ABSTRACT

JERVIS, SUZANNE MARIE. Determining the Impact of Bleaching on the Flavor and Functionality of 80% Whey Protein Concentrate and Determining the Drivers of Choice of Latte-style Coffee Beverages by Choice Based Conjoint Analysis. (Under the direction of Dr. MaryAnne Drake).

Dairy products are ubiquitous in food and beverage applications. Two studies were designed around two different dairy products (80% whey protein concentrate and milk). The first study was designed to determine the impact of bleaching with benzoyl peroxide (BP) or hydrogen peroxide (HP) has on the flavor and functionality of 80% whey protein concentrate (WPC80). Eighty percent whey protein concentrate is utilized for its nutritional value and high functionality. Liquid whey used to make WPC80 largely comes from colored Cheddar cheese manufacture and must be bleached. Norbixin is the carotenoid of interest in whey bleaching. Norbixin recovery was higher for HP bleached WPC80 than BP bleached WPC80 ($P<0.05$). The HP WPC80 was higher in lipid oxidation and protein degradation volatiles as well as cardboard and fatty flavors ($P<0.05$). The BP WPC80 gelled when heated for 10 min; HP WPC80 gelled when heated for 20 min at pH 7. Overall HP bleaching caused more lipid oxidation products and subsequent off flavors than BP bleaching. However, heat stability of WPC80 was enhanced by HP bleaching compared to control or BP bleached WPC80 which may be a function of denaturation of sulfur containing amino acids.

One of the most important product applications of milk is in coffee beverages, where it is utilized as a lightener and flavor source for latte-style coffee beverages. A latte-style coffee beverage is any beverage made with espresso, a milk or a milk-type lighter, and may have additional flavor. The objective of this study was to determine what factors were most influential in consumer choice of latte-style coffee beverages using ethnography and a choice

based conjoint study. Ethnographical data was collected at four of the top producers of latte-style beverages. Attributes measured by the conjoint survey included location of purchase, milk-type, fat content, sweetener-type, and additional flavor. Consumer responses (n=721) from the conjoint survey showed that the most important attributes in determining latte beverage purchase intent were location and milk-type, followed by fat content, sweetener, and additional flavor. Segmentation of respondents based upon patterns in utility scores showed three distinct groups. Segment 1 (n = 185) was influenced by milk-type and sweetener-type. Segment 2 (n = 200) was influenced by a coffee house. Segment 3 (n=336) was calorie and health conscious. Self-defined lactose intolerant (n = 117) consumers preferred a lactose free dairy milk over a lactose free non-dairy milk lightener.

Determining the Impact of Bleaching on the Flavor and Functionality of 80% Whey Protein Concentrate and Determining the Drivers of Choice of Latte-style Coffee Beverages by Choice Based Conjoint Analysis

by
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BIOGRAPHY

Suzanne Marie Jervis was born on August 26th, 1980 to Robert Joseph Gavula and Barbara Marie Gavula. Suzanne grew up in the Pittsburgh Pennsylvania area but moved around quite frequently throughout Pennsylvania and Connecticut. Suzanne attended the University of Pittsburgh in the fall of 1998 and earned a Bachelors of Science degree in Chemistry with a minor in Education. Suzanne went on for an additional year to earn her Secondary Education Teaching Certificate from the University of Pittsburgh. During that same year Suzanne married Matthew Gabriel Jervis on June 14th, 2003.

Suzanne and Matthew both began working as teachers for Caesar Rodney School District in Wyoming DE. Suzanne taught high school Chemistry, Physical Science, and started the AP Chemistry program. Suzanne taught at Caesar Rodney for three years before accepting a position as a Chemistry Teacher at Upper Darby High School in Upper Darby Pennsylvania. After completion of one year at Upper Darby Suzanne left that position for family reasons, and moved to Pittsburgh where she began working as a Research Scientist for H.J. Heinz Company. After completion of one year with the company Suzanne and family relocated to Raleigh North Carolina for a job opportunity for Matthew. Shortly thereafter, Suzanne began working for Nomacorc LLC as a Sensory Analyst. During that time, the 2007 recession hit and Suzanne was let go from her position. Suzanne took that opportunity to go back to school and earn a Masters in Food Science degree which had been a goal of hers since her experience at Heinz.

Suzanne began her Masters career at North Carolina State University under the direction of Dr. MaryAnne Drake studying Sensory and Flavor Chemistry. Suzanne plans to

continue on to earn a PhD in Food Science with specific focus on Sensory and Consumer Studies.

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To my lab mates, you are wonderful young people who kept me feeling young, and old at times too. Thank you for collaborating with me on projects, ideas, innovations; you will all be great successes some day.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1.

Chapter 2.

Chapter 3.

CHAPTER 1: THE IMPACT OF IRON ON THE BLEACHING EFFICACY OF HYDROGEN PEROXIDE IN LIQUID WHEY SYSTEMS

The Impact of Iron on the Bleaching Efficacy of Hydrogen Peroxide in Liquid Whey Systems

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Abstract

Whey is a value added product that is utilized in many food and beverage applications for its nutritional and functional properties. One of the primary sources of whey is from colored Cheddar cheese manufacture which contains the pigment annatto resulting in a characteristic yellow colored Cheddar cheese. The color translates through to the liquid whey and must be bleached. Hydrogen peroxide and benzoyl peroxide are two commercially approved chemical bleaching agents for liquid whey. Concerns regarding bleaching efficacy, off-flavor development and functionality changes have been previously reported for whey bleached with hydrogen peroxide and benzoyl peroxide. It is very important for the dairy industry to understand how bleaching can impact flavor and functionality of dried ingredients. Understanding the mechanism of bleaching for benzoyl peroxide and hydrogen peroxide will aid in this effort. Currently, the precise mechanisms of off-flavor development and functionality changes are not entirely understood. Iron reactions in a bleached liquid whey system may play a key role. Reactions between iron and hydrogen peroxide have been widely studied since the reaction between these two relatively stable species can cause great destruction in biological and chemical systems. The actual mechanism of the reaction of iron with hydrogen peroxide has been of great controversy in the chemistry and biological community. The precise mechanism for a given reaction can vary greatly based upon the concentration of reactants, temperature, pH, and addition of biological material. In this review some hypotheses for the mechanisms of iron reactions that may occur in fluid whey which may impact bleaching efficacy, off-flavor development, and changes in functionality are presented.

Keywords

Iron, Fenton Reaction, Whey, Hydrogen Peroxide, Lactoperoxidase

Introduction

Whey is a value added product that has found widespread use in the functional and nutritionally fortified food and beverage market due to its high protein content and functional properties. The appealing functional properties of whey protein include solubility, water binding ability, gelation, foaming, buffering, and emulsification (Davis and Foegeding 2007). Whey is the liquid by-product of the cheese making process which contains all of the lactose, minerals, and proteins that do not end up in the final cheese product. The principal whey proteins are β-lactoglobulin and α-lactalbumin which account for approximately 80% of the total whey proteins. The other proteins, (in order of decreasing concentration), include bovine serum albumin, immunoglobulins, proteose peptones, soluble caseins, and a variety of minor proteins (lactoferrin, lactoperoxidase, etc) (Schmidt 1983). Whey can be further processed and spray dried into whey protein concentrates (WPC) which can range from 35-89% protein and whey protein isolates which contain 90% of greater protein (USDEC 2008).

Whey from Cheddar cheese production is one of the most common fluid whey sources. Cheddar cheese is largely colored with annatto which is a GRAS pigment from the tropical shrub *bixa orellana*, used to give the Cheddar cheese its characteristic yellow color. Annatto is comprised of two carotenoid pigments, oil soluble bixin and water soluble norbixin. Norbixin is the pigment of interest in coloring Cheddar cheese and it is also the pigment of interest in whey bleaching as the yellow color translates through to the drained whey. Colored Cheddar whey is bleached with either hydrogen peroxide (HP) or benzoyl

peroxide (BP), two commercially approved chemical bleaching agents. BP has GRAS status and has no usage limit but is generally not added above 0.01% or 100 ppm. HP also has GRAS status with a maximum usage level in annatto colored whey of 0.05% or 500 ppm (Kang and others 2010). Differences in the bleaching efficacy of BP and HP in liquid whey systems has been previously reported (Croissant and others 2009; Listiyani and others 2011a; Jervis and others in press). It is important for the dairy industry to maximize bleaching efficacy with minimal effects on the flavor and protein integrity. Whey proteins ideally should have a bland flavor to enhance their ability to be used in a variety of food and beverage products (Drake and others 2009). When whey is bleached, off-flavors can develop and there may be changes in functionality of spray dried finished products. Flavor development from the processing of liquid whey translates into the spray dried product and these flavors can carry through to ingredient applications (Drake 2006; Drake and others 2009; Wright and others 2009). Croissant and others (2009) reported that HP bleached liquid whey and WPC70 from HP bleached whey had higher concentrations of lipid oxidation compounds compared to BP bleached liquid whey and WPC70 from BP bleached whey. Jervis and others (in press) reported distinct volatile and sensory profiles for WPC80 from unbleached, HP bleached, and BP bleached whey. Listiyani and others (2011a) likewise confirmed that volatile and sensory profiles of unbleached, HP bleached and BP bleached WPC34 were distinct.

Bleaching may affect flavor, but is also important in functionality. Grindrod and Nickerson (1966) reported that skim milk had decreased whey protein nitrogen and increased non-protein nitrogen after exposure to a 0.5% and 1.0% HP solution at 49.9C. Increased HP

concentration resulted in an increase in NPN. Cooney and Morr (1971) reported that proteose peptones were the most susceptible to alteration by HP followed by immunoglobulins, β-lactoglobulin and bovine serum albumin; α-lactalbumin was the least susceptible to HP. BP bleached Blue cheese milk was previously reported to have altered electrophoretic patterns of the whey proteins (Washam et al., 1974). This effect was amplified when the cheesemilk was heated.

It is of key importance for the dairy industry to gain the highest bleaching efficacy with minimal off-flavor development and protein conformational changes. Understanding the mechanisms of bleaching and the factors that can affect bleaching and subsequent reactions is of great importance.

Mechanisms of Bleaching

Chemical peroxide bleaching occurs when a radical formed by the breaking of a peroxide bond attacks a conjugated double bond of a color forming compound known as a chromophore rendering the compound colorless (Kang and others 2010; Dannacher 2006). Typically the chromophores to be destroyed consist of extended conjugated π systems (Dannacher 2006). Radicals can attack bonds in one of three ways; hydrogen abstraction, β-scission, and addition however only hydrogen abstraction and addition will directly result in a loss of color (Figure 1) (Sanchez and Myers 2000).

BP and HP are peroxides characterized by the weak O-O peroxide bond. The reactivity of peroxides proceeds in most cases by the hemolytic cleavage of the O-O bond producing free radicals (Sanchez and Myers 2000; Benassi and others 1993). The breaking of the peroxide bond occurs by thermal decomposition or degradation by metal ions. When

the peroxide bond dissociates/decomposes it results in the formation of two peroxide radicals. In order to compare the reactivity of different peroxides it is important to know the bond dissociation energy (BDE) of the O-O bond. The kinetic stability of the radical is also important however the BDE represents the basic chemical behavior of peroxides (Benassi and others 1993).

Benzoyl Peroxide and Hydrogen Peroxide

BP is classified as a diacyl peroxide (ROOR') with the molecular structure as shown in Figure 2. Diacyl peroxides have low O-O bond dissociation energy and are among the best radical-forming compounds. Benassi and others (1993) reported diacyl peroxides to have a higher bond dissociation energy for R-O when R is a phenyl group. This results in breakage of the O-O peroxide bond instead of the R-O bond yielding two benzoyl radical molecules. Because of the low bond dissociation energy, not as much thermal energy is required to degrade BP into two BP radicals. Diacyl peroxides are ideal agents for bleaching because of the lesser amount of energy needed for bond dissociation (Klenk and others 2005). BP can therefore have bleaching efficacy at lower temperatures than other peroxides with higher BDE given no other outside factors for stimulating peroxide bond dissociation (catalysis by metal ions). The bond dissociation energy of the O-O bond has been estimated for diacyl peroxides as 36.9kcal/mol at 298K (Bach and others 1996). Diacyl peroxides decompose faster in polar solvents than non-polar solvents (Sanchez and Myers 2000). Whey is a polar solvent composed of water, sugar, and mineral salts. As such fluid whey is an ideal medium for benzoyl radical production from a polarity standpoint. The

decomposition of diacyl peroxides can also be catalyzed by metal ions such as copper, iron, cobalt, and manganese (Sanchez and Myers 2000).

HP is relatively stable at room temperature and can be used as the generating species for other peroxides (Sanchez and Myers 2000; Benassi and others 1993). HP can decompose into hydroxyl radicals $HO^{\bullet}$ and hydroperoxide radicals $HOO^{\bullet}$. Similar to lipid oxidation, the hydroxyl radical will attack the carotenoid double bond, which is responsible for the color, and cause it to break by hydroxyl radical addition or removal of a hydrogen bonded to the carbon in the double bond resulting in a loss of color (Carlsen and others 2005). The bond dissociation energy of the O-O bond has been reported as 50.5 kcal/mol at 298K (Bach and others 1996). A comparison of BDE suggests BP to be less stable at room temperature (298K) than HP. BP can generate free radicals at 20^{o}C (Sanchez and Myers 2000). Bleaching in whey is conducted under a wide range of temperatures (Listiyani and others 2011a; Listiyani and others in press; Jervis and others in press; Croissant and others 2009). Listiyani and others (2011b) reported HP bleached liquid whey reduced more norbixin pigment at 68^{o}C than at 4^{o}C. There was no reported difference in BP bleaching efficacy with varying temperature; BP showed higher bleaching efficacy than HP at both temperatures. As there was no difference in bleaching efficacy of BP at 4^{o}C versus 68^{o}C, this suggests that there is enough free energy in whey at typical bleaching temperatures for BP to degrade into benzoyl radicals which can then participate in bleaching-type reactions. HP had a significant increase in bleaching efficacy with increased temperature which may be because of the increased BDE of the O-O bond requiring the addition of thermal energy for degradation to occur and subsequent radical formation (Sanchez and Myers 2000; Bach and others 1996;

Benassi and others 1993). HP bleaching has been reported to suffer from low reaction rates at low temperatures as a consequence of the high activation energy required to facilitate oxidation (Dannacher 2006).

Increased bleaching efficacy of HP at elevated temperatures has been previously reported for textile systems however the increase in bleaching temperature resulted in a loss of fabric quality (Maekawa and others, 2007; Dannacher 2006). Bleaching occurred in alkaline solution (pH 10). There appears to be sufficient thermal energy at all bleaching temperatures to decompose BP into free radicals; HP may require more thermal energy or addition of catalysts for complete degradation. Both BP and HP can have catalyzed decomposition by metal cations. However catalysis may not factor into the decomposition of BP as the increased availability of thermal energy appears sufficient for BP degradation into radicals. Metal catalysis may only be significant in regards to HP. HP has been reported to significantly reduce norbixin pigment in liquid whey, WPC70, WPC34, and WPC80, however, HP was not as efficient of a bleaching agent as BP (Jervis and others, pending; Listiyani and others 2011a; Croissant and others 2009). This inefficiency of HP bleaching may be due to insufficient thermal energy for radical production, or possibly the hydroxyl radical not being selective enough in its attack of the carotenoid double bond. HP does have bleaching activity in whey systems and the bleaching activity of the hydroxyl radicals formed from HP degradation may be a result of iron catalyzed reactions.

Iron

Iron is a highly catalytic transition metal and can catalyze the decomposition of HP (Kohler and Jenzer 1988, Medalia and Kolthoff 1949). HP will decompose into water and

oxygen gas at 298K but the reaction is relatively slow. Production of radicals requires the addition of thermal energy or catalytic action by metal cations. When HP decomposes into water and oxygen it is inactivated, however, when the HP is oxidized via transition metal catalysts like iron, radicals can be formed which can result in increased bleaching efficacy. The rate of oxidation reactions will increase as the HP concentration increases and/or catalysis by transition metals (Kohler and Jenzer 1988). It is possible that Fenton-reactions in whey are aiding in the bleaching efficacy of HP.

The Fenton Reaction

The Fenton reaction has been classically defined as the reaction of ferrous iron with HP to form ferric iron, hydroxide ions and hydroxyl radicals.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot} \qquad (1)$$

Fenton reactions are used as one of the most effective ways of reducing organic pollutants in water specifically because of the reactivity of the hydroxyl radical for all types of organic substrates. Fenton reactions reduce organic pollutants by degrading the organic substrates through redox reactions (Pariente and others 2008). The Fenton reaction has also been proposed to be one of the responsible pathways for HP effectiveness as an antimicrobial agent (Barbusinski 2009; Liochev 1999). The antimicrobial effectiveness of HP is due to HP reacting with free iron in a bacterial cell creating free radicals which then can damage DNA and other cellular organs resulting in cell death. The Fenton reaction has been implicated in decreasing the shelf-life of wine through uncontrolled oxidation (Elias and Waterhouse 2010).

