pH, and ion concentration (Huffman, 1996, Jayaprakasha and Brueckner, 1999).

As whey ingredients increase in popularity as food ingredients, flavor concerns grow more important. Unprocessed whey has a very bland and delicate flavor, but when processed and stored, dried whey ingredients can develop undesirable flavors (Carunchia Whetstine et al., 2005; Wright et al., 2008). Undesirable ingredient flavors can be carried into finished products and are a limiting factor in foods which lack a flavor strong enough to overpower or mask that of the whey protein (Drake, 2006; Drake et al., 2008; Wright et al., 2008). The importance of understanding and eliminating the various sources of off flavor in whey protein cannot be overstated.

Annatto

Annatto is a yellow/orange carotenoid often used in the food industry. Annatto, an extract of the tropical tree fruit, *Bixa Orellana,* is named after Francisco de Orellana, an explorer of the upper amazon (Kang et al., 2010). The seed pulp within the fruit contains the actual annatto pigment (Giuliano et al., 2003). Annatto has been used as a colorant and spice for thousands of years. The Aztecs used annatto as a dye to textiles, body dye, and also as a food colorant (Giuliano et al., 2003). In Latin America, annatto is still commonly used as a spice in cochinita pibil, a pork dish with bixa seeds and orange juice (Gerlach and Gerlach, 2002). In the US, the biggest application of annatto is used in the dairy industry and is the primary colorant used to give US Cheddar cheese its yellow color (Emerton, 2008). Annatto was, at one point, reported to be the most commonly consumed natural color additive in the UK (Scotter 2009).

There exist two colorants derived from annatto extract: water-soluble norbixin and oil-soluble ester bixin (Giulano et al., 2003) (Figure 1). Bixin is the primary colorant in the natural seed pulp and is unaffected by pH, but is unstable in light. At temperatures above 100C the cis-bixin converts to trans-bixin (Smith, 2004). The polyene chain in bixin and norbixin is responsible for their instability, especially their susceptibility to different oxidizing agents. Common oxidation sources include oxygen and peroxides, addition of electrophiles including H^+ and lewis acids, and cis to trans isomerization (Scotter 2009). Norbixin is considered a strong colorant and is commonly used in the dairy industry. It is processed by the saponification of the methyl group of bixin in alkaline conditions (Smith, 2004; Giuliano et al., 2003).

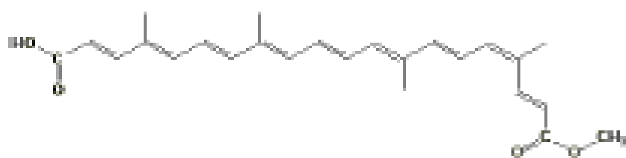


Figure 1: Cis-Bixin

(Smith, 2004)

Figure 2: Trans-Bixin

(Smith 2004)

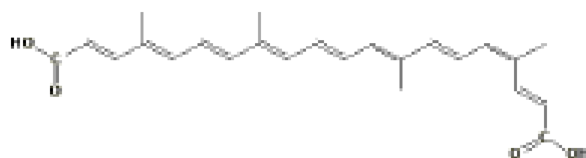


Figure 3: Cis-Norbixin

(Smith 2004)

Figure 4: Trans-Norbixin

(Smith 2004)

Figure 1: Bixin and Norbixin isomers

Despite being used as a spice, little is known about volatile compounds annatto contributes to flavor. It is a concern, however, as it is so widely used in cheese and whey processing. Annatto extraction uses heat, which has been shown to result in a higher level of volatile compounds (Scotter et al., 2002). The cheese making processing is also a heat

treatment which does suggest volatile compound formation from thermal degradation of annatto added to cheese may contribute to cheese and whey flavor (Scotter et al., 2002). Figure 2 shows the reaction pathways for the thermal degradation of a cis-bixin.

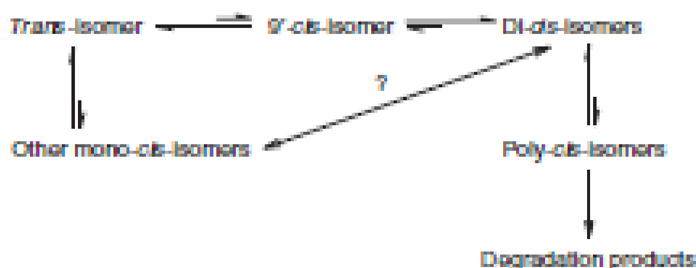


Figure 2: Suggested reaction pathways for the thermal degradation of 9'-cis-bixin (Scotter et al., 2001).

Sensory profiling done of fresh fluid whey from Cheddar and Mozzarella cheese have been shown to be different, with Cheddar being higher in lipid oxidation products than Mozzarella (Liaw et al., 2010). While annatto addition during the Cheddar cheese making process could be one source of flavor difference, Campbell et al. (2010) demonstrated that when identical processes were employed and annatto addition was the single variable, sensory properties of fluid whey and whey protein with and without annatto were not distinct. Volatile compound analysis of fresh liquid wheys with and without added annatto were distinct in limonene and α-pinene (Campbell et al., 2010). Both limonene and α-pinene have previously been found in commercial annatto extracts, which likely explains their increased presence in fresh fluid whey containing annatto (Galindo-Cuspinera et al., 2002).

As stated earlier, the primary colorant used to color yellow Cheddar cheese is annatto. It is currently thought that approximately 20% of the annatto used to color the Cheddar cheese makes it into the whey. The yellow color caused by annatto in the whey is generally undesirable and decolorization must be done to create the color most desired in the finished product (McDonough et al., 1968). In the United States there are two compounds with which it is legal to bleach whey: hydrogen peroxide, covered by 21CFR 184.1366, and benzoyl peroxide, covered by 21CFR 184.1157. Table 1 contains the pros and cons for use in whey bleaching. As can be seen by the table, both hydrogen peroxide and benzoyl peroxide have many cons to their use as bleaching agents. Hydrogen peroxide may not reach a concentration of more than 500 ppm and all residual hydrogen peroxide must ultimately be destroyed by the addition of catalase (US FDA 2011b) Hydrogen peroxide is often used to bleach whey due to the wide range of solid levels and temperatures at which it remains a viable bleaching agent. Hydrogen peroxide, while widely used by industry, causes off flavor formation at a higher level than benzoyl peroxide and its use at high levels is uneconomical. Benzoyl peroxide, while causing off flavor formation at a lower level than hydrogen peroxide, is impossible to export to certain countries because of regulations against the benzoic acid residue it leaves after bleaching. The benefits of using benzoyl peroxide include its effectiveness at lower concentrations and lack of catalase needed for deactivation. In addition, benzoyl peroxide does not pit stainless steel, and is generally less harsh on equipment (Chang et al., 1977).

Table 1: Summary of hydrogen peroxide and benzoyl peroxide characteristics (Kang et al., 2010)

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

Whey Flavor

The flavor of a food product is the single most important factor in determining its success or failure (Morr and Ha, 1991; Drake, 2007). Dairy products commonly enjoy a natural and healthy image, but harsh processing conditions, such as bleaching, can cause off flavor formation. Whey flavor is no exception to this rule. Whey can carry off flavors that originally existed in the milk and those that develop during various processing steps. Storage after spray drying can contribute to stale off-flavors in whey. A sensory language has been developed for whey and whey dried ingredients which includes cardboard, raisin, fatty, soapy, cucumber and cabbage flavors among others (Drake et al., 2003). Flavors found in whey are categorized into two different groups: dairy flavors and nondairy flavors (Carunchia Whetstine et al., 2003). Dairy flavors include sweet aromatic, cooked, and milky flavors. Nondairy flavors include flavors such as cardboard, animal, or cucumber (Carunchia Whetstine et al., 2005). Ingredient flavors carrying through to the final product are a large concern for the food industry which in turn limits the usage of whey.

Flavor variation in whey can be attributed to many different factors reaching back even to the farm. Flavor variability in whey is found between whey batches made from different types of cheeses, milk source and starter culture rotation (Carunchia Whetstine et al., 2003). Seasonal variability exists in milk, primarily seen in milk fat levels due to feed and stage of lactation (Varnam and Sutherland, 1994). Genetics, age of cow and feed can also affect milk composition and flavor (Varnam and Sutherland, 1994, Croissant et al., 2007).

Flavor variation can also be attributed in part to processing steps when making the dried whey ingredients. Pasteurization, membrane filtration, concentration, and spray drying to produce WPC or WPI can all cause additional off-flavors and flavor variability (Drake et al., 2008). Subsequently, any differences in process from facility to facility or from batch to batch can cause variability in flavor. Membrane fouling is a common problem in membrane processing, in which particles, microorganisms, or physicochemical reactions (such as gelation), effectively block the membrane pores (Varnam et al., 1994, Zulewska et al., 2009). Membrane fouling causes differences in flux and subsequent difference in process time. Differences in process time can cause variation between fluid whey batches (Carunchia Whetstine et al., 2005; Wright et al., 2009). Different companies may even use completely different methods to produce the desired whey product. Diafiltration, a membrane process, is used to increase protein concentration by addition of water to retentate (Huffman, 1996; Zydney, 1998). Ion exchange is another method of protein concentration in which the components of the whey are separated by ionic charge rather than molecular size (as in membrane processing) and could be used in the stead of ultrafiltration and diafiltration (Onwulata, 2008). Differing processing times, processes, storage conditions and time/temperature profiles can all contribute to variability in the final product (Drake et al., 2008).

Liquid whey flavor variation can, as stated earlier, be attributed to the cheese starter culture used (Tomaino et al., 2004). Due to possible bacteriophage activity, starter culture strains are often rotated in cheese production (Varnam and Sutherland, 1994). Different

Lactococcus species can differ in lipase activity, proteolytic activity, and production of volatile compound amount and type (Carunchia Whetstine et al., 2003). Since rotation of starter cultures is common and necessary in cheese making, overall flavor of liquid whey can be highly variable. Differences in lipase activity, proteolytic activity and in volatile compound production of Lactococcus species and strains have been reported in wheys from the same manufacturing facilities. Mozzarella liquid whey is distinct from Cheddar whey in flavor and volatile compound profile (Liaw et al., 2011). Cheddar liquid whey also contains higher levels of lipid oxidation products than Mozzarella liquid whey (Liaw et al., 2011).

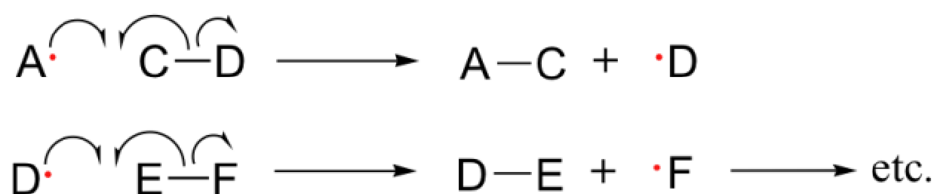
Lipid oxidation products are considered a major source of off-flavors in whey and dried whey ingredients (Carunchia Whetstine et al., 2004, 2005; Wright et al., 2008). While lipids do not generally significantly contribute to the aroma of most foods, due to their low volatility, decomposition of lipids can produce off flavor formation (McClements and Decker, 2008). Oxidation of unsaturated fatty acids causes the formation of a large variety of compounds, including methyl esters, ketones, aldehydes, and free fatty acids (Morr and Ha, 1991; Carunchia Whetstine et al., 2003, 2005). Hydrolytic rancidity and autoxidation are two types of lipid decomposition, with hydrolytic rancidity being the release of free fatty acids and autoxidation being a sequence of chemical changes due to the interaction of oxidative chemicals' interaction with lipids (Frankel, 1998; McClements and Decker, 2008). Oxidation reactions occur during processing, storage and transportation of whey (Campbell et al., 2010; Whitson et al., 2010). Antioxidant addition to Cheddar whey has been studied to determine if flavor variability due to lipid oxidation products can be minimized. Addition of

ascorbic acid or whey protein hydrolysate to liquid whey decreased cardboard flavor and lipid oxidation volatiles (Liaw et al., 2010).