H.J.H. Fenton first described the oxidation of organic substrates by iron(II) and HP in 1894 when he observed the oxidation of tartaric acid by HP in the presence of iron to form a violet colored complex (Dunford 2002, Fenton 1894). Since then the precise mechanism of the Fenton reaction has been under controversial discussion (Barbusinski 2009; Dunford 2002; Koppenol 2001; Liochev and Fridovich 2002). Because of this controversy, the Fenton reaction is commonly referred to as Fenton Chemistry as the precise reaction mechanism has not been agreed upon by the scientific community. What is agreed upon is that the powerful oxidative capacity of iron(II) and HP is dependent mainly upon HP concentration, Fe^{2+}/HP ratio, pH and reaction time (Barbusinski 2009). Regardless of the exact mechanism, HP and Fe^{2+} must be present, so they are referred to as Fenton reagents. There are two reaction pathways that have been proposed for the mechanism of Fenton chemistry, a radical and a non-radical pathway. In all cases where HP is the bleaching agent in a whey system, the HP will be in excess and iron will be the limiting reagent. As such the excess HP will affect the type of Fenton chemistry permissible. It is important to note that all scientists agree that in Fenton chemistry when HP is in excess compared to iron(II), oxygen production and iron(II) regeneration are observed (Deguillaume and others 2005). Understanding the Fenton mechanisms possible in bleached whey systems will aid in the understanding of HP activation and subsequent bleaching efficacy.

Fenton Controversy

Two years after Fenton's death the hydroxyl radical mechanism was proposed for the first time by Haber and Willstätter in 1931 (Barbusinski 2009). They proposed that hydroxyl radicals could be produced by one electron reduction of HP by $HO_2^{\bullet}$ and that hydroxyl

radicals could abstract electrons from hydrocarbons and initiate radical chain reactions. This was later shown to be a very slow reaction and a year later Haber and Weiss suggested hydroxyl radical reduction by one electron reduction of HP by iron(II) (Barbusinski 2009). The Haber Weiss mechanism was modified in 1951 by Barb and others as the following:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + HO^{-} \quad (2)$$

$${}^{\bullet}OH + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \quad (3)$$

$$HO_2^{\bullet} + Fe^{3+} \rightarrow Fe^{2+} + O_2 + H^{+} \quad (4)$$

$$HO_2^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + HO_2^{-} \quad (5)$$

$$HO^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + HO^{-} \quad (6)$$

Reactions 2 – 4 are cycling steps leading to the evolution of O_2 which as mentioned previously is a necessary step when HP is in excess for Fenton chemistry to occur. Step 4 can serve as a regeneration step for iron(II), also necessary for Fenton chemistry when HP is in excess and is considered the rate determining step (Medalia and Kolthoff 1949). Steps 5 and 6 are termination steps (Barbusinski 2009). According to the mechanism proposed by Barb and others (1951), the hydroxyl radical is generated and is responsible for the oxidative reactivity of the Fenton reaction. This mechanism is commonly referred to as the radical pathway or mechanism of the Fenton reaction. The radical pathway has been proposed as the mechanism for the Fenton reaction in wine (Elias and Waterhouse 2010). Fukuzawa and Fujii (1992) reported a radical Fenton pathway for the lipid peroxidation of linoleic acid. Pariente and others (2008) reported the partial degradation of benzoic acid as a result of reactive radicals from the Fenton reaction. A number of compounds not normally oxidized

or oxidized slowly by HP are readily oxidized by HP when iron is present via a Fenton reaction pathway (Medalia and Kolthoff 1949). In this five step sequence, the hydroxyl radical is formed and can participate in bleaching by breaking the conjugated π bonds of norbixin. The oxygen gas formed may also participate in lipid oxidation adding to off-flavor formation.

In 1932 the ferryl ion species FeO^{2+} was proposed by Bray and Gorin as one step in the ferrous ion catalyzed decomposition of HP (Dunford 2002). They connected ferrous and ferric iron through equilibrium, (like O_2 gas production and Fe^{+2} regeneration, explanation of Fe^{+3} generation is an agreed upon condition for Fenton reactions to occur), and proposed the following mechanism which allowed for the formation of O_2 when HP was in excess.

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \quad (7)$$

$$FeO^{2+} + H_2O_2 \rightarrow Fe^{2+} + H_2O + O_2 \quad (8)$$

This reaction mechanism was corroborated by experiments conducted by Cahill and Taube (1952) who reported that the formation of iron(IV) was favored in the reaction of iron(II) and HP. Kremer (1999) concluded that it is difficult to accept the existence of a free radical mechanism because the mechanism does not recognize the formation of any other iron species other than iron(II) and iron(III). Buda and others (2003) reported that a ferryl-oxo complex can be produced as a primary intermediate with HP which supports a non-radical mechanism of the Fenton reaction. The ferryl ion mechanism has been referred to as the non-radical mechanism for the Fenton reaction. Buda and others (2003) proposed the following modification on the Bray and Gorin mechanism:

Initiation: $Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O$

O_2 Production: $FeO^{2+} + H_2O_2 \rightarrow Fe^{2+} + O_2 + H_2O$

Degradation: $FeO^{2+} + Fe^{2+} + H_2O \rightarrow 2Fe^{3+} + 2HO^-$

In the first step of this reaction mechanism, the ferryl ion is produced as an intermediate. In the second step O_2 is produced and the third step represents a possible catalyst termination reaction. Any non-radical mechanism where [HP] >> [Fe^{2+}] has to account for O_2 production. Buda and others concluded that oxygen production can be understood in terms of the FeO^{2+} reaction mechanism and therefore does not constitute evidence for hydroxyl radical intermediates. The ferryl ion is a strong oxidant and may be able to oxidize the conjugated double bond system of norbixin resulting in a loss of color.

During cheese whey bleaching, HP can be added up to 500ppm, a low concentration of HP is not a factor and therefore reaction kinetics of HP generation is a non-issue. The reduction potential of the hydroxyl radical is 2.3V and the high reactivity explains the ability of different iron species and HP to initiate oxidation in a variety of systems (Carlsen and others 2005). One of the questions raised by Bossman and others (1998) is whether or not hydroxyl radical production via the radical mechanism is too slow to compete with direct electron transfer (non-radical mechanism) between the substrate and the higher valent iron species (most likely Fe^{4+}). Increasing the rate of this reaction would mean increasing the concentration of Fe^{+2} as HP is already in excess. UV visible irradiation accelerates both the Fenton reaction (HP/Fe^{2+}) and Fenton-like reactions (HP/Fe^{3+}) however whey is manufactured in a closed system to prevent light catalyzed oxidation reactions so UV irradiation would not be expected to be a contributing factor. Metalloproteins may play a

role as a source of Fe^{+2}. Metalloproteins can serve as ligands to iron(III) which can undergo an efficient inner sphere electron transfer to reduced iron(III) to iron(II) and in the process create hydroxyl radicals with HP. Lactoperoxidase (LPO) may serve as a source of iron(II). In the ground state LPO binds one iron(III) ion that can be readily oxidized to iron(II) in the presence of HP which is the first step in activating the enzyme. In order for Fenton-type reactions to occur regardless of the Fenton-type pathway, it must be determined if Fe^{+2} is present and able to react with HP. In a bleached whey system the HP will be added directly therefore it is only important to discuss how iron(II) will enter the system.

Sources of Fe(II) in Fenton-type Reactions

I. Metalloproteins

In milk there exists a family of iron binding proteins, collectively called the transferrin group, comprised of lactoferrin, serum transferrin and ovotransferrin. They are also known as metalloproteins because of their metal binding ability. These proteins are all glycoproteins with a molecular weight of around 80 kDa (670-690 amino acid residues) and exhibit 50-70% pairwise sequence identity (Baker and others 2002). The proteins have key structural features responsible for their ability to tightly and reversibly bind iron and other divalent and trivalent cations. Lactoferrin (Lf) is comprised of two lobes, the N-terminal and the C-terminal which are comprised of 40% of the same amino acid sequence. Each lobe contains a trivalent binding site with a carbonate ion, and four amino acid ligands (Tyrosine 92, Tyrosine 192, Aspartic acid 60, and Histadine 253) with reversible binding ability (Figure 3) (Baker 1994). The -3 charge contributed by the two tyrosines (Tyr) and one aspartic acid (Asp) balances the +3 charge on the ferric ion; the charge on the carbonate ion is balanced

by the adjacent positive charge on the protein. In transferrins the positive charge comes from the arginine (Arg) side chain and from the N-terminus of the alpha helix. Both the open (no bound Fe) and the closed (bound Fe) structures of Lf and Tf are found in milk (Baker and others 2002). The transferrin (Tf) group is capable of binding divalent cations but cannot bind them as tightly as trivalent cations (Baker and others 2002). Iron is naturally found in the trivalent state when bound to transferrin proteins. The binding affinity of iron to Lf or Tf is high enough that in the presence of Lf or Tf the concentration of free iron cannot exceed 10^{-18}M which prevents precipitation of iron as insoluble hydroxides (Baldwin and others 1984). The iron must therefore be reduced and released by Lf or Tf so that it can participate in Fenton reactions and subsequent bleaching reactions. Iron release can be accomplished in a number of different ways:

1) Decreasing the pH: Will result in protonation of the carbonate ions and weakening of the iron binding site. Human Lf has been shown to retain iron at pH values between 3-4 whereas Tf releases iron at pH values ranging 5-6 (Mazurier and Spik 1980). Similar pH ranges were reported for bovine Lf and Tf as they are structurally similar (Baker 1994). Drained liquid whey typically has a pH $\geq$ 6, therefore pH release of iron is not a viable option by Lf or Tf.
2) Mutagenesis changes to Aspartic acid, Histadine and Tyrosine ligands: This will have a deleterious effect to iron binding capacity resulting in iron release or bound iron(III) that is more easily reduced resulting in its subsequent release (Nicholson and others 1997; Faber and others 1996; Ward and others 1996). The hydroxyl radical has been shown to cause protein confirmation changes (Schmidt

1983; Perlmutter and Brunner 1972; Cooney and Morr 1971; Grindrod and Nickerson 1966). Hydroxyl radicals may be able to damage the binding sites of metalloproteins. Heating of milk at temperatures 60 – 80^{0}C for five minutes was shown to have degradation effects on Lf which may result in a more easily reduced iron (Brisson and others 2007b). Hydroxyl radical action or heat degradation of Lf can degrade the binding site which may result in more easily oxidizable iron which can then participate in Fenton-type reactions and subsequent bleaching of norbixin.

3) Reducing agents or enzymes: Reducing agents or enzymes present in whey can reduce iron(III) to iron(II). Ascorbate is able to reduce iron(III) to iron(II) and is used to catalyze the Fenton reaction in certain antioxidant assays (Caillet and others 2007). Free thiols can catalyze iron(III) reduction in milk. Lactoferrin does not have free thiol groups however β-lactalbumin does. Brisson and others (2007b) reported that free thiol groups can be exposed in whey heated above 80^{o}C. Upon heating, β-lac may expose free thiols that can reduce the iron in Lf (Barbusinski 2009 and Brisson and others 2007a). This elevated temperature is not plausible in standard liquid whey processing. LPO and other native enzymes are also associated with iron(III) reduction (Damodaran 2008; Burkitt 2003; Vercellotti and others 1992).

4) Superoxide radicals O_2^-: Superoxide radical formation is possible in whey systems in the presence of HP by radical generation from transition metals, photosensitizers and UV light. The superoxide radical has been reported to

release iron from ferritin in meat by reducing iron(III) to iron(II) (Thomas and others 1985).

II. Lactoperoxidase

The lactoperoxidase system is a naturally occurring antibacterial system in milk involving the enzyme lactoperoxidase (LPO), thiocyanate (SCN^-), and HP (Seifu and others 2005). LPO and SCN^- are naturally found in milk, HP is added exogenously. LPO is activated by small concentrations of HP (<0.5mM) which then catalyzes the oxidation of thiocyanate generating hypothiocyanate, which is a strong oxidizing agent with antimicrobial properties (Kang and others 2010). The strong oxidizing capacity of hypothiocyanate makes it capable of reacting with carotenoids leading to destruction of conjugation and subsequent color loss of norbixin (Kang and others 2010). Bovine LPO consists of a single polypeptide chain containing 612 amino acid residues (Cals and others 1991) with a molecular weight of approximately 78 kDa (Björck 1990). LPO contains one heme group with the iron content of LPO being 0.07% corresponding to one iron per LPO molecule (Paul and Ohlsson 1985). LPO is very heat stable and survives normal milk pasteurization temperatures (Marks and others 2001). De Wit and van Hooydonk (1996) reported that complete inactivation of LPO in cow milk requires 15s exposure at 78^oC. Maximum LPO activity in milk is at pH 6 which corresponds to the pH of whey at draining from Cheddar cheese making (pH 6.1-6.2) (Kang and others 2010; Kumar and Bhatia 1999). LPO is also the second most abundant enzyme in milk after xanthine oxidase (de Wit and van Hooydonk 1996).

The activation of LPO in its resting state to its ground state involves the reaction of iron(III) bound to LPO with HP to form iron(II) and the hydroperoxyl radical (Seifu and others 2005).

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\cdot} \qquad (9)$$

As soon as HP is added to liquid whey in the presence of SCN^- from the diet of the cows, LPO is activated resulting in the formation of iron(II) bound to the enzyme which may then facilitate Fenton-type reactions with the excess HP (Figure 4) (Seifu and others 2005). The activation of the enzyme involves the formation of ferrous iron which can catalyze the radical pathway of the Fenton reaction. The LPO system generates hypothiocyanate and ferrous iron allowing for bleaching efficacy from hypothiocyanate as well as Fenton-type reactions that may further participate in bleaching efficacy and possible side reactions (e.g. lipid oxidation). Hydroxyl radical generation is a site-specific reaction which occurs at or near the active center of LPO where scavenger molecules may not be able to penetrate. The hydroxyl radical can then react with LPO leading to oxidative cleavage of the porphyrin ring structure of LPO which will liberate the bound iron (Jenzer and others 1987). The LPO basic reaction scheme is represented in Figure 5. Where Compounds I and II are the different configurations of the enzyme during the reaction process. When HP is in excess (>0.5mM) a third intermediate compound III can be formed (Figure 6). Formation of Compound III results in the irreversible inactivation of LPO. In low levels of HP (<0.5mM) Compound III is not formed and Compound II is able to cycle back and reform LPO through the loss of a hydrogen atom and the reduction of iron(IV) to iron(III) (Figure 5) (Seifu and others 2005; Kohler and Jenser 1989). The identity of Compound I has been proposed as shown in Figure 7. This proposed structure has an oxyferryl center allowing for non-radical initiation of Fenton reactions. This oxidation state of iron allows for non-radical catalyzed Fenton reactions which can participate in bleaching of norbixin through oxidation reactions.

If HP is added in sufficient amounts to activate the LPO, LPO can form the hypothiocyanate ion and bleach norbixin. At concentrations of HP sufficient enough to achieve activation, the LPO will regenerate itself resulting in a bleaching mechanism that is cyclical (Kohler and Jenser 1989). At this lower concentration of HP (<0.5mM) Compound III will not be formed and the LPO system can regenerate (Seifu and others 2005; Kohler and Jenser 1989). The lower concentration of HP will result in Fenton-type reactions which may be radical or non-radical mechanisms. Bleaching may result from a combined oxidizing effect of hypothiocyanate and the ferryl ion in Compound I (Figure 7). It is unclear at this point how effective the LPO system would be as a whey bleaching agent of norbixin and what species are responsible for bleaching, however it is clear that current HP bleaching is resulting in the formation of off-flavors and protein degradation compounds that may be the result of multiple iron induced reactions (Listiyani and others in press; Jervis and others in press).

Off-flavor development

Off-flavors in dairy products can be a result of lipid oxidation and/or protein degradation products. As lipids oxidize they form hydroperoxides which are susceptible to further oxidation or decomposition to secondary products such as aldehydes, ketones, acids and alcohols. These compounds adversely affect flavor, aroma, taste, nutritional value and overall quality. Light temperature, enzymes, metals, metalloproteins and microorganisms can all catalyze lipid oxidation (Vercelotti and others 1992). Thermal lipid oxidation by atmospheric oxygen with free radical intermediates of oils and fats involves catalysis by trace transition metals and in biological fluids like milk can involve enzymes like peroxidases. Lipids are

very sensitive to catalysis by trace metals or small concentrations of modified metalloproteins (Carlsen and others 2005). Complexes of iron(II) and other transition metals, are capable of reducing lipid hydroperoxides (Figure 8) (Barbusinski 2009; Liochev 1999). The lipid hydroperoxides are cleaved oxidatively by a transition metal to produce peroxy radicals or reductively to yield alkoxyl radicals. Alkoxyl radicals can further cleave to yield aldehydes and other secondary oxidation products that can result in off-flavors. Aldehydes can subsequently modify proteins through formation of Schiff's bases and for unsaturated aldehydes through crosslinking (Carlsen and others 2005). Below are four proposed mechanisms for iron reactions with hydroperoxides:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\cdot} + OH^{-} \quad (10)$$

$$Fe^{2+} + LOOH \rightarrow FeO^{2+} + LOH \quad (11)$$

$$Fe^{3+} + LOOH \rightarrow FeO^{2+} + LO^{\cdot} + H^{+} \quad (12)$$

$$Fe^{3+} + LOOH \rightarrow FeO^{3+} + LOH \quad (13)$$

Reactions 11 and 12 form the ferryl ion which can catalyze non-radical Fenton reactions resulting in more off-flavor development.

The oxidation of linoleic acid (LA) was studied based upon HP induced lipid peroxidation. Fukuzawa and Fujii (1992) reported that LA without hydroperoxides present had lipid peroxidation strongly induced by HP and iron(II) and proposed the radical Fenton mechanism as most likely responsible for the peroxidation of LA. Aldehydes and alcohols have been found to be products of the oxidation of glycerol by the Fenton reaction (Medalia and Kolthoff 1949). Croissant and others (2009) reported that WPC70 had higher cardboard and fatty/oxidized flavors than WPC70 made from BP bleached whey and unbleached whey.

Volatile analysis confirmed that fluid whey bleached with HP had higher concentrations of DMDS, DMST, heptanal, hexanal, octanal, and pentanal compared to control and BP treated whey. Listiyani and others (2011a) reported WPC34 from HP bleached whey was higher in cardboard flavor and had higher amounts of pentanal, octanal, and DMDS compared to WPC34 control and WPC34 from BP treated whey. Jervis and others (in press) reported higher concentrations of aldehydes for WPC80 from HP bleached whey compared to WPC80 from BP bleached whey and unbleached whey. Metalloproteins and LPO appear to provide the necessary iron(II) as well as other radicals and/or strong oxidants which can attack the electron dense double bonds of the hydrocarbon backbone of lipids. Off flavors from HP bleaching can also occur when there is not sufficient catalytic activity or thermal energy for radical production. The decomposition of HP into water and oxygen gas is kinetically favored although a slow reaction (Dannacher 2006). Oxygen gas can stimulate lipid oxidation. This pathway is favored only when a catalyst is not present. As iron is always present in milk due to the feed source of the cows, catalytic decomposition of HP is going to be a factor in lipid oxidation. It has been previously reported that bleaching action in alkaline solution of laundry was due to the superoxide radical and not the hydroxyl or perhydroxyl radical (Dannacher 2006). In whey, the hydroxyl radical could be involved in lipid oxidation and protein degradation reactions while the superoxide radical is responsible for bleaching, or another oxidizing species. It is of great importance to determine what species are causing bleaching, and what species are causing lipid oxidation so that counter efforts can be explored to maximize bleaching efficacy and minimize off-flavor development.

Fenton Reaction and Functionality

One of the biggest obstacles in whey protein processing is protein denaturation which can lead to functionality changes. Properties of whipping, foaming, and emulsification depend upon the ability of the proteins to unfold and orient at the water-air interface. It is reasonable to assume that processing factors that minimize protein aggregation result in a product with more acceptable whipping, foaming, and emulsification properties (Schmidt 1983). The Fenton reaction plays a major role in the oxidation of membrane lipids and the oxidation of amino acids (Barbusinski 2009). Vercelotti and others (1992) reported free radical oxidation propagated through lipid hydroperoxides and other catalysts initiated reactions responsible for functionality changes of several proteins in muscle foods. The exact mechanism remains unclear however most studies implicate the highly reactive hydroxyl radical (Caillet and others 2007). Amino acids and proteins can be targets for hydroxyl radical damage and these radicals may be important in the deterioration of proteins (Zs-Nagy and Floyd 1984). The hydroxyl radical has also been reported to damage DNA which was hypothesized to be the result of reaction by iron ions associated with the DNA and not by free iron (Liochev 1999). Iron binding proteins like Lf and Tf could be damaged when HP is present.

HP has been reported to have selectivity towards oxidizing sulfur containing amino acids. Schmidt (1983) reported that WPC from HP treated whey had altered methionine and cystine/cysteine concentrations. Perlmutter and Brunner (1972) reported that no changes in composition or physical properties were observed for a model system of β-lactoglobulin in a simulated milk dialysate with 5% lactose that was treated with HP or heated, however when

the model system was treated with HP and heat, all protein sulfhydryls were destroyed partially at 0.01% HP and totally at 0.1% HP. Methionine residues were reduced by 7% at 0.01%HP and 45% at 0.1% HP. Cooney and Morr (1971) reported that the biggest effect on protein alteration of WPC was peroxide concentration followed by reaction time; pH had little effect. The selectivity of HP towards denaturing sulfur containing amino acids may account for the sulfur based off-flavors in whey proteins resulting from dimethyl sufide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and methional (Wright and others 2006). Schmidt (1983) concluded that it is likely that HP will affect gelation properties of WPC because of the importance of disulfides to gel formation. Organic sulfides are readily oxidized by peroxyacids, hydroperoxides, and HP, all of which are more reactive than diacyl peroxides (once decomposition has occurred), suggesting BP lacks the necessary activation energy to break the S-S bond of an organic sulfur compound, like amino acids (Benassi and others 1993). Perlmutter and Brunner (1972) reported turbidity and acrylamide gel electropherograms indicated that HP treatment of β-lac inhibited protein aggregation. Jervis and others (in press) reported that WPC80 from HP treated whey had a higher heat stability temperature than control or WPC80 from BP treated whey. The high heat stability temperature may be a result of HP damaging sulfur containing amino acids preventing the formation of disulfide bonds at lower temperatures and delaying gelation until a longer heat treatment resulted in enough exposed sulfhydryl groups which could then form the disulfide linkages to result in gelation.

Future Work

Experiments need to be conducted to determine the bleaching mechanism of HP as well as what damage, if any, is being done to the whey proteins. Amino acid analysis of liquid whey before and after bleaching can be utilized to determine what amino acids are susceptible to damage from HP bleaching. This technique would be useful in tracking the degradation of methionine and cystine. Amino acid analysis on the final spray dried product would also need to be conducted to determine the amplified effects after concentrating and spray drying. Unbleached controls would be utilized to determine the effects of concentration and spray drying on amino acid degradation in the absence of a bleaching agent.

Fenton chemical reactions can be tracked using Ferrozine which is a compound that reacts only with Fe^{+2} to form a pink-colored complex which can absorb visible light with a maximum at 562 nm and a molar absorptivity of 28,000, and has the added advantage of stability over a wide pH range (Zs-Nagy and Floyd, 1984). After bleaching, reduction of iron and subsequent ability to participate in Fenton reactions could be tracked spectrophotometrically. Increases in Fe^{+2} concentrations throughout bleaching would provide evidence for iron availability to facilitate redox reactions. Fe^{+3} concentrations could also be monitored using spectrophotometric methods (Tarafder and Thakur, 2005; Zs-Nagy and Floyd, 1984).

The role of LPO and Lf could be determined by monitoring bleaching efficacy of HP treated liquid whey from LPO and Lf free milk. The isoelectric points of LPO and Lf are both around 9 and may be separated and filtered out of the milk by displacement

chromatography or other chromatographic methods (Baker and Baker 2005; deWit and van Hooydonk 1996; Carlström and Vesterberg 1967). This would enable researchers to see the compounding effect each of these components has on the total bleaching efficacy of HP. It would also be advantageous to determine if the hydroxyl radical is indeed responsible for bleaching efficacy and/or protein degradation as it has been hypothesized that the superoxide radical is responsible for HP bleaching efficacy of laundry and not the hydroxyl radical (Dannacher 2006). This could be determined using different radical scavengers that are selective towards the different radicals (Dannacher 2006). Determining the radical species of interest will help researchers determine the HP bleaching mechanism, and hopefully find ways to optimize bleaching efficacy while minimizing undesirable side-reactions.

The overall reactivity of HP as a bleaching agent needs to be determined by measuring bleaching efficacy as a function of concentration of HP, pH and temperature, to further pinpoint the bleaching mechanism of HP and its limitations.

Conclusion

Suitable bleaching methods that produce a concentrated whey product free of color and low in off-flavors with minimal protein degradation, will continue to be a priority for the dairy industry. BP bleaching is the best option at present for a chemical bleaching agent however current exporting issues with benzoic acid residue will continue to hinder its use. HP bleaching needs to be optimized for maximum bleaching efficacy with minimal protein damage and off-flavor development. Understanding the mechanism of bleaching will help the dairy industry to design bleaching parameters that account for the thermal stability of HP as well as the compounding factors in a liquid whey system that can affect bleaching, such as

iron reactions. Understanding the bleaching mechanism of HP, specifically the responsible species, will also aid in the mapping of lipid oxidation and protein degradation pathways. Identification of responsible species may lead to minimization of undesired side reactions.

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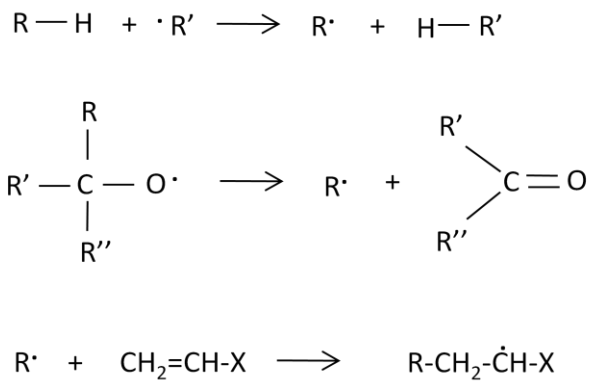

Figure 1. Hydrogen abstraction (top), β-scission (middle), and addition radical reactions (bottom) (Sanchez and Myers 2000)

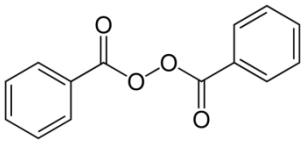

Figure 2. Molecular structure of benzoyl peroxide

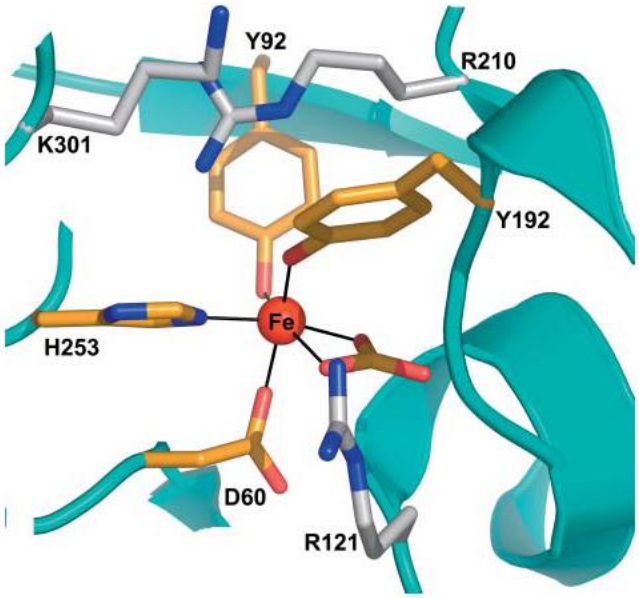

Figure 3. Canonical iron binding site, found in both lobes of all Lfs, shown here for the N-lobe of human Lf. The CO_3^{2-} ion, which binds in bidentate mode to the Fe^{3+} ion, is at the N-terminus of an α-helix, and interacts also with an arginine residue (here Arg121). Two basic residues at the back of the iron site (here Arg210 and Lys301) are more commonly both lysine, in Lfs and Tfs, and are unique to the N-lobe binding sites (Baker and Baker 2005)

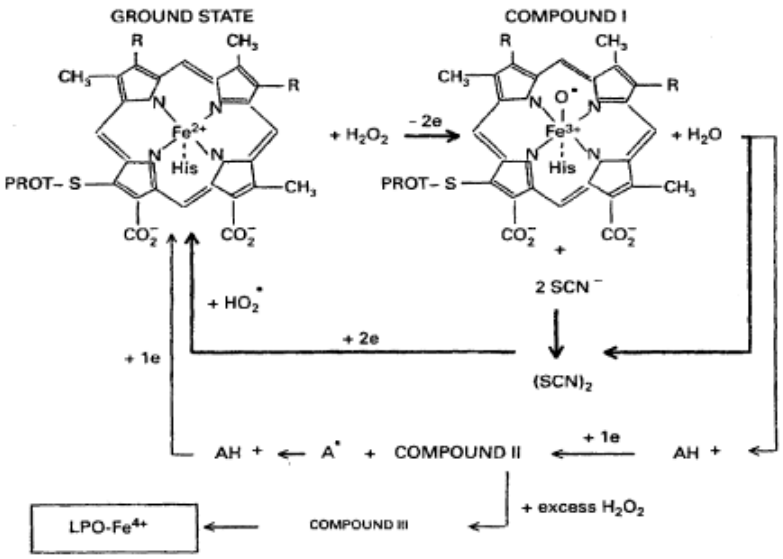

Figure 4. Pathways in the lactoperoxidase-catalyzed reaction mechanism. The normal peroxidatic cycle includes compound I. Insufficient 2-electron donors lead to compound II, and excess of H_2O_2 results in the formation of compound III (de Wit & van Hooydonk, 1996).

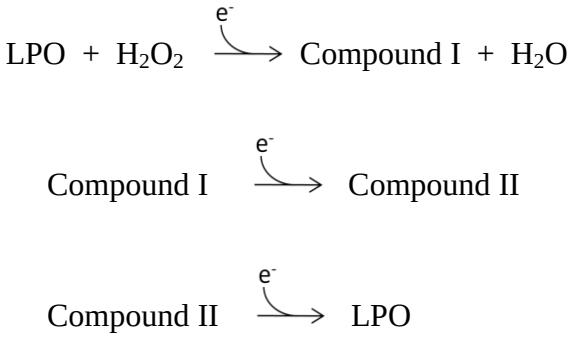

Figure 5. Lactoperoxidase mechanism from activation to reactivation (Kohler and Jenzer 1988)

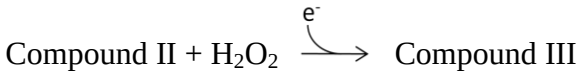

Figure 6. Inactivation of lactoperoxidase system with excess hydrogen peroxide (Kohler and Jenzer 1988)

Figure 7. Proposed structure of Compound I (Kohler and Jenzer 1989; Everse 1988)

$$M^{n+} + ROOH \rightarrow M^{(n+1)} + RO^{\cdot} + HO^{-}$$

Figure 8. General mechanism of transition metals catalyzing hydroperoxides decomposition (Barbusinski 2009)

CHAPTER 2: IMPACT OF BLEACHING WHEY ON SENSORY AND FUNCTIONAL PROPERTIES OF 80% WHEY PROTEIN CONCENTRATE

Impact of Bleaching Whey on Sensory and Functional Properties of 80% Whey Protein Concentrate*

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ABSTRACT

Whey is a highly functional food that has found widespread use in a variety of food and beverage applications. Whey proteins used in such applications are largely derived from annatto colored Cheddar cheese, where the resulting color is undesirable and must be bleached. The objective of this study was to compare two commercially approved bleaching agents, benzoyl peroxide (BP) and hydrogen peroxide (HP), and their effects on the flavor and functionality of 80 % whey protein concentrate (WPC80). Colored and uncolored liquid whey were bleached with BP or HP, ultrafiltered, diafiltered and spray-dried. WPC80 from unbleached colored and uncolored Cheddar whey were manufactured as controls. All treatments were manufactured in triplicate. WPC80 were evaluated by sensory, instrumental analyses, functionality, color, and proximate analysis. HP bleached WPC80 were higher in lipid oxidation compounds than other bleached or unbleached WPC80, specifically hexanal, heptanal, octanal, nonanal, decanal, dimethyl disulfide, and 1-octen-3-one ($P < 0.05$). HP treatments were higher in fatty and cardboard flavors compared with the unbleached and BP bleached samples ($P < 0.05$). WPC80 bleached with BP had lower norbixin concentrations compared to WPC80 bleached with HP ($P < 0.05$). Hunter CIE Lab color values (L* a* b*) of WPC powders were distinct on all 3 color scale parameters and HP bleached WPC80 had the highest L* values. Iron concentration was lower in the HP-bleached WPC80 ($P<0.05$). Proximate values were not different among treatments ($P>0.05$). Functionality testing demonstrated that HP treatments had more soluble protein after 10 min of heating at 90^{o}C at pH 4.6 and pH 7 than the no bleach and BP treatments regardless of additional color. Overall HP bleaching caused more lipid oxidation products and subsequent off flavors than BP

bleaching. However, heat stability of WPC80 was enhanced by HP bleaching compared to control or BP bleached WPC80.

INTRODUCTION

Whey is a highly functional protein ingredient that has found widespread use in many food and beverage applications. The appealing functional properties of whey protein include solubility, water binding ability, gelation, foaming, buffering, and emulsifying properties (Davis and Foegeding, 2007). Whey protein used in most food and beverage applications comes in two forms, whey protein concentrate (WPC), which can range in protein concentration from 35 – 89%, and whey protein isolate (WPI), which contains 90% or greater protein concentration (USDEC 2008). In order to make WPC80 or WPI, liquid whey is ultrafiltered, diafiltered and subsequently spray dried.

There are three sources of flavor variability of whey products that can occur; lipid oxidation, process-induced changes, and fermentation by lactic acid bacteria and other cultures (Cadwallader et al,. 2009). Lipid oxidation products, specifically aldehydes, free fatty acids, and methyl ketones are primarily responsible for off-flavors in dried whey proteins (Carunchia Whetstine et al., 2005; Wright et al., 2009; Whitson et al., 2010). Flavor variability in WPC80 has been attributed to milk source, starter culture, processing, and storage (Carunchia Whetstine et al., 2003; Tomaino et al., 2004, Croissant et al., 2009, Gallardo-Escamilla et al., 2005, Campbell et al., 2010). WPC and WPI have been characterized by cardboard, cabbage, and fatty/oxidized flavors which were attributed to lipid oxidation and protein degradation products (Listiyani et al., 2011; Croissant et al., 2009; Carunchia Whetstine et al., 2005; Wright et al., 2006, Whitson et al., 2010). Whey proteins

ideally should have a bland flavor to enhance their ability to be used in a variety of food and beverage products (Drake, 2006; Drake et al., 2009; Wright et al., 2009).