Free radicals are the primary reactive species causing lipid autoxidation (Frankel, 1998; McClements and Decker, 2008). A free radical is a molecule with an unpaired electron, which cause hydrogen abstraction when they react with lipids. The rate of the oxidation reaction is determined by temperature, oxygen levels, degree of fatty acid composition, and the activity of pro and anti-oxidants (Frankel, 1998; McClements and Decker, 2008). Initiation, propagation, and termination are the 3 steps of autoxidation. Figure 3 is a generic initiation, propagation, termination reaction diagram.

initiation

propagation



termination

Figure 3: Generic initiation, propagation, termination diagram showing the different steps of each (Anonymous 2011).

During inititation an alkyl radical is formed and stabilized by delocalization over the double bonds (McClements and Decker, 2008). Propagation occurs when the oxygen covalently bonds to the fatty acid and forms a peroxyl radical (LOO·) (McClements and Decker, 2008). The peroxyl radical can continue the process by abstracting a hydrogen atom from another lipid molecule. Termination occurs when two radical species react and become a stable, non-reactive molecule (McClements and Decker, 2008). Figure 4 shows a more lipid specific autoxidation process.

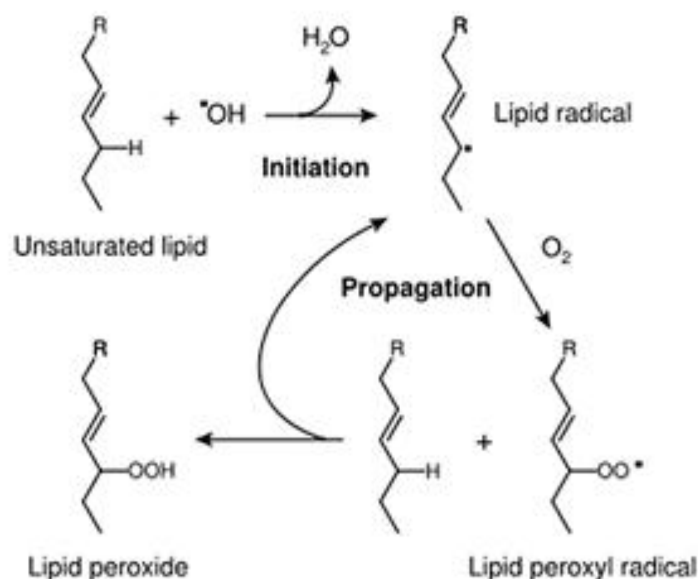


Figure 4: Diagram of lipid peroxidation caused by introduction of a hydroxyl radical species (Clark 2008).

An understanding of lipid oxidation reactions is important when dealing with the processing and storage of liquid whey. While it is likely that lipid oxidation in whey products cannot be completely halted, steps can and should be taken to minimize lipid oxidation in fluid whey and dried whey ingredients.

Whey Processing

Whey processing is an important factor in terms of flavor development. To understand the sources of off flavor in whey protein, one must understand the process of creating whey protein concentrates. Whey protein is far removed from its ultimate source, milk. To make Cheddar cheese, the milk must be received, pretreated, standardized, and heat treated/pasteurized. Calcium chloride and/or color (in the form of annatto) is then added. The milk is then acidified through culture or acid addition and rennet/milk coagulant is added. Once coagulated, the cheese curd is cut, and the curds and whey are cooked to expel remaining whey (Farkye, 2004). After cooking, the whey is separated from the cheese curd for further processing. Liquid whey is not commonly used as a food ingredient (93% water and 0.6% whey protein). Further processing is needed to make whey protein concentrate (WPC, >80% protein w/w) or whey protein isolate (WPI, >90% protein w/w).

Steps included in a normal whey protein concentrate process include holding, clarification/fat separation, pasteurization, ultrafiltration, and spray drying (Huffman, 1996; Varnam and Sutherland, 1994). Holding of whey at any temperature should be kept to a minimum before it is pasteurized due to lipid oxidation and bacterial activity, especially

lactic acid formation which reduces the pH of the whey. Clarification and fat separation is an important step as it removes remaining casein fines from the whey along with remaining fat. Clarification takes place through centrifugation in a separator (Bodyfelt et al., 1988; Varnam and Sutherland, 1994). The protein is then concentrated through ultrafiltration and diafiltration. The ultrafiltration process is a molecular weight exclusion technique which uses a pressure activated membrane to separate the whey protein from the lactose and other low molecular weight molecules (Huffman, 1996). Once desired protein and solids level are achieved, the concentrate is spray dried. Whey protein concentrates above 50% protein composition must also go through a diafiltration step to reach desired protein levels (Huffman, 1996; Onwulata, 2008). Diafiltration is a wash step which dilutes whey solids levels to allow for continued ultrafiltration, decreasing lactose and mineral levels and increasing protein levels (Johnson and Lucey, 2006; Huffman, 1996; Onwulata, 2008).

Whey protein isolates are whey concentrates with a protein composition above 90%. Additional steps are necessary to achieve a higher protein composition, namely, microfiltration and/or lactose hydrolysis. Microfiltration is used to further remove fat which has been concentrated through the ultrafiltration process and lactose hydrolysis is used to remove remaining lactose. Ion exchange is another process commonly used to reach >90% protein levels. It is used before ultrafiltration and it involves lowering the pH below the pI of the whey proteins to give them an overall positive charge. The positively charged protein solution is then run through a tank with negatively charged resin beads (Huffman, 1996). Ion exchange allows for virtually 100% demineralization (Varnam and Sutherland, 1994;

Onwulata, 2008). The positively charged whey proteins attach to the resin beads and the fat, minerals and lactose are washed away. When the resin reaches protein capacity, the pH of the tank is increased and the protein detaches from the resin (Huffman, 1996). Ultrafiltration can then be used to concentrate the protein solution to desired levels.

Ozone

Ozone was first discovered in 1839 by C.F. Schonbein. His discovery was made during a electrolysis experiment when he noticed that sparking in air caused an odor. He called the unknown compound that he smelled ozone, from the Greek *ozein* (to smell) (Perincek et al., 2007). It wasn't until 20 years later when it became clear that ozone was a triatomic allotrope of oxygen. Thomas Andrews proved that ozone could be produced by pure oxygen in 1856 and Soret found that 3 volume of oxygen would produce 2 volumes of ozone (Perincek et al., 2007). Ozone was first commercially used in 1907 in the sanitization of the municipal water supply in Nice, France and then in St. Petersburg, Russia in 1910 (Kogelschatz, 1988).

Ozone has been used for some time to disinfect drinking water in Europe. It has also been used for many other commercial uses including disinfection of bottled water, swimming pools, prevention of fouling of cooling towers, and wastewater treatment (Guzel-Seydim et al., 2003). Ozone received GRAS status for use in bottled water in 1982 and an expert panel decreed that ozone was a GRAS substance for use as a disinfectant or sanitizer for foods

when used in accordance with good manufacturing practices in 1997 (USDA 1997), but has thus far been little used in the food industry.

Ozone is thermodynamically unstable and spontaneously reverts back into O_2 and is therefore unsuitable for storage and transport. It is heavier than air, very reactive, irritating, and pale blue (Perincek et al., 2007). Many of its chemical characteristics are included in Table 2. Due to its highly reactive nature and spontaneous reversion into O^2, ozone can only be generated "in situ" (Iglesias, 2002). Ozone is a strong oxidizing agent from which it gains its wide use in industrial and commercial applications (Iglesias, 2002). Table 3 compares the oxidative potential of many oxidizing agents to that of ozone.

Table 2: Physical properties of ozone (Iglesias, 2002)

Physical Property	Value	Physical Property	Value
Molecular weight	48.0 g/mol	Density, gas (0 °C, 101 kPa)	2.144 $kg.m^{-3}$
Boiling point (101 kPa)	−111.9 °C	Density, liquid (−112 °C)	1358 $kg.m^{-3}$
Melting point	−92.7 °C	Viscosity, liquid (−183 °C)	$1.57*10^{-3}$ Pa.s
Critical temperature	−12.1 °C	Heat of vaporization	15.2 $kJ.mol^{-1}$
Critical pressure	5.53 Mpa		

Table 3: Oxidative potential of ozone and other oxidative chemicals. A higher oxidation potential means a greater affinity for electrons and tendency to oxidize (Iglesias, 2002)

Oxidizing agent	Oxidation potential (mV)
Fluorine	3.06
Ozone	2.07
Permanganate	1.67
Chlorine dioxide	1.50
Hypochlorus acid	1.49
Chlorine gas	1.36

Ozone Generation

In order to generate ozone a diatomic oxygen must be split. The free radical oxygen is then free to react with another diatomic oxygen, which then forms triatomic ozone. Two common methods, ultraviolet radiation (188 nm) and corona discharge, are used to initiate free radical oxygen and generate ozone (Rice et al., 1981).

Corona discharge consists of two electrodes, one of which is the high tension electrode and the other which is the low tension, or ground electrode. Electrons flow between the two electrodes and when they have sufficient kinetic energy to dissociate the oxygen molecule, a molecule of ozone can be formed. Figure 3 is a schematic of a generic corona discharge ozone generator. If ambient air is fed through the gap 1-3% ozone can be produced, although, if pure oxygen is fed through the gap, up to 6% ozone can be produced (Rice et al., 1981).

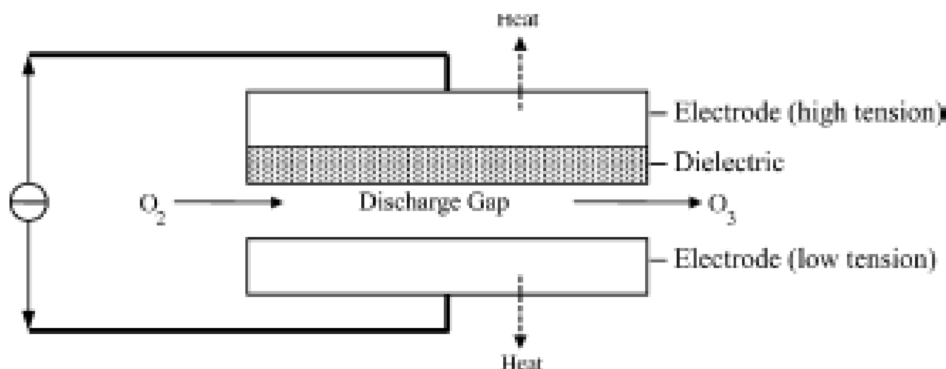


Figure 5: Schematic diagram of a corona discharge ozone generator (Rice et al. 1981).

Antimicrobial effect of ozone

Ozone has been well studied as an antimicrobial agent on a wide variety of organisms including Gram positive and Gram negative bacteria, spores, and vegetative cells (Fetner and Ingols, 1956; Foegeding, 1985; Ishizaki et al., 1986; Restaino et al., 1995). Ozone has been shown to be effective in killing both gram positive *Listeria monocytogenes, Staphylococcus areus, Bacillus cereus,* and *Enterococcus faecalis,* as well as Gram negative *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. Ozone also destroys the yeasts *Candida albicans* and *Zygosaccharomyces bacilli* along with the spores of *Aspergillus niger* (Restaino et al., 1995). The viruses Venezuelan equine encephalomyelitis, hepatitis A, influenze A, vesicular stomatitis virus, and infectious bovine rhinotracheitis virus and several bacteriophage strains have also been destroyed by ozone (Guzel-Seydim 2004). Table 4 shows several common bacteria and the relative effectiveness of ozone in their inactivation.

Table 4: Inactivation levels of several common bacteria by ozone under different conditions (Kim et al., 1999).