A large majority of dried whey protein in the U.S. is manufactured from annatto colored Cheddar cheese whey. Annatto is a natural coloring agent derived from the outer seed coats of the tropical shrub *Bixa orellana* (Scotter 2009). The major carotenoids responsible for the yellow color of annatto are bixin, which is soluble in non-polar mediums, and norbixin, which is soluble in polar mediums. Norbixin is the primary carotenoid derived from annatto used for cheesemilk and the primary colorant in whey. Campbell et al. (2011) recently demonstrated that annatto played no direct role in flavor of dried WPC. However, colored whey is generally bleached in order to achieve a more white-colored dried product suitable for a wide range of applications. Benzoyl peroxide (BP) and hydrogen peroxide (HP) are the two commercially approved agents used in the U.S. to bleach liquid whey. The bleaching step applied in the processing of whey protein may have an effect on the flavor of WPC80. Croissant et al. (2009) reported that hydrogen peroxide bleached liquid whey and WPC70 from HP bleached whey had higher concentrations of lipid oxidation compounds compared to BP bleached liquid whey and WPC70 from BP bleached whey. Listiyani et al. (2011) likewise confirmed that volatile and sensory profiles of unbleached, HP bleached and BP bleached WPC34 were distinct. The impact of these two different bleaching agents on the flavor and functional properties of WPC80 has not been determined. The objective of this study was to characterize and compare the composition, processing, sensory, and functional properties of 80% whey protein concentrate produced from bleached and unbleached Cheddar whey made from milk with and without added annatto color.

MATERIALS AND METHODS

Experimental Design

One batch of whole, raw bovine milk (about 1000 kg) was received from the Cornell University dairy farm on Monday and pasteurized at 72 to 73^{o}C for 16 sec and stored overnight at 4^{o}C. On Tuesday, Cheddar cheese was made, whey was collected from the cheese vat at draining, pasteurized at 72 to 73^{o}C for 16 sec, cooled to 50^{o}C, separated with a centrifugal cream separator and bleached after separation. The whey was cooled and held overnight at 4^{o}C. On Wednesday, the whey was heated to 50^{o}C and ultrafiltered and diafiltered to produce a liquid 80% WPC, cooled to 4^{o}C and held overnight. The liquid WPC was spray dried on Thursday. In each replicate of the experiment there were 6 treatments. For the first 3 treatments within replicate 1, one vat of Cheddar cheese was made in each of 3 weeks with no annatto color added to the milk. In the first week the whey was not bleached, the second week the whey was bleached with benzyol peroxide, and the third week the whey was bleached with hydrogen peroxide. For the second 3 treatments within replicate 1, one vat of Cheddar cheese was made in each of 3 weeks with annatto color added to the milk. In the first week the whey was not bleached, the second week the whey was bleached with benzoyl peroxide, and the third week the whey was bleached with hydrogen peroxide. This set of 6 treatments was replicated 3 times with different batches of raw milk for a total of 18 vats of cheese and 18 batches of 80% WPC.

Cheddar Cheese Manufacture

Raw whole milk for Cheddar cheese production was pasteurized with a plate heat exchanger (Model 080-S, AGC Engineering, Manassas, VA) at 72 to 73°C and a holding time of 16 s and cooled to 4°C and held overnight. The pasteurized milk was weighed into a cheese vat (Model DLHD8SSS, Kusel Equipment Company, Watertown, WI) and heated to 30°C. The milk was inoculated with the starter culture including *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (980 frozen pelleted culture, Chr Hansen, Inc., Milwaukee, WI) at the rate of 0.1 g/kg. The milk was agitated for 5 min and allowed to ripen for 30 min. For the treatments with added annatto (Annatto cheese color - 2X, P/N 70741, Chr Hansen, Inc., Milwaukee, WI), it was added (0.066 mL/kg of milk). The ripened milk, 31°C, was coagulated with double strength chymosin (Chymax Extra, Chr. Hansen Inc., Milwaukee, WI) for 30 min at a rate of 0.099 mL/kg of milk. The coagulum was cut with 1.6-cm wire knives, and the curd and whey was allowed to rest for 5 min and then the curd plus whey was gently stirred for 10 min without added heat. The temperature was increased gradually from 31 to 33°C over 15 min and then from 33 to 38°C over an additional 15 min. The curd was continuously stirred at 38°C until the target whey draining pH of 6.45 was attained. The whey was drained and immediately pasteurized using a plate heat exchanger (with three sections regeneration, heating, and cooling, Model 080-S, AGC Engineering) at 72°C for 16 s. The whey was cooled to 50°C at the exit of the pasteurizer and immediately processed with a cream separator (Model 619, DeLaval, Inc., Kansas, MO) to reduce the fat content. The fat content of the whey before separation was 0.23% $\pm$ 0.01 and after separation was 0.04% $\pm$ 0.004. The whey was mixed and sampled directly before and after cream

separation. After separation, if the whey was not going to be bleached, it was cooled to 4°C with a plate heat exchanger and held overnight at ≤ 4°C. If the whey was going to be bleached, it was heated with a plate heat exchanger to 66°C in a large stainless steel tank. Two different bleaches were used, benzoyl peroxide at 50 ppm (Oxylite Type XX Benzoyl Peroxide 32% by weight, Nelson Jameson, Marshfield, WI) and hydrogen peroxide at 500 ppm (35% HP, FCC grade, Columbus Chemical Industries, Inc., Columbus, WI). When bleaching with benzoyl peroxide, the powdered bleach was mixed with about 30 kg of whey using a high shear mixer and added to the batch of whey. The whey was held for 30 min at 66°C with stirring and then cooled to 4°C. When bleaching with hydrogen peroxide, the hydrogen peroxide was added to about 30 kg of whey, then mixed with the remainder of the separated whey, and stirred for 30 min at 66°C. The HP concentration in the reagent concentrate was tested prior to use with a hydrogen peroxide test strip by diluting the concentrate from 35% (w/w) to 10% (w/w) concentration. The 10% (w/w) concentration was verified with a 10% (w/w) HP test strip (Indigo Instruments, Niagara Falls, NY). If the HP concentration was lower than 10% (w/w), then the actual concentration was calculated and the amount of HP was adjusted to achieve an added level of 500 ppm in the cheese whey. After 30 min, the whey was cooled with a plate heat exchanger to 49 to 52°C and liquid catalase enzyme derived from *Aspergillus niger* (20ppm FoodPro CAT, Pd216626-2.0EN Danisco, Madison, WI) was added and then the whey was mixed for 10 min. The whey was cooled to 4°C with a plate heat exchanger and held overnight at 4°C before spray drying.

WPC Manufacture

Approximately 700 kg of separated whey was heated to 50^{o}C and processed with the UF system in batch recirculation mode using a polyethersulfone spiral wound UF membrane (Model 3838, GEA NIRO Inc., Hudson, WI) with a nominal pore size of 10,000 Da. Before processing the UF membrane was cleaned following the same procedure descibed in Evans et al. (2009). The water flux was typically about 52 kg/m² per hour. Whey was ultrafiltered for about 2 h to achieve a concentration factor of approximately 5 X and the protein content of the retentate measured by infrared spectrophotometer (Lactoscope FTIR, Delta Instruments, Drachten, The Netherlands) was 41% of protein expressed as a percentage of lactose plus fat plus protein in the retentate. The UF retentate was diluted with pasteurized reverse osmosis (RO) water at 50^{o}C to bring the weight back to the original total weight of the starting whey for DF. The membrane was not cleaned before proceeding to the DF stage. The mixture was recirculated through the membrane for 5 min to ensure complete mixing. Then, the diluted UF retentate was diafiltered to achieve a concentration factor of about 11.3 X. Diafiltration was continued until the protein content of the retentate measured by infrared spectrophotometer was 91.2 to 91.6% of protein as a percentage of lactose plus fat plus protein in the retentate. The total time of DF was about 2 h. At the end of processing, the retentate drained from the dead volume of the system was mixed with the retentate in the feed vat, mixed, and sampled. The final liquid retentate protein concentrate was weighed and cooled to 4^{o}C and held overnight until spray drying the next day. After producing the 80% WPC liquid concentrate, the UF system was cleaned as described by Evans et al. (2009). The clean water flux after final cleaning was typically about 52 kg/m^{2} per hour.

Spray Drying

The 80% WPC were spray dried using a Model 1, Niro Atomizer Inc., Columbia, MD. The feed material (about 40 kg) was kept at or below 7°C. The spray dryer was equipped with a FU11 atomizer rotating at 23,000 rpm and the feed rate was 16 kg/h. The inlet temperature was 200°C and the outlet temperature was 95°C. The powder from the first 10 min of the run was discarded. Thereafter, the dried product was collected and packaged every half hour. The total time of the drying run was approximately 3.5 h. The 80% WPC for sensory and functional property testing was packaged in mylar ziplok bags (Sorbent Systems, Los Angeles CA) and shipped to NC State (Raleigh, NC). Upon receipt samples were sub-sampled and stored at -80^{o}C in black whirlpak bags (ULINE, Pleasant Prairie WI).

Chemical Analyses

Milk for cheese making was analyzed using an infrared spectrophotometer (Lactoscope FTIR, Delta Instruments) for fat content and true protein content (Kaylegian et al., 2006). The fat content of unseparated, separated whey, and liquid 80% WPC prior to drying was determined by ether extraction (AOAC, 2000; method 989.05; 33.2.26).

Fresh samples of the final liquid 80% WPC were analyzed for fat, TS, total N, and NPN content using ether extraction (AOAC, 2000; method 989.05; 33.2.26), forced air oven drying (AOAC, 2000; method 990.20; 33.2.44), Kjeldahl (AOAC, 2000; method 991.20; 33.2.11), and Kjeldahl (AOAC, 2000; method 991.21; 33.2.12), respectively. The pH of final liquid 80% WPC was measured with an electrode (model HA 405, Mettler Toledo, Columbus, OH) that was standardized at pH 6.97 and 4.06 at 50°C and kept immersed in 3M

KCl at 50°C between readings to keep its temperature equal to the temperature of the buffers and samples.

The 80% WPC powders were reconstituted to 10% solids and the liquids were analyzed for fat and total N by the methods indicated above. The pH was measured with an electrode (model Electrolyte 9823, Mettler Toledo) that was standardized at pH 7.01 and 4.00 at 22°C. The reconstituted samples were analyzed for total solids content by forced air oven drying (AOAC, 2000; method 990.20; 33.2.44) and moisture content was calculated. Mineral analysis was measured by inductively coupled plasma emission spectroscopy by the North Carolina State Soil Science Department (Raleigh, NC).

Color Analysis of liquid and spray dried 80% WPC

The Hunter L (lightness), a (red-green), b (yellow-blue) values for the fresh 80% WPC powders were determined in duplicate with a MacBeth Color-Eye spectrophotometer (Model 2020; Kollmorgen Instruments, Corp., Newburgh, NY) with Optiview software from the same company. The Hunter values were computed from the diffuse reflectance data in the 360 to 740 nm range, at 20-nm intervals, based on illuminant A. The measurements were done at 23 to 25^{o}C. The powders were reconstituted to 10% (w/v) solids (as described above) and color measurements of these liquids were done using a 1 cm cuvette. The white reference color tile was placed behind the cuvette to reflect transmitted light back through the sample to the detector. This effectively doubled the path length of the cuvette and increased the sensitivity of the method to detect differences in color among treatments. Color measurements on the powders were done by packing the powder into a plastic dish that had a screw cap lid. Color was measured by reflectance through the bottom of the plastic dish.

Norbixin Extraction and Quantification

Norbixin extraction and quantification from WPC80 was conducted using the method from Campbell et al. (2011). Extractions were preformed under lights covered with premium full-spectrum F885 flat sheet filters (Ergomart, Dallas, TX) to minimize norbixin isomerization and degradation (Mercadante, 2008). Chloroform, ethanol, methanol, and water were obtained from EMD Chemicals Inc (Gibbstown, NJ). Glacial acetic acid (17.4M) was obtained from Mallinckrodt Baker (Phillipsburgh, NJ.). WPC (1 g) was weighed into a 50-mL centrifuge tube (Nalgene, Rochester, NY). Samples were rehydrated with 2 mL HPLC grade water and vortexed until no dry powder was visible. To this, 6 mL of 200 proof ethanol was added, vortexed for 30 s and placed under a fume hood for 30 min. After which, 3 mL of chloroform was added and vortexed for 30 s. The samples were then centrifuged at 16,500 x g for 10 min at 4^{o}C (model RC5B, Thermo Scientific) and then the supernatant was removed and placed in a separate centrifuge tube. A second chloroform step was added to this extraction procedure to remove all norbixin from the WPC80. This second extraction was again vortexed and centrifuged after which time the supernatant was combined with the supernatant from the first chloroform extraction. Two milliliters of acetic acid (1% vol/vol) was added to the combined supernatant, vortexed, and centrifuged at 16,500 x g for 10 min at 4^{o}C. The bottom chloroform layer containing the norbixin was collected. All extractions were conducted in triplicate.

Solid-phase extraction (SPE) was conducted on the solvent extracts using a strata-NH_2 SPE column (500 mg/ 3 mL, Phenomenex). Seven milliliters of *n*-hexane (VWR International) was used to condition the column. A 1 mL aliquot of chloroform extract was

transferred onto the SPE column. After the 1 mL was completely on the column, the column was rinsed with 5 mLs of *n*-hexane:diethyl ether [1:1 (vol/vol)] followed by 1 mL of acetone (VWR International). The norbixin was then eluted with 3 mL of methanol:glacial acetic acid [7:3 (vol/vol)]. The final volume of eluent was measured and the norbixin subsequently quantified by spectrophotometry.

The concentration of norbixin was determined spectrophotometrically using a UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Durham, NC). A 0.7mL aliquot of each sample was measured in a 28Q10 Spectrosil quartz cuvette (Starna Cells Inc., Atascader, CA) and measured at 458 nm, the maxima for norbixin (Croissant et al,. 2009). A standard curve was created within the concentration range of 50 μg/kg to 10 mg/kg norbixin. Norbixin powder (45% w/w, Chr. Hansen, Milwaukee WI) was rehydrated in 2.5% potassium hydroxide (VWR International) and then diluted in methanol:glacial acetic acid [7:3 (vol/vol)]. Norbixin concentration was calculated by total solids and correction for dilution during the extraction and SPE processes and by an external standard curve. Measurements on the spectrophotometer were conducted in duplicate.

Descriptive Sensory Analysis

Sensory testing was conducted in compliance with North Carolina State University Institutional Review Board for Human Subjects approval. A trained sensory panel (n = 10, 9 females, 1 male, ages 22 to 50 y) evaluated the flavor attributes of reconstituted WPC80 at 10% solids using a previously published lexicon for dried dairy ingredients (Drake et al., 2003; Drake et al., 2009; Wright et al., 2009; Listiyani et al., 2011).

Each panelist had over 150 h of experience with descriptive analysis of whey and dried whey products using the Spectrum^{TM} descriptive analysis method (Meilgaard et al., 1999; Drake and Civille, 2003). Reconstituted solutions of 10% solids (w/v) were prepared with no overhead lights to avoid exposure to light. Samples were pipetted into 60-mL soufflé cups, lidded, and labeled with a unique three digit code. Samples were evaluated in duplicate by each panelist. Products were presented in a randomized order and scored using paper ballots or computerized ballots using Compusense five release 4.8 (Compusense, Inc., Guelph, Canada).

Volatile Compound Extraction

Head Space Solid-Phase Microextraction GC-MS of WPC powders. Volatile compounds were extracted by HS-SPME and subsequently separated and identified by GC-MS in triplicate using a modified method of Campbell et al. (2011). Spray dried powders were reconstituted at 10% (w/v) solids, with 10% (w/v) NaCl (Fischer Scientific, Fairlawn, NJ). Five milliliters of sample with 5 µL of an 81 ppm 2-methy-3-heptanone in ether (Sigma Aldrich., Milwalkee, WI) internal standard was added to 20 mL autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Sawanee, FL). Samples were injected using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890N GC with 5973 inert MSD (Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 10°C before fiber exposure. Samples were equilibrated at 40°C for 25 min before 30 min fiber exposure of a 1-cm DVB/CAR/PDMS fiber at 31 mm with 4-s pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm.

The GC method used an initial temperature of 40°C for 3 min with a ramp rate of 10°C/min to 90°C then the ramp rate decreased to 5°C/min to 200°C held for 10 min and then increased to 20°C/min to 250°C and held for 5 min. The SPME fibers were introduced into the split/splitless injector at 250°C. An Rtx-5ms column (Rtx-5ms 30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Restek, Bellefonte, PA) was used for all analyses using helium as a carrier gas at a constant flow rate of 1 mL/min. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the quadrupole at 150°C and source at 250°C. Compounds were identified using the NIST 2005 library of spectra and comparison of spectra of authentic standards injected under identical conditions (NIST, 2005). Relative abundance for each compound was calculated using the calculated recovery and concentration of internal standard. Compounds of interest were quantified using external standard calibration curves. Retention indices were calculated using an alkane series (Sigma Aldrich WI) (Van den Dool and Kratz, 1963).