Bacterium	Inactivation (log_{10})	Treatment time (min)	Concentration (mg/liter)	pH	Temp. (°C)	Medium	Reactor type
Bacillus cereus	>2.0	5	0.12		28	O_3 demand-free water	
B. cereus (spores)	>2.0	5	2.29		28	O_3 demand-free water	
Escherichia coli	4.0	1.67	0.23–0.26	7	24	O_3 demand-free water	Continuous flow
E. coli	3.0	19	Initial 2.2, residual 0.06	7.5	16	Raw wastewater	Continuous flow
E. coli	2.0	0.1	0.53	6.8	1	Phosphate buffer	Batch
Legionella pneumophila	>4.5	20	0.32	7	24	Distilled water	Batch
Mycobacterium fortuitum	1.0	1.67	0.23–0.26	7	24	O_3 demand-free water	Continuous flow
Pseudomonas fluorescens	>2.0	0.25					
Salmonella Enteritidis	1.0	0.25	8% (wt/wt)		25	Broiler carcass	Ozone gas
Salmonella Typhimurium	4.3	1.67	0.23–0.26	7	24	O_3 demand-free water	Continuous flow
Staphylococcus aureus	>2.0	0.25		7	25	Phosphate buffer	Batch (bubbling)

Use as a potent antimicrobial agent in food could be a highly effective use of ozone due to its wide range of effectiveness on many different types of food borne pathogens.

The oxidation capacity of ozone is what gives it its antimicrobial capabilities, specifically, its progressive oxidation of vital cellular components (Victorin, 1992). Two mechanisms have been specifically identified as having antimicrobial effect: the first mechanism is the oxidation of sulfhydryl groups and amino acids of enzymes, peptides and proteins to shorter peptides. The second is the oxidation of polyunsaturated fatty acids to acid peroxides (Victorin, 1992). Oxidation of the polyunsaturated fatty acids in the cell envelope of microorganisms causes cell disruption and leakage. In gram negative bacteria,

the lipoprotein and lipopolysaccharide layers are first to be oxidized by ozone. This oxidation results in cell permeability and cell lysis (Kim et al., 1999). In addition to cell envelope, lipoprotein and lipopolysaccharide layer disruption, ozone causes damage to both viral RNA and nucleic acids, with thymine acting as the most sensitive (Kim et al., 1999). The widespread oxidation of internal cellular proteins causes rapid cell death, while chlorine selectively destroys specific intracellular enzyme systems (Mudd et al., 1969; Hinze et al., 1987; Takamoto et al., 1992).

Commercial and Industrial Uses of Ozone

Ozone has been used for many purposes in the food industry, mostly as an antimicrobial agent. Disinfecting recycled poultry chill water is one such use, with the use of ozone meeting the USDA recycling requirements of at least 60% reduction in total microorganisms and similar reduction in coliforms, *E. coli,* and *Salmonella* spp. (Sheldon et al., 1986; USDA 1997). No viable *E. coli* or presumptive coliforms occurred in ozonated poultry water (Waldroup et al., 1993). Ozone also reduced 2 log-units of carcass microorganisms with no signification lipid oxidation, off flavor development, or loss in carcass skin color.

Ozonated water, used as a sanitizer, has also been extensively studied in a food plant setting. Many different methods of plant equipment sanitation exist. Common sanitizing agents used include chlorine derivatives, acid, iodine, and quaternary ammonium compounds (Marriott, 1994). Thermal sanitation is also very effective, but steam and hot water can be

expensive to generate and can damage plant equipment (Troller, 1993). Chlorine derivatives are widely used but they have several disadvantages including producing toxic by-products (Minear et al., 1995). Ozone is a potential alternative to chlorine for use in the food industry as a non-corrosive sanitizing agent with no chemical residue. Ozonated water is as effective as chlorination against dairy surface attached bacteria with a 99% reduction in bacterial populations for both treatments (Greene et al., 1993). In a study performed by Dosti (1998), the bactericidal effectiveness of ozone, chlorine and heat were compared. *P. fluorescens, Pseudomonas fragi, Pseudomonas putida, Enterobacter aerogenes, Enterobacter cloacae,* and *Bacillus licheniformis* were exposed to ozone (0.6ppm) for 1 and 10 minutes, chlorine (100ppm) for 2 minutes, or heat (77°C) for 5 minutes, the 10 minutes ozone exposure was found to have the highest kill rate. The 10 minute ozone exposure reduced mean bacterial populations by 7.3 log units, while heat reduced mean bacterial population by 5.4 log units and chlorine reduced the bacterial population by 3.07 log units.

An ineffective cleaning process can allow for the formation of biofilms on equipment surfaces in which microorganisms accumulate and proliferate. Microorganisms embedded in a biofilm matrix are often resistant to the action of many sanitizers (Dosti 1998). In the case of the biofilm forming *P. flourescens, P. fragi,* and *P. putida,* ozone and chlorine both significantly reduced the biofilm bacteria compared to the control ($p<0.05$), although no significant difference was found between ozone and chlorine except in the case of *P. putida*. Ozone was more effective against *P. putida* than chlorine (Dosti, 1998).

The effectiveness of ozonated water on soiled dairy equipment has also been studied (Guzel-Seydim et al. 2000). Results showed a significant increase in soil removal with ozonated water over warm (40°) water. Ozonated water removed 84% of dairy soil on soiled stainless steel while warm water removed only 51%. This study suggested that the use of ozone as a pre-rinse stage may allow for decreased use of detergent in the dairy industry.

Direct Treatment of Food with Ozone

Treating fruits and vegetables has also been done using ozone (Norton et al., 1968; Rice et al., 1982). Apples treated with ozone had a lower weight loss and spoilage during storage and an increase in shelf life was also seen in oranges. It is thought that the oxidation of ethylene is the method by which shelf life is extended in both apples and oranges (Rice et al., 1982). Blackberries and grapes treated with ozone saw a decrease in fungal deterioration (Beuchat, 1992). Onions have been treated with ozone during which mold and bacterial counts were greatly decreased without effect in chemical composition and sensory quality (Song et al., 2000). The effect of ozone on the microbial load and sensory characteristics of black peppercorns has also been studied (Zhao et al., 1995). Ozone significantly reduced the microbial load of whole and ground black peppercorns. Ozone treatment of ground black peppercorns did cause some oxidation of volatile oils, but had no significant effect on the volatile oils of whole black peppercorns (Zhao et al., 1995). This data suggests that the use of ozone as an antimicrobial agent may be viable in the spice industry. Cheddar cheese surfaces have been exposed to ozone in order to keep mold counts down (Gibson et al.,

1960). A constant high level of ozone did effectively keep mold growth from occurring, but mold populations increased after termination of ozone exposure. It is evident from the several studies above that ozone can be an effective and useful chemical for food processors.

Ozone Use as a Bleaching Agent

Ozone has been used to reduce the yellow color in concentrated gelatin (Cataldo, 2007). For certain industrial uses like photographic use, a clear gelatin is needed. When a concentrated solution of gelatin is treated with a small amount of ozone, the solution of gelatin will change visually from its characteristic yellowish color to a whiter color. The absorption curve of gelatin before ozonation has a cut off range between 380 and 600 nm which accounts for its yellow color. After ozonation the absorption curve cut off range decreases dramatically to 350-450 nm. With oxidation of gelatin at minimal to negligible levels, ozone could be viably utilized as a sterilization and bleaching agent.

Ozone has also been used to bleach cotton fabrics. In an industry where researchers are increasingly looking for processes which can be carried out at low temperatures using small amounts of water in a short duration without the use of harmful chemicals, ozone has become an interesting alternative to hypochlorite and hydrogen peroxide bleaching (Prabaharan et al., 2000). An acceptable degree of whiteness (ready for dyeing) can be obtained by ozone bleaching (Perincek et al., 2007). There was an increase in the whiteness degree of fabrics proportionate with an increase in ozone exposure. Ozone requires a very low quantity of water, requires no harmful chemicals and bleaching is achieved in a very

short time at room temperature without the need for heating or cooling (Perincek et al., 2007).

Azo dyes make up a large amount of the dyes produced today (Silva 2009). While many biological processes, using cultures or fungi have been investigated for color removal of wastewaters, complete mineralization of azo dyes is not achieved by conventional treatment processes (Fu and Viraraghavan, 2001; Santos et at., 2007). Amines, degradation products of such conventional treatment processes, constitute an environmental concern due to their toxicity and hazardous effects (Silva 2009). Ozonation of dye wastewaters has been used as an effective bleaching agent in many instances. Table 5 shows the effect of ozonation on many different azo dyes currently used.

Table 5: Percent color removal of many azo dyes by ozonation (Silva 2009).

	Color removal (%)	COD or (TOC) removal (%)	Reaction time (min)[a]
Azo dyes (mixture)	80–100[b]	40–80 (44–80)	30–40
Dye baths			
(yellow shades)	100	48	20–40
(blue shades)	100	51	
Azo dye (Acid Orange 8)	100	< (25)	30
Industrial (textile) wastewater	86–96	33–39	45
Azo dyes (Acid, Direct and Reactive)	86–97	< (25)	7.36[c]
Azo dye (Acid Red 151)	100	66	180
Dye bath	74–80	(33)	160
Azo dyes (dye bath)	100	48–62	15–34
Azo dyes (Acid Red 27, Orange II)	–	(50–60)	–
Azo dye (Acid Red 88)	≅100	44–63	6
Azo dye (Reactive Red 120)	96–100	52–55	150
Red X-GLR	100	5.7–35	120
Azo dyes	70–100	8–68	40
Dye bath	95	(28–41)	60
Dye bath	73–77	3–11	10
Synthetic wastewater	90	<15	90
Industrial wastewater (azo dyes production)	43	25 (7)	–

[A] Reaction time to reach the reported COD or TOC removal
[B] 100% or complete color removal reported
[C] Hydraulic residence time

Ozonation was very effective in the decolorization of all of the above listed azo dyes. Such information suggests that ozone has the potential to be a great asset in the bleaching in many different industries.

Demand for whey is increasing. As demand increases, the importance of being able to consistently produce a high quality, desirable product increases with it. Flavor defects caused by current bleaching agents are well documented but few studies have been conducted on alternative bleaching agents and methods to reduce norbixin levels in whey. These studies are meant to investigate annatto partitioning into cheese and whey, and the color and flavor effects of using ozone gas as an alternative bleaching agent in whey. A better understanding of norbixin and its characteristics are of great use to the dairy industry to increase the quality of whey and whey ingredients.

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Bleaching Efficacy of Ozone Gas in Liquid Whey and its Effects on Flavor of 80% Whey Protein Concentrate

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Short version of title: Ozone Bleaching of Whey

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

ABSTRACT

Bleaching of whey protein is a necessary commercial practice but recent studies have demonstrated that hydrogen peroxide and benzoyl peroxide bleaching cause off flavors in dried whey ingredients. The objective of this study was to determine the viability of ozone as an alternative bleaching agent in whey protein production. Flavor effects and bleaching efficacy of ozone gas on liquid whey and whey retentate were evaluated in bench top experiments prior to pilot scale manufacture of 80% whey protein concentrate. Cheddar whey and retentate were produced in triplicate. Bleaching variables tested included bleaching temperature (35 and 60°C), ozone (200mg/h in 700mL whey) exposure time (15, 30, and 45 min), and whey solids content (7 and 12%). Bleaching efficacy was evaluated by measurement of norbixin relative to an unbleached control. Based on bench top results, hot bleaching (50°C) of liquid whey with 1h ozone exposure was selected for WPC80 production. To ensure safety, ozone bleaching was performed at a lower level (2.2g/h in 95 L whey) and compared to a control (no bleaching) and hydrogen peroxide (HP) bleaching (250ppm). WPC80 was manufactured in triplicate. Bleaching of retentate with ozone was higher at 35°C compared to 50°C ($p<0.05$); temperature did not affect liquid whey bleaching with ozone ($p > 0.05$). In bench top studies, a 63 % decrease in norbixin content was observed in fluid whey after 45 min ozone exposure. In pilot scale manufacture, WPC80 from HP bleached whey had a 27% norbixin destruction while that bleached with ozone had a 15% reduction. Ozone-treated WPC80 exhibited animal and flour/pasta flavors and HP bleached WPC80 was characterized by cabbage and fatty flavors. These flavors were not

present in the control unbleached WPC80. Higher levels ($p<0.05$) of nonanal and decanal were present in the ozone WPC80 while higher levels ($p<0.05$) of pentanal, DMDS, hexanal, heptanal, 2-pentylfuran, and octanal were present in the HP WPC80 compared to the control WPC80. These results suggest that ozone bleaching does not represent a promising alternative to approved bleaching agents in whey protein production although it could possibly remain feasible at or close to saturation levels.

Key words: whey, retentate, norbixin, bleach, ozone

Introduction

Demand for whey protein as a value-added product has steadily grown worldwide (Tunick, 2008). In 2009, 454.4 million kilograms of dry whey, 332.7 million kilograms of lactose (human and animal), and 188.6 million kilograms of whey protein concentrate were produced (USDA, 2010). By 2010, total US cheese production, excluding cottage cheese, was 10.4 billion pounds; an increase of 42% since 1998.

Flavor is an exceedingly important factor in the success or demise of a food product (Morr and Ha, 1991; Drake, 2007). As such, flavor is of a concern in value-added whey protein products. Off flavors in fluid whey can be formed during the cheese make process and may also be formed during processing steps of fluid whey to dried whey ingredients (Campbell et al., 2011; Carunchia Whetstine et al., 2003, 2005). Lipid oxidation and Maillard browning reactions are responsible for off flavors in dried whey ingredients (Carunchia Whetstine et al., 2005; Wright et al., 2006; 2009; Whitson et al., 2010). Bleaching of fluid whey is of significant interest in off flavor development due to the oxidative properties of currently used bleaching agents, hydrogen peroxide and benzoyl peroxide. Studies have confirmed that chemical bleaching plays a significant role in off flavors of WPC34 and WPC80 (Listiyani et al., 2011; Jervis et al., 2012). Finding alternative bleaching agents with comparable bleaching efficacy to current bleaching methods and less off flavor formation are of interest to the dairy industry.

Ozone was discovered in 1839 by C.F. Schonbein. Used for some time to disinfect drinking water in Europe, ozone received GRAS status in the US in 1982 (USDA 1997).

Ozone is a commonly used gas which has proven to be useful in many different applications, such as bottled water, swimming pools, fouling prevention in cooling towers and wastewater treatment (Guzel-Seydim et al., 2003). The use of ozone as a bleaching agent has been explored in other products. Examples include use in concentrated gelatin solutions to reduce yellow color and to bleach cotton fabric (Cataldo, 2007; Perincek et al., 2007). A small amount of ozone, when applied to a concentrated gelatin solution has been shown to reduce the natural yellow color, to a more desirable white color (Cataldo, 2007). Ozone, due to its strong oxidative potential, likely reacts with impurities in the gelatin which cause yellowing. In addition to gelatin, the ability of ozone to bleach cotton fabrics has also been examined. Ozone bleaching of cotton uses lower temperatures, lower water usage and a lack of harmful chemicals (Prabaharan et al., 2000). The GRAS status of ozone and its ability to effectively bleach at low concentrations without the need for the addition of harmful chemicals could be very useful to the dairy industry. The objective of this study was to determine the viability of using ozone gas as a bleaching agent of liquid whey.

MATERIALS AND METHODS

Experimental Design Overview

Two experiments (experiment I and II, figures 1 and 2) were included in this study. The purpose of experiment I was to determine the effect of time, temperature and the solids level of liquid whey on the bleaching efficacy of ozone gas at above saturation levels.

Cheddar cheese whey was manufactured in the NCSU Dairy Pilot Plant. After pasteurization and fat separation of whey, an aliquot of liquid whey was taken for bleaching while the remainder was ultrafiltered and diafiltered to 12% solids (w/w) and 80% (w/w) protein retentate. A control of the liquid whey was taken and the remainder was either cooled to 35°C or heated to 60°C. Once the respective temperatures were reached, the ozonation process began. A high level of ozone was bubbled into liquid whey for 15, 30, and 45 min. A similar procedure was conducted to bleach liquid whey retentate. Four trials of experiment I were performed.

The purpose of experiment II was to test ozone bleaching of liquid whey at below saturation levels and to compare the bleaching efficacy and flavor profile to liquid whey bleached with 250 ppm hydrogen peroxide, a traditional chemical bleaching application. Again, Cheddar cheese liquid whey was produced, pasteurized, and fat separated. In experiment II, the liquid whey was heated to 50°C and bleached with either ozone or hydrogen peroxide or recirculated (control) for 1 h. After bleaching, liquid whey was ultrafiltered and diafiltered to 22% solids, 80% protein and spray dried to produce WPC80. Experiment II was replicated four times.

Liquid whey and WPC80 production

Raw whole milk, 98 kg, was obtained from the North Carolina State University Dairy Research and Education Farm (Raleigh, NC). Milk was vat pasteurized (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC) at 63°C for 30 min. After

pasteurization, milk was immediately cooled to 31°C and inoculated with freeze dried lactic acid starter culture (50 DCU/454 kg, Choozit MA 11, Danisco, New Century, NJ). Milk was agitated and ripened for 30 min. After the initial ripening period, calcium chloride solution (50% (w/v), Dairy Connection Inc., Madison, WI) was added (0.39 mL/kg milk) as was double strength annatto color (4% norbixin w/w, Danisco, New Century, NJ) (15 mL/454 kg milk, diluted 20 times in deionized (DI) water). Agitation and ripening of milk continued for another 30 min. After this period, milk was coagulated with double strength recombinant rennet (Dairy Connection Inc., Madison, WI; 0.09 mL/kg milk, diluted 80 times in DI water). The milk was allowed to coagulate for 30 min without agitation. The coagulum was cut with 0.95-cm wire knives and allowed to rest for 5 min. After resting, the curd and whey were gently stirred for 10 min. The curds and whey were slowly heated from 31°C to 39°C over a period of 30 min during which pH and titratable acidity were monitored. Whey was drained from curds at pH 6.40. The whey was strained to remove cheese particles and pumped into a batch pasteurizer where it was pasteurized at 63°C for 30 min. After pasteurization, hot whey was separated in a hot bowl separator (Model JF 125 EAR, Clair, Althofen, Austria). Fresh liquid whey (2 L) was collected immediately following fat separation for ozone bleaching. Remaining liquid whey was weighed and retained for WPC80 production and placed in a stainless steel fermentor (F3-14, Tri-clamp models, Blichmann Engineering, LLC).

The retentate for experiment I was concentrated using an ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) with five polyethersulfone cartridge

membrane filters (Model P2B010V05, nominal separation cutoff: 10,000 kDa, surface area: 0.5m^2). The UF membranes were fed liquid whey by peristaltic pump (Model 77410-10) with model 77601-00 pump heads using silicone tubing (Model 96440-73). During circulation and ultrafiltration the temperature of the whey retentate was held at 50°C using a stainless steel heating coil (PAC Stainless LTD, Seattle, WA) with hot water (82°C). The UF process was conducted until a concentration factor of approximately 2 times was achieved, at which point diafiltration was initiated by adding approximately 33% of the total beginning weight of whey back in the form of DI water (Campbell et al., 2011). The UF process was continued until the retentate was again concentrated by a factor of 2 times. At this point, DI water equal to approximately 17% of total beginning liquid whey weight was added to the retentate. Total DI water addition was equal to 50% of the total beginning whey weight. The retentate was then run through the UF process until approximately 12% solids (w/w), 80% protein (w/w) was achieved. Total percent solids of retentate was analyzed using the Smart System 5 moisture/solids analyzer (CEM, Matthews, NC). Protein content was determined by the Sprint™ Protein Analyzer (CEM, Matthews, NC).

For experiment II, spray dried WPC80 was produced. Liquid whey and retentate production steps remained identical except that the retentate was ultrafiltered to a solids content of 22% (w/w) to assist in the spray drying process. Once 22% solids, 80% protein was achieved, the liquid retentate was spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark). Spray drying took approximately 2 h. The WPC80 powders were stored in mylar bags at -80°C until analyses.

Bleaching of Liquid Whey and Retentate

For experiment I, liquid whey was divided into two parts in preparation for bleaching. A sterile sample cup (VWR International, West Chester, PA) was filled with 90 mL of liquid whey and stored at -20°C as a negative control. Two samples (700 mL) were placed in 2 L pyrex beakers and then placed at 35°C or 60°C. Upon reaching 35°C or 60°C, respectively, an ozone generator (Model OZX-300U, Enaly M&E ltd., Shanghi) was activated, producing approximately 200 mg/h ozone into the samples through an 80 micron diffusion stone. Samples (90 mL) were collected following ozone treatments of 15, 30, and 45 min and stored at -20°C. These processes were repeated for bleaching of WPC80 retentate.

For experiment II, fresh liquid whey was collected into bleaching vats (~95 L each) immediately following fat separation. The liquid whey control was covered and recirculated at 50°C, mimicking bleaching conditions for 1 h. 250 ppm hydrogen peroxide (250 ppm, 30% v/v, Columbus Chemical Industries, Inc., Columbus, WI) was added to a separate 95 L liquid whey sample at 50°C and held for 1 h, after which 20 ppm catalase (FoodPro CAT, Danisco, New Century, NJ) was added to cease hydrogen peroxide activity. Ozone (~2.2g/h) was continuously bubbled through 80 micron diffusion stones into another 25 liquid whey sample using 4 ozone generators (3 model OZX-300U, 1 model 1000BT-12, Enaly M&E Ltd., New Jersey) for 1 h at 50°C. Once bleached, ultrafiltration of liquid whey commenced as previously described.

Proximate Analysis

The solids of liquid whey and retentate were conducted using a forced-air drying oven (AOAC, 2000; method number 990.20; 33.2.44). Total nitrogen of liquid whey, retentate, and WPC80 was determined by the NCSU Analytical Services Laboratory (Raleigh, NC) using the Kjeldahl method (APAC, 2000; method number 991.20; 33.2.11); crude protein was calculated by multiplying the total nitrogen by 6.38. Total fat content of the liquid whey was determined by the Pennsylvania modified Babcock method (AOAC 2000; method number 989.04), while the fat content of retentate and WPC80 was determined by Modified Mojonnier ether extraction (AOAC, 2000; method number 989.05; 33.2.26 and AOAC, 2000; method number 932.06; 33.5.08). Mineral analysis (phosphorus, calcium, potassium, magnesium, sodium, iron, and sulphur) was performed in duplicate by the NCSU Analytical Services Laboratory (Raleigh, NC) using a dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009).

Norbixin Extraction and Quantification

Norbixin extraction was performed on all liquid whey, retentate, and WPC80 as described by Campbell et al. (2011). All samples underwent a solvent extraction followed by norbixin quantification by UV-VIS spectrophotometry (UV-1700 PharmaSpec, Shimadzu Scientific Instruments, Columbia, MD) at 458 nm. A standard curve of 0 to 10 mg/kg norbixin (45% w/w. Chr. Hansen, Milwaukee, WI) was used for quantitation. The norbixin standard was dissolved in 2.5% potassium hydroxide (J.T. Baker) and added to a 7:3 solution

of methanol:glacial acetic acid (VWR International, West Chester, PA). All samples were extracted in triplicate.

Hunter L*a*b*

The color of the liquid whey, liquid retentate and WPC80 powders was measured using a Minolta Chroma meter (CR-410, Ramsey, NJ). The Hunter CIE Lab color scale was used. For experiment I, 10 mL of liquid whey or retentate was placed into a 60 mm x 15mm polystyrene petri dish (Beckton Dickinson, Franklin Lakes, NJ) in duplicate and analyzed. Each petri dish was also measured in duplicate. For experiment II, 10 grams of WPC80 powder was placed into the petri dish and the aforementioned methods were used for analysis. A factory-supplied white calibration plate was used to calibrate the Chroma meter.