Quantification of specific volatile compounds identified in samples was conducted using five point external standard curves ranging from 0.50 μg/kg to 2 mg/kg (ranges varied between these two extremes depending on the compound of interest), integrated to internal standard, 81ppm 2-methyl-3-heptanone. Fresh egg white protein from organic, cage-free, antibiotic free, no omega three fatty acid additions, free range eggs was purchased from a local grocery store (Whole Foods, Raleigh, NC) and used as the protein medium for the external standard curves. The egg whites were evaluated by HS-SPME-GCMS to ensure they were free of target compounds. Egg whites were prepared by separating whites from yolks, combining egg whites from multiple eggs, and stirring to make a uniform solution.

External standard curves were prepared by pipetting 4.0 mL of egg whites (approximately a 10% protein solution w/v) into 20 mL autosampler vials with steel screw top lids lined with silicone septa faced in Teflon with spiked amounts of target compounds and 10 μL internal standard. HPLC grade water was used to bring the total solution volume to 5.0 mL. Sodium chloride was added to the vials prior to the addition of egg white protein so that the total solution was 10% (w/v) sodium chloride.

Functional Properties

Foam Generation An Artisan Kitchen Aid Mixer (Kitchen Aid, St. Joseph's, MI, USA) with a 4.5 quart stationary bowl and a rotating wire beater was used for foam formation. Solutions of protein (10% w/v) were rehydrated for 6 h at room temperature (20-24^{o}C) on a stir plate set to 200rpm. Solutions were refrigerated overnight at 4^{o}C and brought up to 25^{o}C. Solutions were then adjusted to pH 7 using 1N HCl or 1N NaOH (VWR International). All solutions were prepared in triplicate. A 200 mL sample of solution was whipped at speed 10 (beater rpm of 752) for 19 min and 36 sec (Davis et al., 2007).

Yield Stress. Yield stress was measured using vane rheometry (Pernell et al., 2000; Davis et al., 2007). A Brookfield 25xLVTDV-ICP (Brookfield Engineering Laboratories, Inc. Middleboro, MA) viscometer was used at a speed of 0.3rpm. The vane used was 10mm in diameter and 40mm in length. Maximum torque response (M_o) was recorded for each foam in triplicate. Torque measurements were used to calculate yield stress using the following formula published by Duzy et al. (1983; 1985) and Steffe et al. (1996).

$$\tau_o = \frac{M_o}{[(h/d) + (1/6)]((\pi d^3)/2)}$$

where τ_o is yield stress, and h and d are the height and diameter of the vane. Torque was measured in triplicate per solution.

Overrun Overrun measurements were conducted after yield stress. Foam was removed from the bowl using a rubber spatula in a circular pattern and filling a weighing dish (100mL) in triplicate. The mean value was used to calculate overrun and air phase fraction using the following equations:

$$\text{Overrun} = \frac{(\text{wt. 100mLsolution}) - (\text{wt. 100mLfoam})}{\text{100mLfoam x 100wt.}}$$

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

All treatments were measured in triplicate (Davis et al., 2007; Wilde, 2000; Dickinson, 1999).

Stability Foam drainage was measured after overrun using the methods of Phillips et al. (1990) and Luck et al. (2001). Foam stability was measured by the time it took for half of the pre-foam mass to drain through a whole in a whipping bowl. The mass of foam removed during the overrun measurements was subtracted when calculating the pre-foam mass. The starting time for these measurements was taken immediately after foam formation (Davis et al., 2007).

Solubility Solutions of 10% protein (w/v) were rehydrated for 6 h at room temperature (20-24^{o}C) on a stir plate set to 200 rpm. Solutions were refrigerated overnight at 4^{o}C and brought up to 25^{o}C. Solutions were then adjusted using 1N HCl or 1N NaOH to pH 3, 4, 5, 6, or 7 and brought to 100 mL total volume with deionized water. Turbidity and solubility were measured on the uncentrifuged solutions. Solutions were then centrifuged at 16,500 x g for 10 min using a (model RC5B, Thermo Scientific) and then the supernates were measured for turbidity and solubility.

Solubility was measured by the Micro bicinchoninic acid (BCA) assay using a kit from Thermo Fisher Scientific/ Pierce (Rockford, IL). Protein solutions before and after centrifugation were diluted 1:100 in deionized water. Solutions were added to a working reagent in a ratio of 1:8 and pipetted in triplicate into a 96 multi-well plastic plate. Deionized water with working solution was used as a reference blank. The plate was put on a shaker for 30 sec and incubated at 37^{o}C for 30 min. The plate was brought to room temperature and read on a Tecan Safire plate reader spectrophotometer at absorbance 562nm (Durham, NC). Analysis was performed in quadruplicate. Solubility was calculated using the following solution:

$$\text{Protein solubility} = 100 - (((\text{Abs}_{\text{before}} - \text{Abs}_{\text{after}}) / \text{Abs}_{\text{before}}) * 100)$$

Turbidity was measured using a Hach 2100AN Turbidimeter (Loveland, CO). Samples were pipetted into glass cuvettes and measured in quadruplicate. Turbidity was calculated using the following equation:

% Turbidity = (((Turbidity$_{before}$ – Turbidity$_{after}$)/ Turbidity$_{before}$))* 100)

Heat Stability Solutions were prepared the same as for solubility with the exception that all solutions were made at 5% (w/v) and 10% (w/v) protein. Solutions were raised to pH 7 using 1N NaOH and heated in a water bath at 90^{o}C for either 0, 10, 20 or 30min. Samples were immediately placed in an ice bath until the sample was back to 25^{o}C. Turbidity and solubility measurements were taken before and after centrifugation and calculated as addressed previously. The supernatant was then collected and brought to pH 4.6 using 1N HCl. Turbidity and solubility were measured on the pH 4.6 supernatant before and after centrifugation. All solutions were measured in quadruplicate.

Statistical analyses

To determine if there were significant differences in color or composition due to annatto or bleaching treatment, all data were analyzed by ANOVA using the Proc GLM procedures of SAS (SAS version 8.02, 1999-2001, SAS Institute Inc., Cary, NC). A GLM model was used to determine if flux changed with the time of processing run UF and during DF between treatments. Time was treated as a continuous variable in the split-plot ANOVA models. Distortion of the ANOVA by multicollinearity in the model was minimized by centering the time of running using a mathematical transformation (Glantz and Slinker, 2001). Time was transformed as follows: time transformed = running time – [(last time – first time) / 2] for each bleaching treatment. This transformation made the data set orthogonal with respect to time. The complete GLM models with all terms and interactions for all

statistical analyses will be shown in the data tables. The GLM model for analysis of composition, color, chemical composition, descriptive analysis, and instrumental analysis data was the same as the whole plot for the ANOVA model used to analyze the UF flux data. Fisher's least significant difference was conducted as a post hoc test. Principal component analysis was applied to the correlation matrix of sensory and volatile component data to visualize how bleaching agent differentiated WPC80 based upon sensory attributes and/or volatile components.

RESULTS

Mineral and Composition For all 6 treatments the unseparated whey and separated whey mean values for total solids, fat and crude protein were not different ($P > 0.05$) (Table 1). Compositional differences were also not detected in spray dried treatments ($P > 0.05$) (Table 2). Both WPC HP treatments had lower iron compared to other WPC ($P < 0.05$) (Table 3). There were no other consistent trends observed among the other minerals among treatments.

Color Bleaching whey with added annatto with BP or HP increased L values of liquids and powders (Table 4). HP treatments, regardless of annatto color, had higher L values than BP treatments with and without annatto ($P<0.05$). The b-values for annatto colored HP and BP treatments were lower than annatto with no bleached, liquid and powder ($P<0.05$). BP with annatto color had lower b values in liquid and powder compared to HP and no bleach treatments with annatto added ($P<0.05$). HP with annatto color had lower b values compared to no bleach with annatto color ($P<0.05$).

Annatto Extraction Both the BP and the HP treatments reduced norbixin concentration ($P < 0.05$) (Table 5). WPC with or without annatto bleached with BP was not different from

control WPC with no added annatto. Hydrogen peroxide reduced norbixin 44% compared to the total norbixin concentration of the no bleach annatto control. BP reduced norbixin 92% compared to the total norbixin concentration of the no bleach annatto control.

Sensory Analysis Descriptive analysis of rehydrated WPC80 differentiated the samples based upon treatment (Table 6, Figure 3). The HP bleached samples were higher in cardboard and fatty flavors and had lower intensities of cooked/milky and sweet aromatic flavors compared to the BP and the no bleach samples ($P < 0.05$). The colored no bleach WPC80 were lower in cardboard flavor than the uncolored no bleach WPC80 ($P < 0.05$).

Volatile Component Analysis WPC80 with and without annatto bleached with HP was characterized by higher concentrations of hexanal, heptanal, octanal, nonanal, decanal, dimethyl disulfide, and 1-octen-3-one compared to no bleach and BP bleached WPC80 with and without annatto ($P < 0.05$) (Table 7, Figure 3). All of these compounds have been previously identified in whey and are associated with lipid oxidation or protein degradation products (Croissant et al,. 2009, Evans et al,. 2010, Listiyani et al,. 2011, Whitson et al., 2010, Wright et al., 2006). BP with color was significantly higher in 2-pentyl furan ($P < 0.05$).

Functionality None of the treatments foamed therefore yield stress, overrun, and stability could not be measured. Solubility by pH with no heat treatment was not different among treatments within a specific pH value ($P > 0.05$) (results not shown). Solubility results at selected pH with varying time of heating represented as heat stability indicated that both colored and uncolored HP WPC at 10% (w/v) protein were more soluble after 10 min at 90^{o}C than colored or uncolored BP or unbleached WPC at pH 7 and pH 4.6 ($P < 0.05$) (Figures 1,

2). Turbidity measurements showed similar trends in protein solubility and the Micro BCA measurements at pH 7 and pH 4.6 (results not shown). No differences in heat stability were observed at 5% (w/v) protein at pH 7 and pH 4.6 ($P > 0.05$) (results not shown).

DISCUSSION

Bleaching Efficacy

Norbixin recovery as well as color measurements indicated that BP was a better bleaching agent than HP. Benzoyl peroxide removed 92% of the norbixin in colored WPC compared to only 44% removal by HP. Listiyani et al. (2011) reported 50 ppm BP bleached WPC34 had less recovered norbixin than 500 ppm HP bleached WPC34. Higher b* values were also noted for colored HP compared to colored BP. The b* value is associated with the blue to yellow spectrum where a more positive b value is more closely associated with yellow and a negative b value is more closely associated with blue. The b values of HP colored WPC for liquid and powder were higher than the b values of the BP bleached colored WPC. This result suggests that the colored HP bleached WPC were more yellow than the colored BP bleached WPC. The L values for the colored and uncolored HP samples for both liquid and powder were higher than colored and uncolored BP and no bleach treatments. The L value is associated with white and black with more positive L values associated with white. This result signifies that the hydrogen peroxide created a brighter colored whey product compared to BP bleaching but not a less yellow product. Bleaching is the chemical degradation of molecular moieties responsible for absorbing visible electromagnetic radiation resulting in an increase in the total reflectance of the substrate making it appear brighter (Dannacher 2006). It is possible that the bleaching mechanism for hydrogen peroxide is different from benzoyl

peroxide, namely the hydrogen peroxide attacks the norbixin pigment but also attacks other conjugated double bonds of compounds in the whey affecting the brightness of the product, whereas the benzoyl peroxide mechanism of bleaching appears focused on the norbixin pigment. Croissant et al. (2009) reported that the L value for HP bleached WPC70 powder was higher than the L value for BP bleached WPC70; the b value for the BP WPC70 was lower than the HP WPC70. Listiyani et al. (2011) reported no differences in the L or b values for HP bleached and BP bleached WPC34.

Sensory and Volatile Component Analysis

HP and BP bleached WPC80 were distinct by sensory and volatile compound analysis. HP treatments were higher in lipid oxidation and protein degradation compounds determined both by volatile and sensory analysis compared to BP or control unbleached WPC. HP WPC80 were described as cardboard and fatty, both of which are attributed to lipid oxidation and protein degradation compounds (Wright et al., 2009; Whitson et al., 2010; Tomaino et. al., 2004). Whitson et al. (2010) attributed cardboard flavor to pentanal, heptanal, nonanal, 1-octen-3-one and dimethyl trisulfide. HP treatments had the highest heptanal, nonanal, and 1-octen-3-one concentrations. Dimethyl trisulfide was identified in all samples but was not significantly higher for HP samples; pentanal was not detected in WPC. The most abundant volatile in the hydrogen peroxide WPC was hexanal. Hexanal is an indicator of lipid oxidation and can be formed by multiple pathways (Frankel 2005). BP WPC also had hexanal and other key lipid oxidation and protein degradation volatiles, but concentrations were not as high as those in HP WPC. BP bleaching of WPC appears to favor the formation of 2-pentyl furan. The large production of two vastly different quantities of compounds

suggests that the two bleaching agents either oxidize free fatty acids through similar pathways but that the reaction rate of HP is much higher than that of BP, or that the two bleaching agents oxidize free fatty acids through different pathways to end up with different concentrations of lipid oxidation products. Croissant et al. (2009) reported higher concentrations of hexanal, DMTS, heptanal, and octanal in HP bleached WPC70. Listiyani et al. (2011) reported a higher concentration of octanal in HP bleached WPC34. Both Croissant et al. (2009) and Listiyani et al. (2011) reported the same key lipid oxidation and protein degradation compounds in HP and BP bleached WPC as identified in this study. Dimethyl disulfide and dimethyl trisulfide are formed from protein degradation of sulfur containing amino acids.

Oleic and linoleic acids are two of the main unsaturated free fatty acids that form lipid oxidation products in whey. Milk fat is composed of approximately 25% oleic acid and 2% linoleic acid however the phospholipid component of the milk fat globule membrane is 6% linoleic acid (Frankel 2005) and this fraction is amplified in whey proteins. When auto-oxidized, oleate forms 8-, 9-, 10- and 11- hydroperoxides which are further cleaved on the ester side to produce heptanal, octanal, nonanal, and decanal aldehydes. As these four aldehydes were significantly higher in HP bleached treatments compared to BP or control WPC, this is one possible mechanism for their formation. Hexanal can be produced through the cleavage of the 12-hydroperoxide formed from the photo-oxidized linoleic acid (Frankel 2005) or by the breakdown of unsaturated aldehydes (Schieberle and Grosch 1981). As BP also formed these aldehydes it then stands to reason that BP creates less of the starting material or produces different products from the same starting materials, namely the 8-, 9-,

10- and 11- hydroperoxides. Colored BP formed a significantly higher amount of 2-pentylfuran which has been proposed to be formed from the carbon 10-hydroperoxide of linoleic acid (Min et al., 2003).

Each bleaching agent has the potential to form different lipid oxidation products based upon different chemically favored pathways. The reactivity of peroxides proceeds in most cases by the hemolytic cleavage of the O-O bond producing free radicals (Benassi et al., 1993). This occurs by thermal decomposition or degradation by metal ions. When the peroxide bond dissociates/decomposes it results in the formation of two peroxide radicals. In order to compare the reactivity of different peroxides it is important to know the bond dissociation energy (BDE) of the O-O bond. The kinetic stability of the radical is also important however the BDE represents the basic chemical behavior of peroxides (Benassi et al., 1993). Benassi et al. (1993) reported peroxides with the structural formula ROOR' to have a higher bond dissociation energy for R-O when R is a phenyl group, as in benzoyl peroxide. This results in breakage of the O-O peroxide bond which is weaker than the R-O bond yielding two benzoyl radical molecules. The bond dissociation energy of the O-O bond of diacyl peroxides, such as benzoyl peroxide, has been estimated as 36.9kcal/mol at 298K (Bach et al., 1996). The bond dissociation energy of the O-O bond in hydrogen peroxide has been reported as 50.5 kcal/mol at 298K (Bach et al., 1996). Hydrogen peroxide is more stable than benzoyl peroxide at the same temperature meaning BP will break into radicals more readily than HP. The decomposition of HP into radicals will require additional thermal energy or a transition metal catalyst (Benassi et al., 1993).

Hydrogen peroxide can break down into two types of free radicals, ·OOH and ·OH, where the hydroxyl radical (OH) is one of the most reactive species known (McClements and Decker 2008). Benzoyl peroxide produces benzoyl radicals by homolytic cleavage of the oxygen-oxygen bonds yielding two benzoic acid molecules. The free radicals will then abstract a hydrogen from a fatty acid to form a fatty acid radical (L·) known as an alkyl radical. This first step is known as initiation. Once the alkyl radical is formed the structure is stabilized by delocalizing the lone electron across the conjugated double bonds of the carbon chain. This delocalizing of the lone electron causes the formation of double bonds across the carbon chain which can result in cis and trans formations. The changing of the configuration from cis and trans can be important for the final products formed from the lipid oxidation. The next step in the process is called propagation where oxygen from the atmosphere or triplet oxygen is added to the alkyl radical. In this step the alkyl radical can form peroxy radicals, hydroperoxides, and new hydrocarbon radicals. The radicals then can attack other fatty acids continuing the radical formation. The process ends with the termination step where two radicals will interact to form aldehydes, ketones, alcohols and hydrocarbons formed from the β-scission reaction. Factors that can affect the decomposition of hydroperoxides in the termination step include temperature of oxidation, metal catalysts, stability of volatile products and competing secondary reactions (Frankel, 2005). All of the possible different combinations of reactions are responsible for the final distribution of volatile compounds from each bleaching treatment.