Instrumental Volatile Compound Analysis

Instrumental volatile compound analysis was performed on rehydrated, spray dried WPC 80 produced in experiment II. Selected volatile compounds were extracted and identified by head space-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME GC-MS) as described by Campbell et al. (2010). The GC used was an Agilent 6890N with 5973 inert MSD with a ZB-5ms (30m x 0.25mm ID x 0.25µm) column (Phenomenex, Torrance, CA). Sample injection was automated using a CTC Analytics CombiPal Autosampler (CTC Analytics, Alexandria, VA). Spray dried WPC80 were rehydrated to 10% solids (w/v) using HPLC water (EMD Chemicals Inc., Gibbstown, NJ),

and 5 mL were added to 20 mL SPME vials (MicroLiter Analystical Supplies, Inc., Suwanee, GA) along with 10% (w/v) salt. Each sample was prepared in triplicate. 10 µL of 81 ppm 2-methyl-3-heptanone in methanol was added to each vial to act as an internal standard. Equilibration took place over 25 min at 40°C during which the samples were pulse agitated at 250 rpm. A DVB/Carboxen/PDMS 1 cm fiber (Supelco, Bellefonte, PA) was used for all samples. Compounds were identified using the National Institute of Standards and Technology (2002) 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 in the GC-MS under the same conditions.

Descriptive Sensory Analysis

Descriptive sensory analysis (DA) was performed on rehydrated WPC80 (10% w/v) from experiment II. All sensory testing was performed in compliance with North Carolina State University Institutional Review Board for Human Subjects guidelines. An established lexicon for dried dairy ingredients was used (Drake et al., 2003, 2009; Wright et al., 2009). Samples were evaluated by eight trained panelists, each with more than 100 h experience each in the Spectrum™ descriptive analysis method. Approximately 25 mL of rehydrated WPC80 were added to 59 mL cups (Solo Cup Company, Champaign, IL). Each sample was

given a 3-digit code and tasted in duplicate by each panelist. All data was collected using paper ballots or Compusense five, version 5.2 (Compusense, Guelph, Canada).

Results and Discussion

Proximate Analysis

The composition of the whey produced in experiment I was consistent ($p>0.05$). In liquid whey, fat averaged 0.59% ±0.39% (w/w), and solids was 6.55% ±0.22% (w/w). Minerals were also not distinct ($p>0.05$). Minerals measured included phosphorus (0.48±0.01%), calcium (0.56±0.01%), magnesium (0.11±0.02%), potassium (2.1±0.07%), sulfur (0.17±0.01%), sodium (0.59±0.01%), and iron (0.76±0. 02ppm). In retentate, fat averaged 0.76±0.07%, and solids 12.4±0.01%. Minerals in retentate were phosphorus (0.55±0.05%), calcium (0.98±0.08%), magnesium (0.07±0.00%), potassium (0.69±0.05%), sulfur (1.1±0.07%), sodium (0.19±0.01%), and iron (11.9±1.5ppm).

The composition of the WPC80 produced in experiment II was consistent among treatments (control, hydrogen peroxide bleached, and ozone bleached powders) ($p>0.05$). Powders did not differ from each other in fat composition and averaged 5.66±0.6%. Protein and moisture were also not different ($p>0.05$) with an average content of 81.12±1.85% protein and an average moisture content of 5.47±1.28%. Levels of phosphorus (0.46±0.02%), calcium (0.77±0.06%), magnesium (0.06±0.00%), potassium (0.60±0.05%), sodium (0.18±0.01%), and iron (21.0±14.1ppm) were also not different ($p>0.05$).

Norbixin Analysis

Ozone is a strong oxidizing agent with an oxidation potential much higher than that of hydrogen peroxide (Iglesias, 2002). Due to its propensity to form radicals, it is highly reactive with many different compounds including the conjugated bonds of the chromophore of norbixin. In experiment I, ozone at saturation levels (200 g/h/600 mL liquid whey) significantly decreased norbixin content in liquid whey and retentate at both 35 and 60°C ($p<0.05$). Norbixin was decreased by 49% in liquid whey treated at 35°C and reduced by 63% at 60°C after 45 min of ozone exposure. In retentate, a 75% reduction occurred at 35°C and 33% at 60°C. This experiment served as proof of concept for ozone bleaching of norbixin within liquid whey when ozone was at or above saturation levels. In a practical application this amount of ozone would be destructive to more than just the norbixin, likely causing protein degradation and unacceptable levels of lipid oxidation which would cause high levels off-flavor formation. Safety issues are also important to keep in mind when dealing with ozone gas. Ozone is considered an air contaminant and ozone levels are regulated in the workplace by OSHA. Currently, a maximum of 0.1ppm ozone ($0.2 mg/m^3$) (OSHA 2012) is allowed for general industry. To bleach at saturation levels, an unsafe amount of ozone would have to be used in a system open to air in a sizeable scale-up of liquid whey production.

In experiment II, WPC80 were evaluated for bleaching efficacy from liquid whey bleaching at lower levels of ozone (2.2 g/h/95 L) relative to the volume of whey bleached than those used in experiment I for safety and reasons of practicality. The lower level of

ozone exposure destroyed 15% of the norbixin in liquid whey compared to 31% destruction by hydrogen peroxide treatment. While both hydrogen peroxide and ozone destroyed a significant amount of norbixin compared to unbleached whey the lower level of ozonation per volume of whey caused a much lower degree of norbixin destruction than saturation levels used in experiment I.

Hunter L*A*B*

B* value results were consistent with the annatto extraction results (Table 1-3). b* value measures the degree of blue and yellow in a sample, with a higher value indicating a higher yellow color. A decrease in b* value suggests effective bleaching of norbixin. For experiment I, no differences were found in b* value between temperature treatments of liquid whey ($p<0.05$). As expected, the b* value decreased as ozone exposure time increased ($p<0.05$). The results for retentate were similar, with no difference between temperature treatments. In experiment II, both hydrogen peroxide and ozone significantly reduced b* value of rehydrated WPC80, with hydrogen peroxide reducing b* value more than ozone ($p<0.05$). L*a*b* is a rapid method for measuring color differences in samples, but is less sensitive to changes than direct norbixin extraction and measurement. While no previous study has been performed comparing the decrease in yellowness in liquid whey bleached by HP to liquid whey bleached by ozone, L*a*b* has been extensively used to compare bleaching efficacy of different bleaching agents and conditions (Croissant et al., 2009;

Listiyani et al., 2011; Campbell et al., 2011). The relationship between bleaching efficacy and the decrease in b* is consistent with previously published papers.

Sensory and Volatile Analysis

Lipid oxidation is of primary concern when analyzing flavor of liquid whey. Many lipid oxidation compounds tend to have very low flavor thresholds and exist well above detection levels in dairy products, such as whey (Whitson et al., 2010). Lipid oxidation products are present in fresh Cheddar liquid whey and it is likely that lipid oxidation is initiated during the cheese making process (Tomaino et al., 2004; Liaw et al., 2010). Sensory differences between liquid whey bleached by different bleaching agents have been reported in several studies (Croissant et al., 2009; Campbell et al., 2011; Listyani et al., 2011). Both hydrogen peroxide (250 ppm) and ozone bleaching treatments had a significant effects ($p>0.05$) on flavor profiles of WPC80 (Table 4). Hydrogen peroxide bleaching increased the aroma intensity and cardboard flavor and decreased sweet aromatic flavor ($p<0.05$). Ozone treatment decreased the sweet aromatic flavor and was also unique in its inclusion of flour/pasta and animal flavors in WPC80. The control WPC80 was lower in cardboard and aroma intensity compared to HP bleached WPC80 and higher in sweet aromatic compared to both ozone and HP bleached WPC80 ($p<0.05$). A sweet aromatic flavor and low cardboard flavor in unbleached WPC is consistent with previous studies (Campbell et al., 2011; Jervis et al., 2012). The inclusion of flour/pasta and animal flavors in ozone bleached liquid whey is of note due to its uncommon occurrence in Cheddar WPC80. It is likely that due to its

high oxidative potential ozone is oxidizing components of whey not oxidized by HP, thereby causing unique off flavors not normally seen.

Volatile compound analysis of WPC80 was also distinct (Table 5). Ozone WPC 80 had higher levels of nonanal (11.23 ppb vs 4.14 ppb), e-2-nonenal (0.18 ppb vs 0.12 ppb), butyric acid (0.003 ppb vs ND), and decanal (0.13 ppb vs 0.08 ppb) than the hydrogen peroxide bleached WPC80 ($p<0.05$), while hydrogen peroxide WPC80 was significantly higher in octanal (0.99 ppb vs 0.60 ppb), hexanal (49.37 ppb vs 33.49 ppb), 2-heptanone (0.79 ppb vs 0.37 ppb), and DMDS (3.98 ppb vs 0.16 ppb) ($p<0.05$). In all other compounds, no significant difference was detected between the two WPC80. HP WPC80 was higher than the control unbleached WPC80 in 2-pentylfuran (4.14 ppb vs 2.394 ppb), e-2-nonenal (0.12 ppb vs 0.05 ppb), octanal (0.99 ppb vs 0.27 ppb), hexanal (49.37 ppb vs 19.05 ppb), hexanoic acid (0.15 ppb vs 0.10 ppb), 2-heptanone (0.79 ppb vs 0.44 ppb), DMDS (3.98 ppb vs 0.22 ppb), and phenylethanal (0.21 ppb vs 0.06 ppb). Ozone WPC80 was higher than the control in nonanal (11.23 ppb vs 2.39 ppb), 2-pentylfuran (2.33 ppb vs 0.89 ppb), e-2-nonenal (0.18 ppb vs 0.05 ppb), octanal (0.60 ppb vs 0.26 ppb), hexanal (33.49 ppb vs 19.05 ppb), 1-octen-3-one (0.17 ppb vs 0.09 ppb), phenylethanal (0.24 ppb vs 0.06 ppb). The unbleached control WPC80 was significantly higher than hydrogen peroxide (250ppm) and ozone bleaching treatments in butanal (10.80 ppb vs 7.47 ppb vs 7.46 ppb), pentanal (0.47 ppb vs 0.32 ppb vs 0.33 ppb), b-ionone (0.03 ppb vs 0.008 ppb vs 0.007 ppb), DMS (0.159 ppb vs 0.11 ppb vs 0.08 ppb), diacetyl (0.48 ppb vs 0.09 ppb vs 0.05 ppb), and 2-methylbutanal (10.78 ppb vs 7.35 ppb vs 7.40 ppb) ($p<0.05$). Bleached WPC80 had increased lipid oxidation products

compared to the control unbleached WPC80. While both hydrogen peroxide and ozone increased oxidation in fluid whey and dried WPC80, they were distinct in volatiles and sensory flavors, suggesting a possible difference in bleaching pathway. This is consistent with the descriptive analysis results, which displayed distinct differences in flavor of WPC80. Ozone is likely going beyond lipid oxidation and causing a greater degree of protein degradation. Even at the relatively low levels used (2.2 g/hr/95 L) and with limited bleaching efficacy compared to HP, the oxidative strength of ozone appears to make it less selective in its oxidative activity.

Conclusions

Ozone gas is an effective whey bleaching agent at 200 mg/h/700 mL, but high levels are likely to cause unacceptable off flavor formation in spray dried products based on the descriptive analysis and volatile analysis results of WPC80. Experiment I demonstrated that ozone at or above saturation levels could destroy a high level of norbixin (~63%) in liquid whey. Experiment II showed that reducing ozone to below saturation levels dramatically decreased bleaching efficacy, below that of hydrogen peroxide. Despite drastically reduced bleaching efficacy, volatile compound and sensory analyses demonstrated increased lipid oxidation compounds and off flavors in ozone bleached WPC80 compared to unbleached WPC80. Volatile and descriptive analysis results also suggest a decreased selectivity in the oxidative activity of ozone compared to HP, producing a distinctly different product. It is likely that the degree of off flavor formation caused by the amount of ozone needed to bleach at equivalent levels to hydrogen peroxide would be unacceptable in WPC80.

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TABLES AND FIGURES

Table 1: Experiment I b* values means for liquid whey bleached by ozone at 35°C or 60°C for 15-45 min.

Treatment	b*
35°C 15min whey	14.90[ab]
35°C 30min whey	14.56[bc]
35°C 45min whey	13.17[d]
60°C 15min whey	15.27[a]
60°C 30min whey	14.29[c]
60°C 45min whey	13.04[d]

[a-d] Means in the same column that do not share a common superscript are different ($p<0.05$)

Table 2: Experiment I b* value means for liquid WPC80 retentate (~12% solids) bleached by ozone at 35°C or 60°C for 15-45 min.

Treatment	b*
35°C 15min retentate	38.59[a]
35°C 30min retentate	35.90[b]
35°C 45min retentate	33.93[c]
60°C 15min retentate	38.11[a]
60°C 30min retentate	35.62[b]
60°C 45min retentate	33.47[c]

[a-c] Means in the same column that do not share a common superscript are different ($p<0.05$)

Table 3: Experiment II b* value means for rehydrated (~10% solids)spray dried WPC80 powders samples bleached by ozone at 50°C for 1 hour.

Treatment	b*
Control	17.67^{a}
OZ	15.59^{b}
HP	14.94^{c}

$^{a\text{-}c}$ Means in the same column that do not share a common superscript are different ($p<0.05$)

Table 4: Descriptive analysis means for rehydrated (~10% solids) spray dried WPC80 with or without bleaching.

Treatment	Aroma Intensity	Sweet Aromatic	Cardboard	Flour/Pasta	Animal	Astringent Mouthfeel
Control	2.0[b]	1.5[a]	1.9[b]	ND	ND	2.2[a]
HP 250	2.5[a]	0.5[b]	2.7[a]	ND	ND	2.4[a]
Ozone	1.9[b]	ND	2.0[ab]	1.7[a]	0.7[a]	2.2[a]

[a,b] Means in the same column that do not share a common superscript are different ($p<0.05$)
ND – Not detected
Control – Unbleached WPC80 recirculated for 1 h
HP 250 – WPC80 bleached with 250 ppm hydrogen peroxide for 1 h
Ozone – WPC80 bleached with 2.2 g/h ozone gas for 1 h

Table 5: Relative abundance of selected volatiles (ppb) in rehydrated (~10% solids) spray dried WPC80 with or without bleaching.

Compound	Con	HP	OZ
Nonanal	2.40[b]	4.14[b]	11.23[a]
2-pentylfuran	0.89[b]	2.96[a]	2.33[a]
e-2-octenal	0.28[a]	0.28[a]	0.16[a]
e-2-heptanal	0.54[a]	0.49[ab]	0.28[b]
Butanal	10.80[a]	7.47[b]	73.46[b]
Pentanal	0.47[a]	0.32[b]	0.33[b]
Isobutyraldehyde	0.12[a]	0.09[a]	0.17[a]
e-2-nonenal	0.05[c]	0.12[b]	0.18[a]
Decanal	0.10[ab]	0.08[b]	0.13[a]
b-ionone	0.03[a]	0.01[b]	0.01[b]
Octanal	0.27[c]	0.99[a]	0.60[b]
1-octen-3-ol	0.53[a]	0.47[a]	0.50[a]
Hexanal	19.05[c]	49.37[a]	33.49[b]
Heptanal	1.03[a]	0.89[ab]	0.65[b]
Butyric Acid	0.01[a]	ND	ND
Hexanoic Acid	0.01[b]	0.15[a]	0.13[ab]
DMS	0.16[a]	0.11[b]	0.08[b]
Diacetyl	0.48[a]	0.09[b]	0.05[b]
3-methylbutanal	0.43[a]	0.23[b]	0.32[ab]
2-methylbutanal	10.78[a]	73.35[b]	7.40[b]
Ethylbutyrate	0.15[a]	0.09[b]	0.11[ab]
2-heptanone	0.44[b]	0.79[a]	0.37[b]
DMDS	0.22[b]	3.98[a]	0.16[b]
DMTS	0.06[a]	0.28[a]	0.07[a]
1-octen-3-one	0.09[b]	0.12[ab]	0.17[a]
ethyl hexanoate	0.07[a]	0.02[a]	0.03[a]
Phenylethanal	0.06[b]	0.21[a]	0.24[a]
Methylmercaptan	0.05[a]	0.06[a]	0.05[a]

[a,b] Means in the same row that do not share a common superscript are different ($p<0.05$)

ND – Not detected

Con – Unbleached WPC80 recirculated for 1 h

HP – WPC80 bleached with 250 ppm hydrogen peroxide for 1 h

OZ – WPC80 bleached with 2.2 g/h ozone gas for 1 h

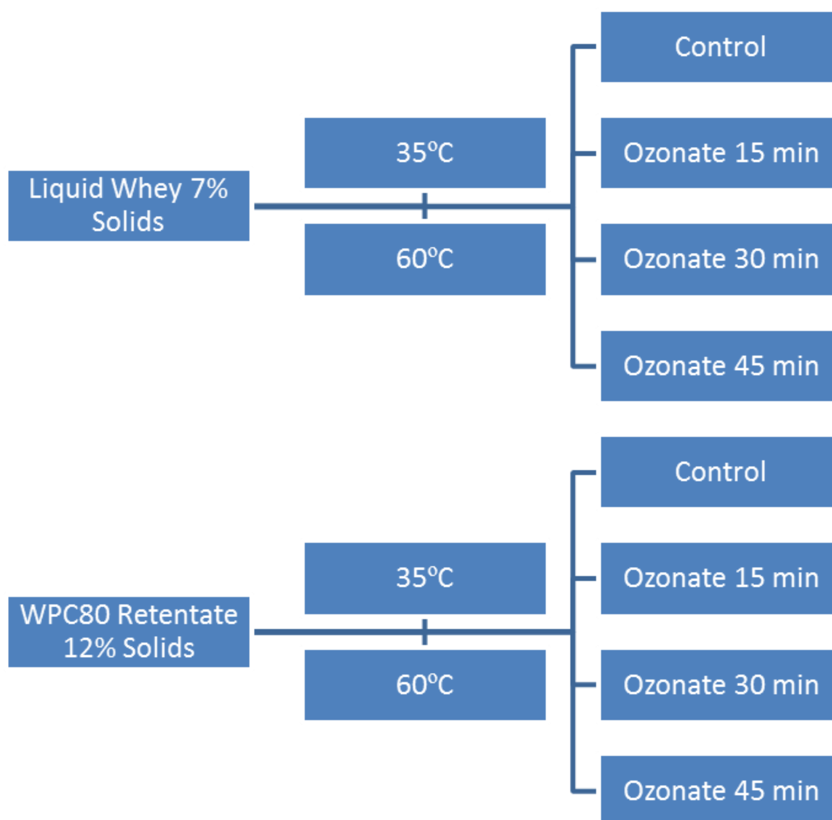

Figure 1: Experiment I. The effect of solids level and temperature on the efficacy of ozone bleaching of liquid whey.

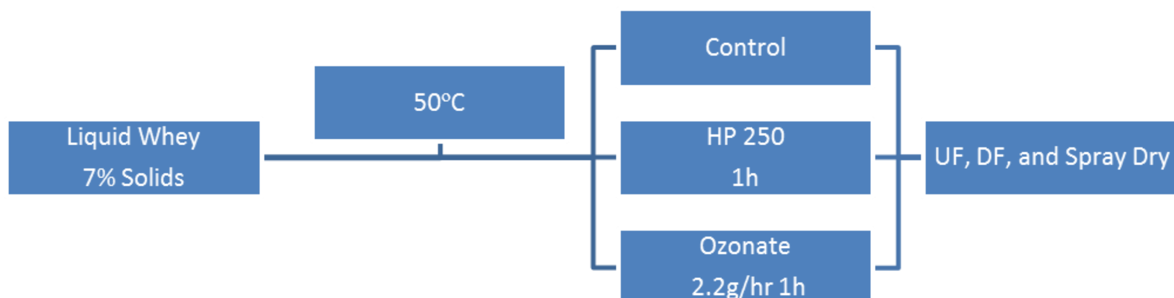

Figure 2: Experiment II. A comparison of ozone bleaching to industry standard bleaching agent, hydrogen peroxide, of liquid whey followed by manufacture of spray dried WPC80.
HP 250 = hydrogen peroxide at 250 ppm

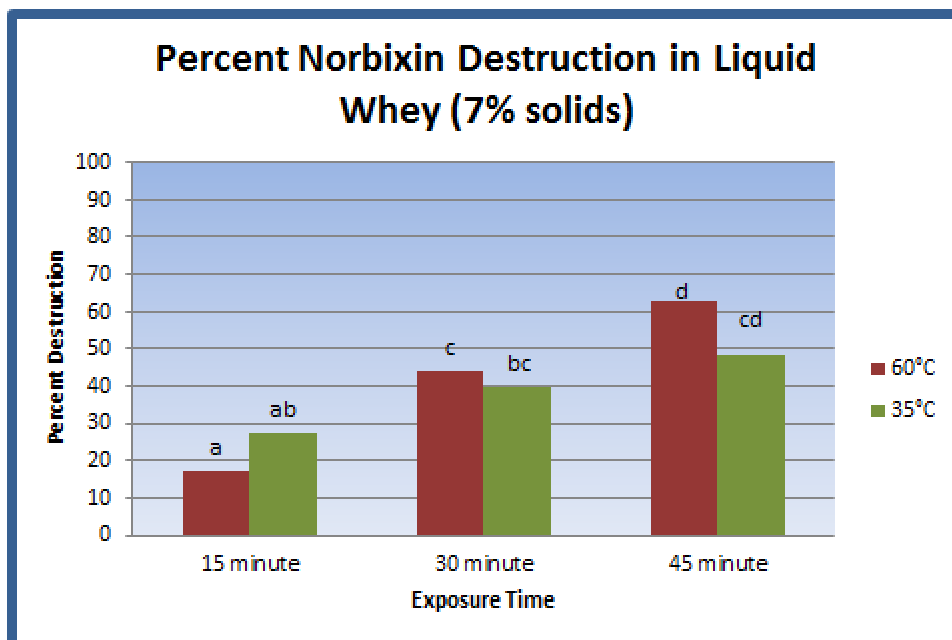

Figure 3: Experiment I percent norbixin destruction in liquid whey (~7% solids) by ozonation (200mg/h) at 35 or 60ºC for 15, 30, or 45 min.

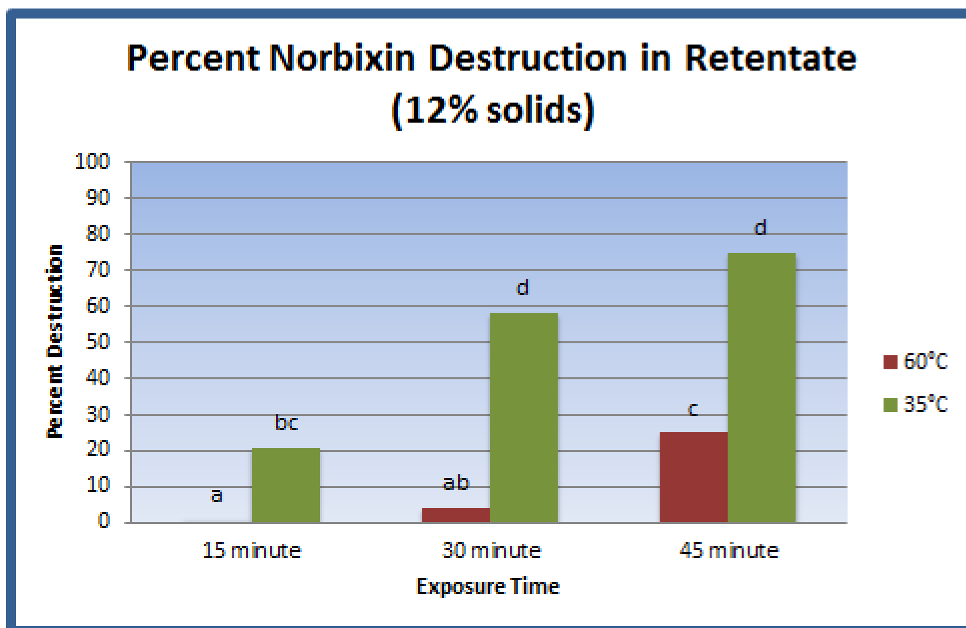

Figure 4: Experiment I percent norbixin destruction in liquid WPC80 retentate (12% solids) by ozonation (200mg/h) at 35 or 60ºC for 15, 30, or 45 min.