Iron concentration of HP WPC with and without color was lower compared to the unbleached and BP treatments. Listiyani et al. (2011) also reported iron levels to be lower

for HP bleached WPC34 compared to control WPC34 but not lower than BP bleached WPC34. There was a lower final concentration of iron in the HP WPC80; it is possible iron is catalyzing HP decomposition allowing for radical production. Hydrogen peroxide creates more active radicals, (the hydroxyl radical), the more reactive radicals could explain the increased off-flavor development of the HP bleached treatments. It is possible that the decrease in iron from the HP WPC80 was due to iron participating in a Fenton-type reaction. The classic Fenton reaction occurs when ferrous iron reacts with hydrogen peroxide to form ferric ions, and in the process produces hydroxyl radicals (Damodaran et al., 2008). This reaction could increase the overall concentration of the hydroxyl radicals which would then accelerate lipid oxidation. Peroxidation of linoleic acid was reported to be catalyzed by Fenton reagents HP and Fe^{+2} (Fukuzawa and Fujii, 1992; Damodaran et al., 2008). Lipid oxidation has a lag period before off-flavors are developed. The presence of prooxidants, like iron, can decrease the lag phase which increases the formation of off-flavors. The decrease of iron in the final HP bleached WPC80 product could also be due to protein denaturation, such as lactoferrin, resulting in a release of ferric ions and therefore the iron passes directly into the permeate during filtration due to damage to the binding sites (Damodaran et al., 2008).

Functionality

At pH 7 without heating, all WPC80 had almost entirely soluble protein. After ten min of heating, both the colored and uncolored no bleach and BP WPC80 gelled, therefore turbidity and solubility could not be measured. The colored and uncolored HP WPC80 had a significant amount of soluble protein after 10 min of heating. After 20 min of heating both

HP treatments gelled. The supernate of the no heat WPC and the ten min heated HP WPC were adjusted to pH 4.6 and again measured for turbidity and solubility. As would be expected at this pH, the solubility of all treatments decreased. The most interesting observation was that the 10 min heated HP WPC80 still had soluble protein after the pH adjustment. The pH of 4.6 was chosen because insoluble protein material at pH 4.6 was shown to contribute to gelation (Puyol et al., 1999). The insoluble material is generated through processing steps. It is a measure of denatured protein.

The more damage done to the protein during bleaching or other processing steps, the more aggregation and possibly gelation would be observed from heating. Because the unbleached WPC80 gelled with the same heat treatment as BP, it can not be concluded at this point that BP denatures the protein more compared to HP. It can, however, be concluded that the HP and BP bleaching treatments affect the protein differently. BP was previously reported to alter the electrophoretic patterns of whey proteins from Blue cheese made from milk bleached with BP (Washam et al., 1974). This effect was amplified when the cheesemilk was heated. Grindrod and Nickerson (1966) reported that HP in skim milk resulted in a decrease in whey protein nitrogen and an increase in non-protein nitrogen after exposure to a 0.5% and 1.0% HP solution at 49.9C. The increased HP concentration resulted in an increase in NPN. Cooney and Morr (1971) reported that proteose peptones were the most susceptible to alteration by HP followed by immunoglobulins, β-lactoglobulin, bovine serum albumin; α-lactalbumin was the least susceptible to HP. Schmidt and others (1983) reported HP treated WPC had altered methionine and cystine/cysteine concentrations which was suggested to affect gelation properties due to the importance of disulfide bonds. If HP

bleaching was not damaging the protein during processing then it would stand to reason that the unbleached WPC80 would have also exhibited similar behavior to the HP bleached WPC. This result was not observed. What seems more likely is that the HP damaged the protein differently than BP and the no bleaching WPC80 by possibly creating smaller fractions of protein which were unable to align quickly and gel within the 10 min of heating. After a whey protein is heat denatured, the unfolded protein will aggregate and form a network based upon electrostatic interactions, disulfide bonds, and hydrophobic interactions. If the HP denatured the protein into smaller fragments, it would take more time for the fragments to aggregate and align to form a gel. If sulfur containing amino acids are being damaged, this could affect disulfide bond formation and subsequent flavor development. HP appears to improve the heat stability temperature of the whey protein which may enhance functionality; however it is unclear if there is damage to the amino acids which would then affect the nutritional value. Further investigation into the effects each bleaching method has on protein should be explored.

CONCLUSIONS

Both hydrogen peroxide and benzoyl peroxide are viable bleaching methods for Cheddar cheese whey. Higher off flavor intensities and lipid oxidation associated with HP bleaching may limit application of whey proteins. Benzoyl peroxide creates less off-flavors and bleaches more efficiently than HP; however its limited use for export products greatly hinders its ability to be used commercially. BP at present is not an approved bleaching agent for whey products in China or Japan. Hydrogen peroxide may improve the heat stability of rehydrated WPC80 potentially enhancing its functionality in heat treated products.

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Tables and Figures

Table 1. Mean (n = 3) composition (% by weight) of the liquid 80% whey protein concentrate with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm) before spray drying.

	Unseparated whey			Separated whey		
Treatment	Total solids	Fat	CP	Total solids	Fat	CP
No color	6.87	0.23	0.91	6.71	0.04	0.9
No color + BP	6.85	0.21	0.91	6.7	0.03	0.9
No color + HP	6.9	0.23	0.93	6.73	0.04	0.91
Annatto	6.79	0.22	0.92	6.71	0.04	0.92
Annatto + BP	6.87	0.23	0.92	6.72	0.04	0.92
Annatto + HP	6.87	0.23	0.91	6.71	0.04	0.91
R - square	0.55	0.78	0.64	0.67	0.61	0.65
SE	0.039	0.005	0.01	0.012	0.002	0.009

No differences in means within the same column were detected ($P > 0.05$).
CP = crude protein (total nitrogen x 6.38); pH of the liquid WPC.
SE = Standard error.

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

		Dry basis		Wet basis	
Treatment	Moisture	Fat	CP	Fat	CP
No color	3.64	4.57	80.72	4.14	73.06
No color + BP	2.80	4.54	80.98	4.11	73.30
No color + HP	3.23	4.78	81.21	4.33	73.50
Annatto	3.89	4.84	81.25	4.38	73.54
Annatto + BP	2.64	4.65	80.15	4.21	72.54
Annatto + HP	2.76	4.61	80.31	4.17	72.69
R - square	0.76	0.64	0.88	0.64	0.88
SE	0.41	0.08	0.40	0.07	0.37

No differences in means within the same column were detected ($P > 0.05$).
CP = crude protein (total nitrogen x 6.38).
SE = Standard error.

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

	Phosphorous	Potassium	Calcium	Magnesium	Iron	Sodium	Sulfur
	-wt%-	-wt%-	-wt%-	-wt%-	-mg/kg-	-mg/kg-	-wt%-
No Color	0.356 c	0.564 b	0.516 a	0.062 a	10.8 a	2560 b	1.09 ab
No Color BP	0.38 ab	0.597 ab	0.538 a	0.061 a	10.2 a	2580 b	1.11 a
No Color HP	0.368 abc	0.577 ab	0.505 a	0.060 a	6.45 b	3030 a	1.09 ab
Annatto	0.364 abc	0.546 b	0.518 a	0.063 a	11.0 a	2740 ab	1.11 a
Annatto BP	0.381 a	0.529 b	0.538 a	0.060 a	9.63 a	2950 a	1.10 ab
Annatto HP	0.363 bc	0.682 a	0.500 a	0.061 a	7.50 b	2780 ab	1.07 b
R - square	0.32	0.53	0.61	0.27	0.79	0.58	0.39
SE	0.004	0.021	0.008	0.001	0.32	65.9	0.006

[a,b] Means in the same column not sharing a common superscript are different ($P < 0.05$).
SE = Standard error

Table 4. Mean (n = 3) color (L, a, and b-values) of liquid and spray dried 80% whey protein concentrate (WPC) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm).

	L-value		a-value		b-value	
Treatment	Liquid	Powder	Liquid	Powder	Liquid	Powder
No color	46.80^{d}	88.08^{c}	-0.82^{b}	1.75^{c}	4.88^{c}	7.76^{c}
No color +BP	53.75^{c}	88.59^{c}	-1.83^{c}	1.51^{c}	4.06^{cd}	7.22^{cd}
No color + HP	58.20^{a}	91.19^{a}	-4.18^{d}	0.53^{d}	0.27^{e}	6.39^{df}
Annatto	46.04^{d}	86.74^{d}	1.45^{a}	3.59^{a}	14.39^{a}	15.33^{a}
Annatto + BP	54.12^{c}	88.16^{c}	-1.55^{c}	1.63^{c}	5.82^{c}	8.54^{c}
Annatto + HP	56.44^{b}	90.13^{b}	-1.72^{c}	2.45^{b}	10.86^{b}	12.82^{b}
R - square	0.98	0.98	0.99	0.99	0.98	0.98
SE	0.52	0.17	0.13	0.09	0.55	0.35

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

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

No Color	No Color BP	No Color HP	Annatto	Annatto BP	Annatto HP	R - squared	**SE**
0.66 d	0.55 d	0.92 cd	18.90 a	1.45 cd	10.63 b	0.98	0.24

[a,b] Means in the same row not sharing a common superscript are different ($P < 0.05$).

SE = Standard error

Table 6. Means (n = 3 replicates with 10 panelists) sensory attributes[1] of 80% whey protein concentrate (WPC) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm) reconstituted to 10% solids.

	Aroma Intensity	Sweet Aromatic	Cereal/ Oatmeal	Cardboard	Fatty	Astringency	Cooked/ Milky
No Color	1.7 a	1.2 ab	1.3 a	1.5 c	ND	2.1 a	1.4 ab
No Color BP	1.6 a	0.8 b	0.8 a	1.9 bc	ND	2.0 a	0.6 b
No Color HP	2.1 a	ND	ND	2.4 a	1.4 a	2.2 a	ND
Annatto	1.9 a	1.8 a	0.9 a	1.0 d	ND	2.0 a	1.7 a
Annatto BP	1.7 a	0.7 b	ND	1.8 c	0.9 a	2.2 a	0.7 b
Annatto HP	2.1 a	ND	ND	2.3 ab	1.3 a	2.1 a	ND
R- squared	0.97	0.96	0.94	0.97	0.97	0.66	0.99
SE	0.044	0.096	0.11	0.063	0.082	0.073	0.012

[a,b] Means in the same column not sharing a common superscript are different ($P < 0.05$)

[1]Intensities were scored on a 0 to 15 universal scale where 0 = none and 15 = very high intensity (Meilgaard et al., 1999).

Dried whey ingredient intensities usually fall between 0 and 4 on this scale (Drake et al., 2003; Wright et al., 2009).

Attributes not listed were not detected (ND).

SE = Standard error

Table 7. Mean (n = 3) concentrations of selected aroma-active compounds (µg/L) of spray dried 80% whey protein concentrate (WPC) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm) isolated using solid-phase microextraction.

	No Color	No Color BP	No Color HP	Annatto	Annatto BP	Annatto HP	R - square	SE
2,3-Butanedione	0.505 bc	0.621 ab	0.349 c	0.777 a	0.690 ab	0.508 bc	0.30	0.06
Acetic Acid	0.175 b	0.216 ab	0.209 ab	0.266 ab	0.248 ab	0.288 a	0.45	0.022
3-Methylbutanal	0.556 a	0.492 a	0.700 a	0.734 a	0.601 a	0.617 a	0.46	0.057
2-Methylbutanal	1.03 ab	0.821 b	1.49 a	1.21 a	0.932 b	1.20 ab	0.37	0.11
***DMDS**	0.151 b	0.196 b	0.633 a	0.157 b	0.218 b	0.497 a	0.74	0.15
Toluene	6.76 a	3.12 bcd	2.11 d	4.05 bc	4.86 b	2.57 cd	0.51	0.43
1- pentanol	1.06 b	1.10 b	3.84 a	1.32 b	1.41 b	3.76 a	0.70	0.21
***Hexanal**	31.1 b	90.5 b	796 a	44.5 b	114 b	682 a	0.84	58.3
Z-4-Heptenal	0.016 b	0.002 b	0.122 b	0.122 b	0.414 a	0.159 b	0.38	0.056
***Heptanal**	1.74 d	10.2 cd	52.0 b	3.34 cd	18.0 c	76.9 a	0.78	3.8
α-Pinene	0.702 b	0.708 b	0.594 b	1.26 a	0.655 b	0.592 b	0.21	0.13
Benzaldehyde	0.326 a	0.252 a	0.181 a	0.184 a	0.169 a	0.146 a	0.82	0.024
DMTS	0.014 a	0.020 a	0.049 a	0.058 a	0.021 a	0.072 a	0.76	0.008
***1-octen-3-one**	0.182 c	0.211 c	0.559 b	0.210 c	0.289 c	0.893 a	0.68	0.046
β-Pinene	0.355 ab	0.297 b	0.306 b	0.460 a	0.371 ab	0.428 ab	0.43	0.028
β-Myrcene	0.252 d	0.546 b	0.355 cd	0.282 d	0.718 a	0.415 c	0.66	0.031
***2-Pentyl furan**	21.5 c	320.4 a	168 b	24.7 c	391 a	146 b	0.83	10.5
2,3-Octanedione	0.634 b	0.696 b	0.767 b	1.73 a	0.842 b	0.982 ab	0.41	0.18
Decane	12.4 b	11.6 bc	8.68 c	17.4 a	14.5 ab	12.1 b	0.61	0.65
***Octanal**	0.081 c	1.69 b	11.0 a	0.138 c	2.62 b	9.65 a	0.84	0.91
Limonene	8.46 ab	7.67 b	0.184 c	13.0 a	5.61 b	0.208 c	0.63	0.99
o-Cresol	0.011 c	0.069 a	0.055 ab	0.030 bc	0.084 a	0.080 a	0.57	0.007
2-E-Octenal	2.97 d	3.98 bcd	5.34 abc	5.83 ab	3.75 cd	6.29 a	0.50	0.41
p-Cresol	0.004 a	0.001 a	0.002 a	0.002 a	0.005 a	ND	0.32	0.001
***Nonanal**	1.71 e	7.05 d	22.2 b	4.22 d	14.2 c	28.2 a	0.82	1.16
E,2-Nonenal	0.022 a	0.007 a	0.008 a	0.019 a	0.013 a	0.024 a	0.40	0.004
***Decanal**	0.176 d	0.234 cd	0.420 ab	0.161 d	0.323 bc	0.513 a	0.74	0.022
E-E-2,4-Nonadienal	ND	ND	0.007 a	0.002 ab	ND	0.004 ab	0.45	0.001
Copaene	0.126 a	0.117 a	0.100 a	0.113 a	0.102 a	0.103 a	0.87	0.006
Spathulenol	0.080 a	0.082 a	0.066 a	0.122 a	0.110 a	0.092 a	0.62	0.012

[a,b] Means in same column not sharing a common superscript are different ($P < 0.05$).

[1]ND = Not Detected

Compounds with an asterix (*) are integrated to a five point external standard curve. All R^2 values are greater than 0.934.