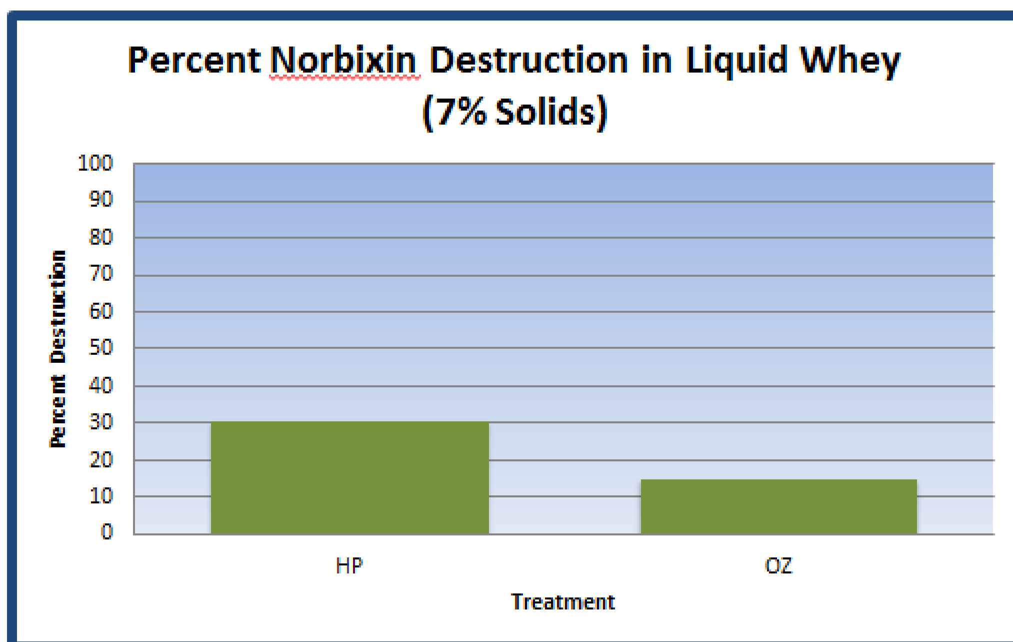

Figure 5: Experiment II percent norbixin destruction in liquid whey (7% solids) caused by ozonation (2.2g/h) at 50°C for 1h.

Norbixin Partitioning in Full Fat and Fat Free Cheddar Cheese

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

ABSTRACT

Whey protein is an important commercial product for the dairy industry and a large portion of it is manufactured from colored Cheddar cheese whey. The Cheddar cheese colorant, annatto, is also present in whey and must be removed by bleaching. A better understanding of the primary colorant in annatto, norbixin, is crucial in order to produce effective methods for its removal from liquid whey. The objective of this study was to determine norbixin partitioning in cheese and whey from full fat and fat free Cheddar cheese. Full fat and fat free Cheddar cheeses and wheys were manufactured from colored pasteurized milk in quadruplicate. Three different norbixin levels (7.5, 15, and 30 mL annatto/454 kg milk milk) were used for full Cheddar cheese manufacture and one norbixin level was evaluated in fat free Cheddar cheese (15 mL annatto/454 kg milk). Norbixin extractions were performed on the milk, the unseparated cheese whey, and the pressed cheese. Norbixin was extracted by solvent extraction and column purification and quantified by high performance liquid chromatography. An average of 10.4% of the norbixin added to the full fat cheese milk was recovered in the whey and 82.0% was recovered in the cheese. Similarly, 11.9% of the norbixin added to skim milk was recovered in the skim milk cheese whey and 76.2% was recovered in the fat free cheeses. Level of norbixin addition to cheese milk and fat content had no impact on norbixin recovery in cheese or whey ($p>0.05$). These results suggest that fat content has little impact on norbixin binding or entrapment during the cheese make process.

Introduction

Demand for whey protein as a value-added product has steadily increased over the years (Tunick 2008). According to the USDA, more than 183.6 million kg of WPC was produced in 2010 (USDA 2012). Whey and dried whey ingredients are an exceptionally important commodity for the dairy industry. Whey is appealing in many ingredient applications due to its various functional properties, including water binding ability, gelation, foaming, buffering, and emulsifying properties (Davis and Foegeding, 2007).

In 2010, US production of Cheddar cheese was more than 1.4 billion kg (USDA 2012). In the US, a large majority of dried whey protein is manufactured from Cheddar cheese whey colored by annatto, a coloring agent extracted from the seed of the *Bixa orellana* shrub (Scotter 2009). The major carotenoids responsible for the yellow color of annatto are norbixin and bixin, although norbixin is the primary carotenoid used in cheese making (Giuliano et al., 2003). While annatto itself has been proven to have no direct effect on the flavor of dried whey protein concentrate (WPC), it must be removed from fluid whey to produce a desirable spray dried product (Campbell et al., 2010). Currently, benzoyl peroxide (BP) and hydrogen peroxide (HP) are the two chemical bleaching agents approved by the FDA (US FDA 2009). The use of chemical bleaching agents has many detrimental effects including off flavor development, chemical residues, and changes in whey protein functional properties (Listiyani et al., 2011; Kang et al., 2012; Jervis et al., 2012).

Off flavor development in WPC, due to lipid oxidation is of special concern when dealing with oxidative chemical bleaching agents. Aldehydes are primarily responsible for

off flavors in dried whey proteins (Carunchia Whetstine et al., 2005; Wright et al., 2009, Whitson et al., 2010). Common off flavors caused by lipid oxidation products in WPC are cardboard, cabbage, and fatty-oxidized flavors (Listiyani et al., 2011; Whitson et al., 2010). Investigating a method to reduce or eliminate unnecessary and harsh processing steps is important to add overall value to WPC as an ingredient. To be used in a variety of different products, it is increasingly important to produce whey proteins with as bland a flavor as possible (Drake et al., 2009; Wright et al., 2009). It is important to know the effects of various processing steps on norbixin concentration of whey. Many studies have been conducted on the effects of whey processing on whey protein flavor and bleaching efficacy (Croissant et al., 2009; Listiyani et al., 2011; Listiyani et al., 2012; Campbell et al., 2010), but to our knowledge, a study of the norbixin partitioning differences between cheeses with differing fat levels has not been performed. Specific knowledge on norbixin partitioning will enable development of optimal bleaching conditions or alternative bleaching approaches.

Relatively little is known of the partitioning and binding characteristics of norbixin during the cheese making process. Previous studies have postulated that approximately 20% of the norbixin added during the cheese making process partitions into the whey, but little research has been performed in the past 30 years which proves this hypothesis (Barnicoat 1950, Chapman et al., 1980). While it is often assumed that norbixin is contained within the serum phase of the whey it is also thought that it may be bound to the retinol-binding site of β-LG (Cho et al., 1994; Govindarajan and Morris, 1973; Hammond et al., 1975; Zhu and Damodoran, 2012). Recently it has been postulated that annatto associates with the milk fat

globule membrane portion of whey (Zhu and Damodoran, 2012). The objectives of this study were to investigate norbixin partitioning into cheese and liquid whey using modern methods and instrumentation, in order to better understand norbixin characteristics during the cheese making process.

Materials and Methods

Experimental Design Overview

Two experiments were conducted in this study. For experiment I, three sets of Cheddar cheese were produced using unseparated whole pasteurized milk with three levels of annatto added used (7.5 mL, 15 mL and 30 mL/455kg of milk). The purpose of experiment I was to determine norbixin partitioning between cheese and whey when differing amounts of annatto was added. For experiment II, Cheddar cheese was manufactured from pasteurized, fat separated skim milk and 15 mL/455kg of milk annatto added. The purpose of experiment II was to determine if there were differences between norbixin in cheese and whey in fat free and full fat cheeses. Full fat and fat free Cheddar cheeses were manufactured in the NCSU Dairy Pilot Plant. Samples taken for norbixin extraction and measurement included milk after addition of annatto, Cheddar cheese and unseparated, unpasteurized whey. Four trials of all four cheeses were conducted.

Cheddar cheese and liquid whey production

Four sets of cheeses were manufactured for each experiment. For experiment I, raw whole milk, 98 kg, was obtained from the North Carolina State University Dairy Research and Education Farm (Raleigh, NC). Milk was vat pasteurized (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC) at 63°C for 30 min. After pasteurization, milk was immediately cooled to 31°C and inoculated with freeze dried lactic acid starter culture (50 DCU/454 kg, Choozit MA 11, Danisco, New Century, NJ). Milk was agitated and ripened for 30 min. After the initial ripening period, calcium chloride solution (50% (w/v), Dairy Connection Inc., Madison, WI) was added (0.39 mL/kg milk) as was double strength annatto color (4% norbixin w/w, Danisco, New Century, NJ) (7.5, 15, or 30 mL/454 kg milk, diluted 20 times in deionized (DI) water for full fat, 15 mL/454 kg milk, diluted 20 times in DI water for fat free cheese). Agitation and ripening of milk continued for another 30 min. After this period, milk was coagulated with double strength recombinant rennet (Dairy Connection Inc., Madison, WI; 0.09 mL/kg milk, diluted 80 times in DI water). The milk was allowed to coagulate for 30 min without agitation. The coagulum was cut with 0.95-cm wire knives and allowed to rest for 5 min. After resting, the curds and whey were gently stirred for 10 min. The curds and whey were slowly heated from 31°C to 39°C over a period of 30 min during which pH and titratable acidity were monitored. Whey was drained from curds at pH 6.40. The cheese make procedure for experiment II was identical except that after pasteurization, hot milk was separated in a hot bowl separator (Model JF 125 EAR, Clair, Althofen, Austria) to produce skim milk and cream. The separated skim

milk was then immediately cooled to 31°C and inoculated with freeze dried lactic acid starter culture. Remaining cheese make steps were identical to those performed with the full fat cheese make.

For both experiment I and II, once a pH of 6.40 was reached, whey was strained to remove cheese particles and a 1 L sample was taken for analysis. The cheddaring methods were modified from Nair et al. (2004). During Cheddaring, trenched curd loaves were turned every 20 min to a final pH of 5.2 before milling. Curd was milled manually with knives and salted (2.7% salt w/w based on curd weight). Salted milled curds were pressed for 16 h at 2.8 kg/cm^2 (40 psi) and then vacuum sealed and stored at 4C. All cheeses were manufactured in quadruplicate.

Proximate Analysis

The solids of liquid whey, full fat and fat free cheese were conducted using a forced-air drying oven (AOAC, 2000; method number 990.20; 33.2.44). Total nitrogen of liquid whey, full fat and fat free cheese was determined by the NCSU Analytical Services Laboratory (Raleigh, NC) using the Kjeldahl method (APAC, 2000; method number 991.20; 33.2.11) (AOAC Method 920.123); crude protein for each was calculated by multiplying the total nitrogen by 6.38. Total fat content of the liquid whey was determined by Modified Mojonnier ether extraction (AOAC, 2000; method number 989.05; 33.2.26 and AOAC, 2000; method number 932.06; 33.5.08). Total fat content of full-fat Cheddar cheese was determined by Mojonnier method (AOAC 933.05). Fat in the fat free cheese was determined

by the same method but with an additional 2 mL of 12M HCl (VWR International, West Chester, PA) acid for enhanced digestion (Nelson and Barbano, 2004) . Mineral analysis of the full-fat and fat free cheeses (phosphorus and sodium) was performed in duplicate by the NCSU Analytical Services Laboratory (Raleigh, NC) using a dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009). Sodium chloride in the cheese was determined using a salt analyzer (SAT-500, DKK-TOA Corp., Tokyo, Japan).