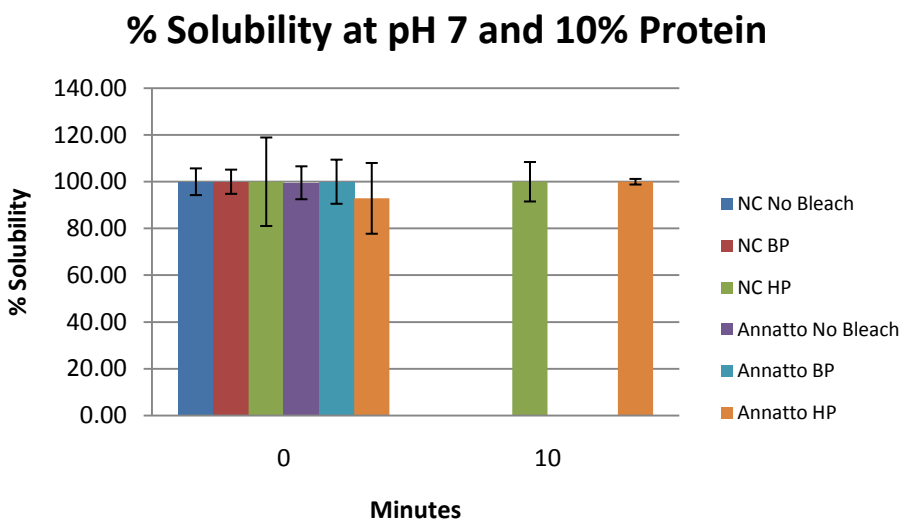

Figure 1. Percent solubility of WPC80 at pH 7 and 10% (w/v) protein heated for 0, 10, 20, and 30 min at 90^{o}C. WPC were manufactured with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm).
*Solutions not represented gelled therefore could not be measured

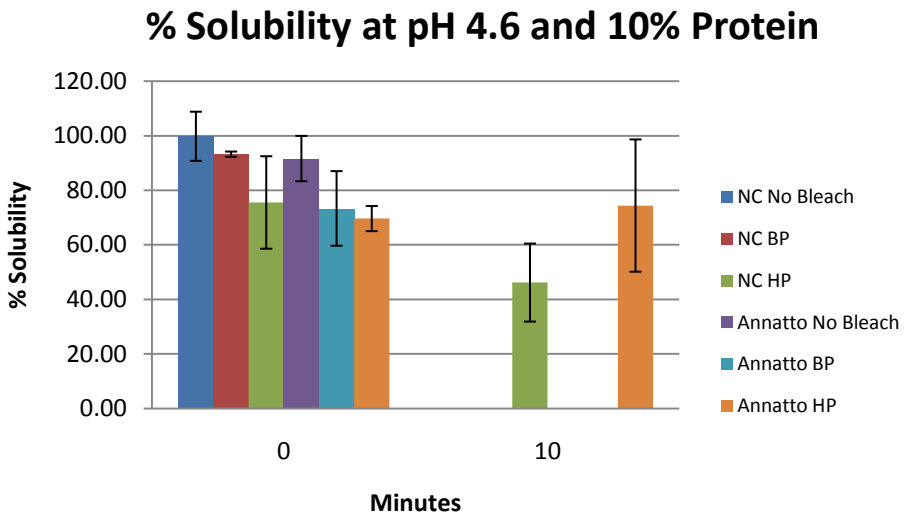

Figure 2. Percent solubility of WPC80 at pH 4.6 and 10% (w/v) protein heated for 0, 10, 20, and 30 min at 90°C. WPC were manufactured) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm).
*Solutions not represented gelled therefore could not be measured

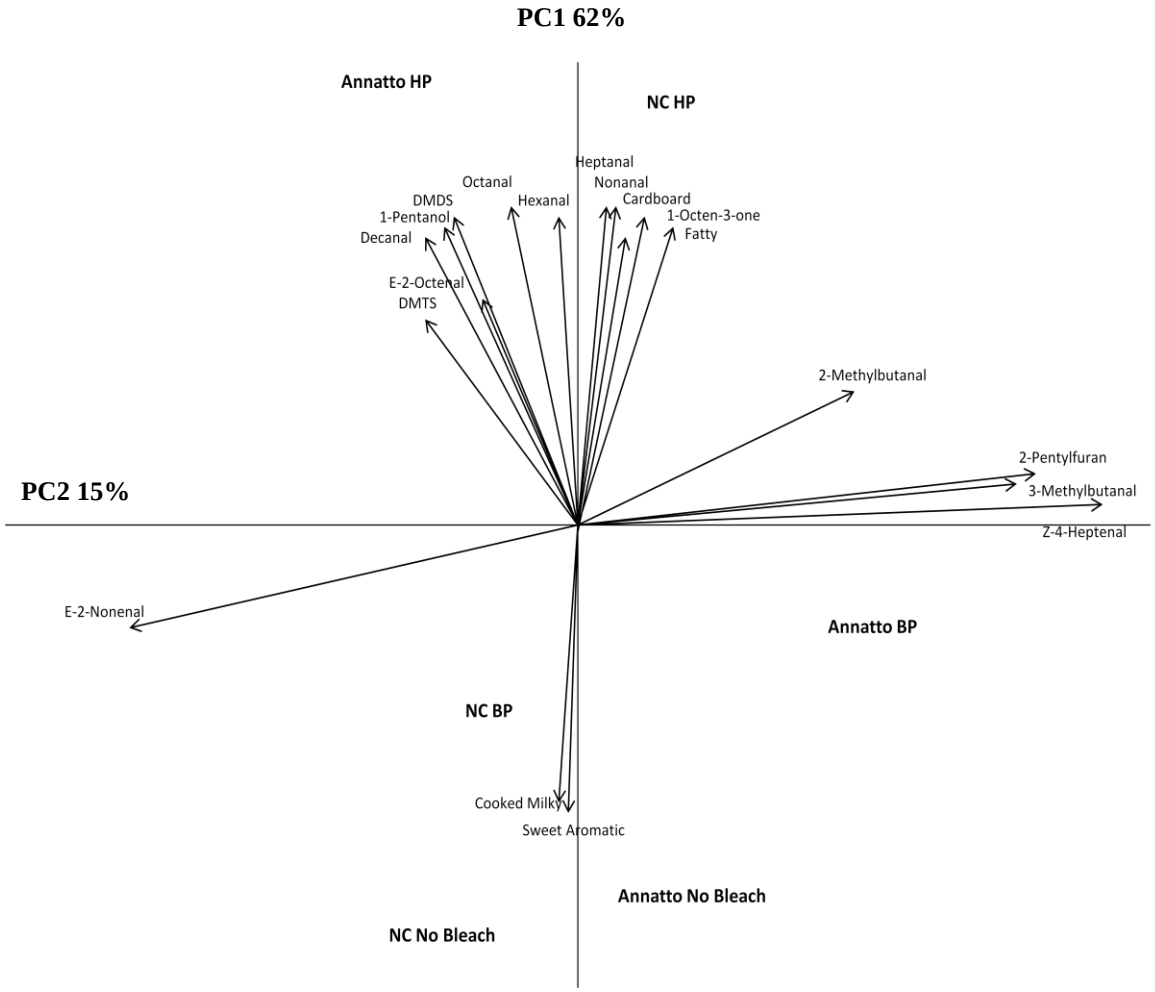

Figure 3. Principal component biplot of sensory attributes and selected lipid oxidation volatile components of 80% whey (WPC) with and without annatto color added to the milk followed by either no bleaching, bleaching with benzoyl peroxide (50 ppm) or bleaching with hydrogen peroxide (500 ppm).
PC1 and PC2 = principal components 1 and 2

CHAPTER 3: DETERMINING THE DRIVERS OF CHOICE OF LATTE-STYLE COFFEE BEVERAGES BY CHOICE BASED CONJOINT ANALYSIS

DETERMINING THE DRIVERS OF CHOICE OF LATTE-STYLE COFFEE BEVERAGES BY CHOICE BASED CONJOINT ANALYSIS

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RUNNING TITLE: Conjoint analysis of Latte-style coffee beverages

ABSTRACT

The objective of this study was to determine what factors were most influential in consumer choice of latte-style coffee beverages using ethnography and a choice based conjoint study. Ethnographical data was collected at four of the top producers of latte-style beverages. Attributes measured by the conjoint survey included location of purchase, milk-type, fat content, sweetener-type, and additional flavor. Consumer responses (n=721) from the conjoint survey showed that the most important attributes in determining latte beverage purchase intent were location and milk-type, followed by fat content, sweetener, and additional flavor. Segmentation of respondents based upon patterns in utility scores showed three distinct groups. Segment 1 (n = 185) was influenced by milk-type and sweetener-type. Segment 2 (n = 200) was influenced by a coffee house. Segment 3 (n=336) was calorie and health conscious. Self-defined lactose intolerant (n = 117) consumers preferred a lactose free dairy milk over a lactose free non-dairy milk lightener.

PRACTICAL IMPLICATIONS

Coffee is a multibillion dollar business and determining drivers of choice will greatly enhance how companies target consumers. Choice based conjoint analysis is a robust technique designed to collect large amounts of information of choice behavior in a format that is similar to how consumers really make choices. Observations of consumers making the choices in real world situations help in understanding the data collected in a conjoint study. Coffee companies will be able to utilize the results to better target the attributes that are driving consumer choice.

KEY WORDS

Coffee, Ethnography, Choice Based Conjoint, Lactose Intolerance

INTRODUCTION

Coffee is a multibillion dollar business with specialty coffee drinks becoming more popular everyday. Starbucks is the leader in the coffee beverage market with McDonalds, Dunkin' Donuts and Caribou as some of the top competitors (Starbucks Company Marketing Plan 2010, Flight 2007, and Caribou Coffee Company Inc. Annual Report 2010). Latte-style coffee beverages are some of the most popular beverages sold as specialty coffee drinks and are ubiquitous among specialty coffee drink manufacturers. A latte is defined as any coffee drink made with espresso, milk or a milk-type lightener, and may have an additional flavor added. Espresso differs from coffee in that it is more concentrated as it is prepared by forcing hot water with high pressure through the coffee grounds. This definition is an agglomeration of how a latte is defined at top grossing coffee retailers (Starbucks, Dunkin' Donuts, McDonalds, and Caribou). Lighteners and flavoring choices differ across manufacturers however all lattes are made with espresso, have a lightener, and offer the option of additional flavors.

When a consumer purchases a latte they first are choosing where to go to buy the latte. They have the choice of a coffee house, (Starbucks, Caribou, etc.), a fast food restaurant (Dunkin' Donuts, McDonalds, etc.), a sit down restaurant (IHOP, Panera, etc.), a convenience store (Sheetz, Wawa, Circle K, etc.), or a grocery store. The consumer then can choose the fat content and lightener-type of milk in the latte based upon the milk used as the lightener source. At present the only lightener options available to consumers are a) whole

milk (Starbucks, McDonalds), b) 2% milk (Starbucks, Caribou, Dunkin' Donuts), and c) fat free milk (McDonalds, Starbucks, Caribou, Dunkin' Donuts), and d) shelf-stable soy milk (Starbucks, Caribou). Lactose free milk is currently not available in specialty coffee restaurants as an alternative for lactose intolerant consumers. Consumers can choose to increase the sweetness of the beverage using sugar (white, raw, honey), no calorie sweeteners (Equal, Splenda, Sweet n' Low), and natural no calorie sweeteners (Stevia, Truvia). Consumers can also choose flavor syrups that can be sweetened with high fructose corn syrup, sucrose, glucose, or sugar free alternatives.

Conjoint analysis has been a method employed by marketers for years to determine what attributes drive choice. One of the first to use conjoint analysis was Green and Srinivasan (1978) who defined conjoint analysis as any technique that estimates consumer preference by evaluating all possible products defined by varying levels of product attributes. One of the most popular methods of conjoint analysis is choice based conjoint (CBC) (Sawtooth Software Inc. 2008). Choice based conjoint analysis is chosen widely because it measures consumer responses by presenting questions that reflect how consumers actually make decisions and therefore the method of questioning is more representative of a real world situation. It is also able to measure interactions between all attributes which can be very important in determining if an attribute alone has an effect on choice or if there is an effect when two attributes are presented together (Sawtooth Software Inc. 2008).

In choice based conjoint analysis, consumers are presented with different product concepts, which is an example product that contains one level of each attribute, and are asked to choose which concept is the most appealing. Each choice task includes a "none of these"

option so that consumers may indicate their lack of choice for the product concepts presented. Based upon consumer responses, how much value or utility each feature of the product has to the consumer can be determined. In a choice based conjoint there are attributes, which are the features of a product, and levels, which are the variances of each attribute. In a CBC, no more than six attributes and no more than nine levels for each attribute are recommended as the amount of information the consumer has to process can be quite daunting (Green and Srinivasan 1990, Sawtooth Software 2008).

Conjoint analysis has been employed in a variety of food and beverage applications (Nelson *et al.* 2005; Childs *et al.* 2008; Moskowitz *et al.* 2004; Beckley and Ashman 2004). Childs *et al.* (2009) used a choice based conjoint survey to determine that flavor was the most important attribute for consumption of fat reduced Cheddar and Mozzarella cheese followed by texture. Melo *et al.* (2010) examined acceptance of milk chocolate product concepts with different sweeteners for diabetic and nondiabetic consumers, and reported the sugar claim to be more important to diabetic consumers than non-diabetic consumers, and within that claim, sugar free or sugar reduced were the most important levels. Deliza *et al.* (2009) used choice based conjoint analysis to determine if irradiation of papaya negatively impacted choice and reported that a label indicating irradiation did not negatively impact choice of papaya.

It is important to understand not only what factors are driving choices of products, but also why those factors drive choice. Conjoint analysis with ethnographical observations can be used to answer both of these questions. Ethnography is the study of behavior through observations with the goal of describing the nature or behavior of the consumer.

Observations of consumers purchasing latte beverages can help understand choice behavior by telling the story behind the choices made in the conjoint. If consumers are reading, working, socializing at one venue, and taking their coffee to-go at another venue, then this yields information on why the consumers may go to these locations. To have a successful ethnographical study the observer must study the consumer in a natural setting, in the case of latte beverages, where the consumer buys lattes, and become a part of the coffee consumer experience (Elliott and Jankel-Elliott 2003). Agar (1996) described the essence of ethnographic methods consisting of participant observation in which one is directly involved in community life, observing and talking with people as you learn from them their view of realty.

It is important to determine what about a latte coffee beverage itself is important in determining choice and whether or not it acts in conjunction with the atmosphere or culture of where the beverage is being purchased. Determining what attributes of a specialty coffee drink drive consumer choice will be crucial for companies to produce products that complement what consumers determine to be important attributes. The objective of this study was two fold, first to determine the drivers of choice of latte-style beverages and second, to determine if lactose free milk was a viable lightener option over non-dairy based lactose free lighteners. A choice based conjoint was chosen for this study because the number of attributes and levels in the study were all below six. Interactions were not an initial concern; however it was desirable to know if they existed. Ethnography was used to assist in interpretation of conjoint survey results.

MATERIALS AND METHODS

Ethnography

Ethnographical data was collected at four top grossing coffee restaurants (Starbucks, Caribou, Dunkin' Donuts, McDonalds) in the form of observations. There is no set method for collecting ethnographical data however it is generally accepted that in order to understand the topic of study, the researcher must become a part of the culture (Elliott and Jankel-Elliott 2003). To do this the observers purchased latte-style coffee beverages and sat among the consumers to be presented as an average coffee consumer. The observers took notes on laptop computers. Five locations of each coffee restaurant-type were chosen for observations around the Raleigh, NC area. At each location for each restaurant-type, a one hour observational period was conducted for a total of 10 h of observation per restaurant-type. Observations were equally distributed among the early morning coffee consumption hours (7:00 – 9:00am), mid-morning (9:00-11:00am), mid-afternoon (2:00 – 4:00pm), evening (5:00 – 7:00pm), and late evening (7:00pm – 9:00pm). Locations within restaurant-type were randomly chosen for each evaluation time point.

If a restaurant had a drive-thru option, observers positioned themselves so that they could see the drive-thru window and what was prepared for that customer. During the observational period, a checklist of behaviors was collected as well as a free-flow format of observations where the observer freely typed about the behavior and atmosphere of the location (Table 2). At the end of the observation the data was analyzed for frequency of observations and general "feel" of the location. All observations for a restaurant-type were combined to tell the general "story" of the restaurant-type. The results of the observations

were used to help develop the behavior questions of the conjoint survey as well as deciding what levels were important for each attribute. Ethnographical data was also used to understand the conjoint survey results.

Conjoint Analysis

An online survey was created using SSI Web (Sawtooth Software version 7.0.22, Sequim, WA). Five attributes with corresponding levels were chosen based upon what products were currently available to consumers at the four highest producers of made-to-order latte-style coffee beverages (Table 1). Levels of product attributes that were not available at all possible locations were included as it was useful to determine if those products were available, how much utility consumers would ascribe to them.

Locations were chosen based upon the broad categories of places consumers were able to purchase made-to-order latte-style coffee beverages in the Raleigh, NC area. Each level was defined for the consumer using examples so that there was no confusion between levels (e.g. fast food restaurant – Dunkin' Donuts). Milk/lightener-type represented the two types of lighteners currently available (milk and lactose free non-dairy based) as well as the currently unavailable option of lactose free milk. Again each level was presented with examples so that consumers understood the difference between each level. Fat content and sweetener-type reflected what was available at all locations but not all options were available within one location. Sweetener-type was presented with examples of what each level represented. At all locations the option of additional flavor was available. It was not important to evaluate the type of flavor (caramel, raspberry, hazelnut, etc) as the variety of flavors available would exceed the amount of levels allowed in a CBC and was not the

primary focus of this study. Therefore only two levels were presented for this attribute, additional flavor and no additional flavor.

The survey was designed with fourteen choice tasks with three product concepts per task with a "none of these" option for each choice task. In each choice task, the consumers were presented with three product concepts and a fourth "none of these" option. Each product concept was a random combination of levels for each attribute with each attribute represented in every product concept. This study used a balanced overlap design and 300 versions of the survey were created. Once the survey was constructed it was uploaded to an internet web-server. Participants (n=767) were recruited through email listservs that contained a web link that directed them to the survey. Consumers were first guided through a demographic screener. The purpose of the screener was to first gain basic information about the population taking the survey, and second to eliminate any participants who were below the age of 18 y, did not buy coffee drinks at least once a month, or only consumed black coffee. After the demographic questions, consumers began the second part of the survey which asked questions about coffee consumption and purchase behavior questions. The third part of the survey was the CBC survey. Once consumers completed the entire survey they were entered into a drawing to receive a twenty dollar gift card to a local shopping store.

A market simulator (Sawtooth Software, Sequim WA) was used to compare ideal products that differed based upon lactose free lightener options. Utility scores of consumers with self-defined mild to severe lactose intolerance (n=117) were loaded into the market simulator with the only difference being one product used a lactose free milk lightener, and

the other used a lactose free non-dairy based lightener. This was done to determine the effect of the product claim based upon the lightener option to those consumers most likely to choose a lactose free lightener. The market simulator provided two models showing product shares of preference and purchase likelihood (Melo *et al.* 2009). The product shares of preference provided an overall percentage of preference out of 100% for each product.