Norbixin Extraction and Quantification

Norbixin extraction was performed on full-fat and fat free milk, unseparated and unpasteurized liquid whey, and full-fat and fat free cheeses using the method performed by Li et al. (2012) and Campbell et al. (2012). All samples (6 mL liquid whey, 2 mL full-fat and fat free milk, 0.5g full-fat and fat free cheese) underwent a solvent extraction followed by norbixin quantification by HPLC (Waters 1525 Binary Pump, Waters, Milford, MA). Full-fat and fat free cheese samples were first finely grated and then underwent the same solvent extraction. All samples were extracted in duplicate. Norbixin was quantified using the method performed by Campbell et al. (2012).

Quantification of Norbixin in Annatto Solution

The annatto solution used to color the cheeses in all cheese makes was diluted 1:100,000 (w/v) in 3:7 glacial acetic acid: methanol (w/v) and injected onto the HPLC as described by Li et al. (2012).

Mass Balance of Norbixin

A mass balance of norbixin in the full-fat and fat-free milk, cheese, and whey was performed. When performing a mass balance of a carotenoid using an extraction procedure involving SPE and an HPLC, one must take into account every step of the extraction procedure. For experiments I and II, the weight of the whole or skim milk and weight of the cheese was recorded. The weight of the whey portion was extrapolated by subtracting the final weight of the cheese from the total weight of the milk. The mass balance of the system is given by:

$$M_{nor} = (CV_{post}V_{tpre}M_{tot}) / (V_{cpre}M_{s})$$

Where M_{nor} is the total mass (µg) of norbixin in the milk, cheese, or whey respectively, C is the norbixin concentration (µg/L) of the SPE solution injected onto the HPLC, V_{post} is the total volume (L) of the SPE solution injected onto the HPLC, V_{tpre} is the total volume (L) of the extract solution pre-placement onto the SPE column, M_{tot} is the total mass (kg) of milk, whey, or cheese used in the cheese make procedure, V_{cpre} is the volume (L) of the extract

solution (pre-SPE) placed onto the SPE column, and M_s is the mass of the sample of milk, whey, or cheese from which the norbixin is extracted. The function of this formula is essentially to find the mass of the norbixin extracted from a sample and calculate the total norbixin in the complete mass of milk, whey, or cheese from which the sample was taken. Exact weights and volumes are critical when performing this type of mass balance as error from the extraction procedure has the potential of affecting the end result. However, the extraction efficiency can be taken into account and used to calculate a truer mass of norbixin in the sample.

Using the previous equation one may calculate the mass of norbixin in milk. The relative difference between the calculated mass of norbixin in milk, found through the extraction procure, and the actual amount of norbixin added to milk gives the extraction efficiency. Extraction efficiency was determined by the following equation:

$$E = (M_{ann})100 \, / \, M_{nor}$$

where E is percent extraction efficiency, M_{ann} is the mass (μg) of norbixin added to the milk, and M_{cal} is the mass (mg) of norbixin calculated to be in the milk through the extraction procedure.

Correcting for extraction efficiency allows one to find a true norbixin loss during the cheese make procedure. It is important to track that loss, as differences in loss between trials may indicate differences in the cheese make procedure. It is also important to know if

differences in norbixin addition or fat content, as determined by experiment I and II, have an effect on the amount of norbixin lost during the cheese make procedure. As stated earlier, norbixin is sensitive to both heat and light, both of which it was exposed to during the pilot scale cheese manufacture. As such, a modest amount of norbixin destruction during cheese manufacture was expected (de Oliveira et al., 2007). Percent norbixin loss was determined by the following equation:

$$L = M_{ann} - ([M_{wnor} + M_{cnor}]100 / E)$$

where L is percent norbixin loss, M_{ann} is the mass (μg) of norbixin added to the milk, M_{wnor} is the total mass (μg) of norbixin in whey, M_{cnor} is the total mass (μg) of norbixin in the cheese, and E is the percent extraction efficiency. Using the percent norbixin loss during the procedure, one may very simply determine the mass of the norbixin lost using the following equation:

$$M_{lnor} = LM_{ann}$$

where M_{lnor} is the mass (μg) of norbixin lost, L is the percent norbixin lost, and M_{ann} is the mass (μg) of total norbixin added to the milk.

With simple and straightforward calculations it is very possible to accurately determine the partitioning characteristics of norbixin by following it through the cheese make

process. It is critical that one determine the accurate weights and volumes associated with the extraction procedure, as even small variations allow for a large degree of error.

Statistical Analysis

Data was analyzed by analysis of variance with means separation (ANOVA) (XLSTAT, version 2011.5.01, Addinsoft, New York, NY). Differences between the sample means were analyzed by Tukey's honestly significant difference (HSD).

Results

Proximate Analysis

The composition of the whole milk was consistent ($p>0.05$). Fat averaged 3.70% ±0.25% (w/w), and solids was 12.03% ±0.26% (w/w). In liquid whey from whole milk, fat averaged 0.26% ±0.15% (w/w), and solids was 6.61% ±0.17% (w/w) ($p>0.05$). Sodium (0.59±0.01%) in liquid whey was also not distinct ($p>0.05$). In full fat Cheddar cheeses, fat averaged 33.51±2.20%, and solids 63.57±1.74% ($p>0.05$). Sodium chloride in the cheese was 1.35±0.09%.

Fat content of fat free cheese, milk, and whey were at levels below the requisite 0.5g/serving necessary to be considered legally "fat free" by the code of federal regulations (US FDA 2011c). The composition of the fat free milk averaged 0.13% ±0.08% (w/w) fat, and solids was 8.98% ±0.20% (w/w) ($p>0.05$). The composition of the whey produced in the fat free Cheddar cheese makes averaged 0.03% ±0.02% (w/w) fat, and solids was 6.54%

±0.08% (w/w) ($p>0.05$). Sodium (0.55±0.01%) was measured and not distinct ($p>0.05$). In fat free Cheddar cheese, fat averaged 2.84±0.78%, and solids 54.79±2.2%. Sodium chloride in the cheese was found to be 1.62±0.11%.

Norbixin Extraction and Mass Balance

Extraction efficiency from milk was 90% in the 1/2x trials, 89% in the 1x trials, 89% in the 2x trials, and 88% in the fat free trials (Table 1) and was not different across all trials ($p>0.05$). The overall average extraction efficiency was 89%. This efficiency is comparable to that reported by Bareth et al. (2002). Bareth et al. (2002) reported a recovery of 91.0% ± 2.4% norbixin from Cheddar cheese. Loss in efficiency is likely due to several factors, including human error, incomplete solvent separation of norbixin from sample, and SPE column inefficiency. The purpose of extracting norbixin from the milk after addition of annatto was to find the extraction efficiency and correct for error caused by lack thereof. The milk after addition of annatto was used to determine the overall extraction efficiency for each individual trial due to its unadulterated state, whereas both cheese and whey go through periods of pH change, mechanical work, and heat and light exposure.

With extraction efficiency taken into account 9.4% of annatto leached into the whey for the 1/2x treatments, 12% during the 1x treatments, 10% during the 2x treatments, and 12% during the fat free treatments (Table 1). These were found to not be different ($p>0.05$). The average partition rate for norbixin into whey across all trials was 10%. 83% of norbixin was recovered in 1/2x cheese, 81% in 1x cheese, 82% in 2x cheese, and 76% of norbixin

added to milk was recovered in the fat free cheese (Table 1). Again, these values were not different ($p>0.05$). An average of 81% of norbixin added to milk was recovered in the cheese. These values are contrary to the widely held belief that 20% of norbixin added to cheese milk ends up in the cheese whey (Barnicoat, 1950; Chapman et al., 1980).

A norbixin loss of 7.4%, 4.8 mg, was calculated in the 1/2x trials, 7.8%, 10.01 mg in the 1x trials, 7.5%, 19.44 mg in the 2x trials, and 12%, 12.92 mg norbixin was lost during the fat free trials (Table 1). More norbixin was lost during the fat free cheese make procedure than all three full fat cheese treatments ($p<0.05$). Norbixin loss is likely due to heat and light abuse during the cheese making process. It is well established that both light and heat have destructive effects on norbixin (de Oliveira et al., 2007) and these are the likely culprits of norbixin destruction during the cheese make procedure. The greater norbixin loss during fat free cheese manufacture may be due to the lack of opacity in the fat free whey due to lack of fat. Opacity in the whey, caused by fat, may protect norbixin from exposure to light, while the clearer fat free whey has a greater portion of norbixin directly exposed to light during the cheese making process. The norbixin loss relationship between full fat treatments is linear likely due to the fact that the same proportion of norbixin is exposed to light during the cheese make procedure, irrespective of total amount of norbixin added.

While percent norbixin partitioning into whey does disagree with previous research stating that 20% of norbixin leaches into whey (Barnicoat, 1950; Chapman et al., 1980), the research done in this paper does not completely disagree with that which was previously shown. Chapman et al. (1980) stated that anywhere between 18 and 26 percent of norbixin

went into the whey, but was only taking into account the recovered norbixin. The current study reports percent norbixin in whey compared to the amount initially added to milk. Over the course of cheese manufacture norbixin is lost to heat, light, and extraction inefficiencies. If only recovered norbixin was taken into account, a higher percentage of norbixin would be reported leached into whey. Again, the objective of the current study was to investigate norbixin partitioning into cheese and liquid whey using modern methods and instrumentation, in order to better understand norbixin characteristics during the cheese making process. Chapman et al. (1980) stated that the annatto solution used in their study was a relatively impure preparation. Differences in instrumentation and materials, especially the annatto used, could very well explain the differences in findings (10% norbixin partition rate into whey as opposed to 18-26% norbixin partition rate into whey) between this study and those performed 30 years in the past. It is possible that the current study and Chapman et al. (1980) are both correct, but represent a shift in norbixin partition rate due to differences in the annatto solution.

Conclusions

The solvent extraction efficiency of norbixin from milk was not affected by added annatto levels or by fat content. In full fat Cheddar cheese, a smaller proportion of the annatto added to the cheese milk is present in the cheese whey than was previously thought. Only norbixin loss during cheese manufacture was distinct between full fat and fat free cheeses. A higher percent norbixin loss in fat free cheese may be due to the loss of opacity to a lack of fat,

meaning that the norbixin was exposed to light to a higher degree during fat free cheese manufacture than norbixin in full fat cheese manufacture.

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TABLES AND FIGURES

Table 1: The percent total recoverable norbixin partitioned into the whey and cheese and the percent total norbixin recovered by solvent extraction. Comparisons were made between treatments full fat 1/2x (7.5mL annatto/1000lbs milk), full fat 1x (15mL annatto/1000lbs milk), full fat 2x (30mL annatto/1000lbs milk), and fat free 1x (15mL annatto/1000lbs milk).

Treatments	Extraction Efficiency (%)	Norbixin Loss (mg)	Norbixin Loss (%)	Norbixin in Whey (%)	Norbixin in Cheese (%)
1/2x	89.54a	4.8	7.44a	9.39a	83.17a
1x	89.26a	10.01	7.76a	11.48a	80.76a
2x	88.62a	19.44	7.54a	10.44a	82.02a
Fat Free 1x	88.18a	12.92	11.96b	11.85a	76.18a

[a-b] Means in the same column that do not share a common superscript are different ($p<0.05$)