Statistical Analyses

Individual utility scores were extracted by Hierarchical Bayesian (HB) estimation and rescaled using a zero-centered differences method (Sawtooth Software, Sequim WA). The zero-centered differences method standardizes all attribute utility scores so that easy comparisons are able to be made. Individual utility scores were analyzed using Latent class analysis (Sawtooth Software, Sequim WA) and used to determine segmentation of groups. Latent class analysis was also used to measure 2 and 3-way interactions among attributes. Importance scores were determined by calculating the utility score range of each attribute and dividing by the total utility range multiplied by one hundred (Orme 2010). Root Likelihood Values (RLH) were analyzed and used to remove respondents with an RLH value of 0.333 and lower (Sawtooth Software 2009). The RLH value is a prediction of respondent choices in a CBC and corresponds to the number of product concepts. In a survey with four product concepts (including the none of these option) consumer responses should be predictable 25% of the time (0.250). RLH of lower than 33% was chosen as an overestimation. Removal of lower RLH panelists was done prior to utility estimation. A one-way ANOVA with Fisher's least significant difference as the post hoc test was used for analysis of the zero-centered utility scores for the total population (n=721) using XLSTAT

Addinsoft version 2010.5.02 (New York, NY). Latent Class analysis was performed with Sawtooth Software SMRT (Sequim, WA). Market Simulator analysis was performed with Sawtooth Software SMRT version 4.20 with Chi Square analysis using XLStat. PCA analysis of clusters was performed using XLSTAT. Demographic and behavior questions were analyzed for frequency of choice. All statistical analyses were carried out at a 95% significance level.

RESULTS AND DISCUSSION

Ethnography

Starbucks was loud, filled with noise from incoming customers, baristas preparing drinks, taking orders, music playing, and customers socializing. The early morning hours were primarily filled with consumers getting coffee drinks on their way to start their days. Most customers were dressed in business attire and many modified their orders by adding their own lighteners and additional sweeteners after the beverage was prepared by the barista. More black coffee was ordered in the first few hours of the store being open than later in the day. On average, thirty-five black coffees were ordered from 7:00-9:00am and only an average of seventeen from 9:00am – 11:00am. As the morning progressed, the consumer attire changed to casual and many stayed to socialize or read. Customers utilized the free Wi-Fi access and worked on laptop computers. There was a steady flow of traffic throughout the day but the morning hours (7:00 – 9:00am) were the peak time. In the evenings, more indulgent beverages were purchased and less black coffee. Only 10-20% of the total coffee purchases made after 6:00pm were black coffee. Coffee purchases and socializing kept the store busy into the late evening hours (8:00 – 9:00pm).

Caribou offered similar options to consumers as Starbucks but an entirely different atmosphere. In Caribou the environment was quiet except for soft music that played throughout the store. Like Starbucks, the majority of the traffic flow was in the morning but more consumers stayed and worked either alone or in groups. An average 25% of the morning hour patrons (7:00-9:00am) stayed to work where only an average of 4% of Starbucks consumers stayed to do work. The Caribou restaurant was filled with large tables with multiple chairs and many of the locations observed (n=3) had separate rooms that could be reserved for meetings. Free Wi-Fi access was also offered at Caribou, and more consumers were observed with laptop computers at all hours at Caribou than any other coffee restaurant-type. On average, three consumers worked on laptop computers at Caribou at all observation periods where the average for Starbucks was one. Laptop use was not observed at McDonalds or Dunkin' Donuts. Black coffee that was modified at the condiment table as well as indulgent beverages was purchased throughout the day at Caribou. Individual customers stayed longer at Caribou (1hr +) than at Starbucks although rarely were extra beverages ordered; they stayed to continue working. During the ten hours of observation at Caribou, three different interviews were observed. This activity was not observed at any other coffee restaurant-type. Caribou created a relaxed atmosphere with their décor of a mountain lodge and their large comfortable chairs and tables.

Both McDonalds and Dunkin' Donuts offered Wi-Fi access, McDonalds was free, Dunkin' Donuts was not. At McDonalds almost all of the coffee purchases occurred before 11:00am even though coffee beverages can be purchased all day long. An average of ten consumers purchased coffee before 11:00am whereas only an average of two consumers

purchased after 2:00pm. Some McDonald's restaurants used for observations were modified McCafés (n=2). McCafés are designed to look more like a coffee house. Unlike Starbucks and Caribou, consumers coming to order coffee and socialize were not observed nor were observations of customers coming to do work in the restaurant. The primary coffee consumer at McDonald's ordered black coffee and modified it themselves with lightener and sweetener at the condiment table. Some customers would order the made-for-you lattes, mochas, and cappuccinos but this was not observed as much as the black coffee consumer (30% of coffee purchases were lattes/mochas/cappuccinos). More families with children (59% of total families observed at all locations were at McDonalds) were observed at McDonalds than any other coffee restaurant-type. Families ordered breakfast and the parents sometimes ordered a coffee beverage for themselves. The majority of consumers ordered through the drive-thru window (62%).

Dunkin' Donuts had the majority of the coffee consumer traffic in the morning hours which was generally accompanied with a donut purchase. An average of fifteen consumers purchased coffee from 7:00-9:00am which dropped to an average of two consumers after 2:00pm. The most popular morning beverage purchased was iced coffee followed closely by black coffee, both of which were modified with lightener and sweetener. The drive-thru was the preferred method of coffee purchase as the majority of consumers who made their purchases inside the restaurant did so while ordering donuts (76% of in restaurant purchases purchased donuts and coffee). As the morning progressed (after 11:00am) the coffee consumer frequency dropped. The majority of the evening and late night consumers ordered donuts or ice cream (n=2 Dunkin' Donuts restaurants used for observations also sold Baskin'

Robin's ice cream) but not coffee beverages. On two observations (2 h total) no coffee beverages of any kind were purchased after 5:00pm.

Conjoint Analysis

A total of 756 respondents were surveyed; after removal of RLH values 0.333 and below, 721 total respondents were used for the data analysis. Demographic information for the total sample size surveyed is presented in Table 3. A utility score (also known as a part worth utility) represents the attractiveness of each feature in a conjoint study, where the higher the utility score the more that attribute or level drove choice. Individual utility scores can be generated and used for future analysis in market simulators or in cluster analysis. Hierarchical Bayesian estimation is used to estimate individual utility scores and has been described favorably by researchers as the technique that has the ability to estimate individual part worths given only a few choices by an individual (Sawtooth Software 2009). Latent Class analysis is also a useful technique for determining if groups with similar preferences exist within the population (Sawtooth Software 2009).

Estimation of the part-worth utilities for the total population surveyed (n=721) revealed consumers had the most utility for coffee houses as a location for purchasing coffee beverages, followed by a fast food restaurant, a sit-down restaurant, a convenience store, and a grocery store ($P<0.05$) (Figure 1). A milk-based lightener had the most utility followed by a lactose free milk-based lightener and then a lactose free non-dairy-based lightener ($P<0.05$). Reduced fat had the most utility followed by fat free and then full fat ($P<0.05$). Natural no calorie sweetener had the most utility followed by sugar, no calorie sweetener, and then no sweetener ($P<0.05$). Flavor had more utility over no additional flavor ($P<0.05$).

When consumers were asked in the coffee purchase and consumption part of the survey which location they primarily went to for coffee beverage purchases, 58.5% selected coffee houses followed by fast food restaurants, sit-down restaurants, convenience stores, and then grocery stores (Table 4). The results of the CBC were in alignment with the survey questions where respondents were asked directly about their location preferences. Respondents indicated that convenience and price were more important than location (Table 4). Fast food restaurants offer latte beverages at lower prices than coffee houses however, the utility of fast food restaurant as a location for latte purchase was lower than a coffee house, this suggests there are other factors influencing coffee purchase at coffee houses that factor into the overall utility of a coffee house as a location. Many coffee houses are now offering drive-thru purchasing for a more convenient purchasing experience. Observations of consumers at coffee houses (Caribou and Starbucks) showed consumers coming in to purchase beverages and leaving right away in the early morning hours (7:00am – 8:00am) and as the day progressed consumers were more likely to stay and drink their beverages in the coffee house. The frequency of early morning consumers coming and going suggests that consumers are taking their coffees and lattes to work with them. Observations coupled with the CBC results suggest that consumers are able to have a convenient experience of purchasing a beverage quickly, and also have the utility of the overall coffee house experience. Morning (7:00am – 8:00am) observations of consumers at Starbucks and Caribou revealed that many consumers were dressed in business attire when ordering a beverage. The majority of early morning McDonalds and Dunkin' Donuts consumers used the drive-thru. When consumers were asked what they are doing most of the time when

consuming coffee drinks, 30.7% said they were at work and 17.9% said they were in the car (Table 4).

Fat reduction (reduced or fat free) had higher utility than full fat (Figure 1). Reduction in fat sates the desire for a healthier option without completely compromising a full fat flavor and mouthfeel. Fats are very important in the development of flavors because fats act as solvents for flavor compounds and with heat can generate flavor reactions (Cheetham 2010.) Fat free options are becoming increasingly popular with the advent of sugar-free flavor syrups. Consumers are able to sacrifice fat content and still have flavor from flavor syrups without the added calories of the sugar-based syrups. The higher utility ascribed to reduced fat and fat free are in agreement with the surveyed responses from the consumption/purchasing behavior section of the survey. When respondents were asked if they agreed having a healthy balanced diet was important to them, 84% agreed or strongly agreed (Table 4). Only 20% of the respondents surveyed indicated they were currently on some type of diet however 74% indicated they were concerned about what they ate/drank (Table 4).

A milk-based lightener had the highest utility as a lightener source followed by lactose free milk. Respondents were asked to self-identify lactose intolerance status (Table 4). Out of the total 721 respondents, 604 indicated they had no intolerance to lactose or only occasional intolerance. Only 3.6% of respondents indicated they used lactose free milk as a lightener and 10.7% used soy milk for their coffee beverages (Table 4). Soy milk is the only lactose free option currently available in coffee beverage restaurants. Palacios *et al.* (2009) conducted consumer testing of cow's milk versus soy milk and reported that consumers

preferred cow's milk over soy milk. They also reported that lactose-free cow's milk was preferred over soy based beverages. Consumers with no physical need to switch to a non-dairy based lightener are much less likely to try lactose-free alternatives. The remaining respondents (n=117) indicated that they had mild to severe intolerance. A market simulator was run on this population comparing two beverages comprised of the levels of the attributes with the highest utility scores, (coffee house, reduced fat, natural no calorie sweetener, flavor), that differed only by the lightener source. Beverage A used a lactose-free milk based lightener and beverage B used a lactose free non-dairy based lightener. Beverage A with a lactose free milk-based lightener was preferred over the non-dairy option (56% vs. 44%) ($p<0.05$). This result suggests that offering lactose-free milk as a lightener source in lattes would be preferred more often than lactose free non-dairy based lighteners (e.g. soy milk) by the lactose intolerant community.

A natural no calorie sweetener had the highest utility followed by sugar, no calorie sweeteners, and then no sweetener. Thirty percent of respondents indicated they used no additional sweetener in their coffee and 25% didn't want their coffee beverages to be sweet at all. No sweetener had the lowest utility in the conjoint analysis (Figure 1). Many latte-style coffee beverages are sweetened by the flavor syrups used which may not require additional sweeteners to be added. The word "natural" has a positive connotation to consumers. Melo *et al.* (2010) reported product concepts of chocolate made with natural sweeteners to have higher utility than artificial or a combination of sweeteners. Natural no calorie sweeteners such as Truvia or Stevia, have been shown to have a negative aftertaste and lingering bitterness (Abelyan *et al.* 2004; Cardello *et al.* 2007). Melo *et al.* (2009)

reported chocolates made with Stevia had increased bitterness and bitter aftertaste. In a conjoint study, products are evaluated for their concept alone. A latte beverage with a natural no calorie sweetener was the most appealing to the consumer however the actual product itself may not be optimized based upon the flavor. Other conjoint studies have reported conjoint results not translating to consumer acceptance testing (Melo *et al.* 2010; Cheng *et al.* 1990).

The option of flavor was more appealing than no additional flavor (Figure 1). As mentioned previously, flavor is the most important attribute of a food or beverage product. The option of more flavor will therefore have a positive connotation to the consumer. Respondents indicated flavor as the most important feature of a coffee beverage, although this question referred to the overall flavor of the coffee beverage and not just the additional flavor added through flavor syrups (Table 4). Overall the attribute that was most important in the latte purchase experience was location followed by lightener choice. Fat content, sweetener content, and additional flavor all had the same level of importance (Figure 2).

Interactions were observed between location and flavor and between fat content and sweetener ($P<0.05$). Coffee houses, fast food restaurants, and sit-down restaurants were all perceived as places to have coffee beverages with additional flavors whereas convenience stores and grocery stores were not. This is most likely due to the availability of made-to-order latte beverages at convenience stores and grocery stores where machines are used to make the pre-formulated latte beverages instead of a barista. Fat free and reduced fat had the highest interaction effect with no calorie sweeteners and sugar ($P<0.01$). Respondents wanted reduced fat beverages that were still sweet. Respondents were willing to sacrifice

flavor and mouthfeel from fat content, but not flavor from additional sweeteners. There is a group of consumers not willing to consume additional calories through sugar-based sweeteners and desired no calorie sweeteners in conjunction with the reduction in fat.

Segmentation of respondents based upon patterns in utility scores showed three distinct groups (Table 5). Segment 1 were the "indulgent consumers" (n = 185). The indulgent consumers were influenced by lightener-type and sweetener, specifically milk-based and sugar (Table 5). Segment 1 was the only group to have a higher utility for no additional flavor than additional flavor (Table 5). Segment 1 had a large percentage of 25-29 yr olds suggesting that the younger coffee consumers can indulge with less concern for calorie and fat content. Segment 1 also drank coffee/espresso beverages less frequently than either Segment 2 or Segment 3, used whipped cream more and if given the option would choose cream or half and half as a lightener source (Table 4). Consumers in this age group were observed more in the evening hours where as during the morning hours the ages were more evenly distributed. The evening hours were past dinner and perhaps the coffee beverages were seen as a sweet treat or a dessert.

Segment 2 (n = 200) were the "coffee house" consumers and they were influenced by location; coffee house (Figure 2 and Table 5). Segment 2 had more people ages 30 – 44 yrs who purchased coffee early in the morning (7:00am – 8:00am) and consumed coffees in the car on the way to work. Observations of consumers suggested that the majority of consumers at coffee houses (Starbucks, Caribou) purchased coffee beverages in the early morning hours and left (7:00-9:00am). Consumers at fast food restaurants (Dunkin' Donuts, McDonalds) also purchased their coffee beverages and left presumably to go to work or start their day.

Fast food restaurant coffee consumers were more concerned with getting the beverage quickly and getting on with their day where as coffee house consumers used the coffee house experience as part of their day. Early in the morning consumers of all ages came to purchase coffee beverages but as the morning progressed (8:00am – 10:00am) a slightly older group of consumers arrived and stayed to socialize or read, usually the newspaper. In the evenings the coffee houses were primarily filled with younger consumers studying, working, or socializing. Fewer consumers in Segment 2 consumed coffee beverages while out running errands compared to segments 1 and 3, suggesting again that the act of going to a coffee house for a coffee beverage purchase was part of the experience and not necessarily done on the way to do something else (Table 4). Segment 3 consumers (n=336) were the "health conscious" consumers. This segment was driven by fat reduction and no calorie sweeteners, specifically natural no calorie sweeteners, and also by additional flavor (Figure 2 and Table 5). Segment 3 was comprised of more men and more people ages 45 – 54 yr who were more concerned with having a balanced diet, what they consumed, and how much they consumed (Table 4). All segments had the highest utility for coffee house as a location however this particular attribute was the most distinguishing for Segment 2 (Figure 2). Consumers who had obviously just exercised, usually bike riding based upon their dress and equipment, were observed coming to coffee houses (Starbucks only) to have a coffee beverage. Consumers post-exercise were not observed at any other coffee restaurant location.

Conclusion

Latte-style beverages are a growing trend that is no longer associated with the specialty coffee consumer, but has found widespread popularity with the average coffee

consumer. The culture developed by coffee houses is the most influential factor in the latte purchase experience and will continue to be so as long as coffee houses continue to foster environments conducive to socializing and studying/working. Indulgent beverages will continue to appeal to a specific segment of consumers concerned with flavor. Calorie and fat reduced beverages are key to targeting consumers concerned with their health/diet. Lactose intolerant consumers prefer lactose free milk as a lightener option over lactose free non-dairy based alternatives. The latte beverage experience is driven by the experience of the latte purchase as well as what makes up the latte itself. Consumers are driven by how they feel when buying the beverage, the atmosphere created by the location, as well as what options are available to them to customize a beverage based upon lightener-type, fat content, sweetener-type, and additional flavors.

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Tables and Figures

Table 1: Attributes and levels for conjoint analysis

Attribute	Level	Example
Location	Coffee House	Starbucks, Caribou
	Fast Food Restaurant	McDonalds, Dunkin' Donuts
	Sit Down Restaurant	Panera, IHOP
	Convenience Store	Sheetz, Circle K
	Grocery Store	Harris Teeter, Kroger
Lightener Type	Milk Based	Regular Milk
	Lactose Free Milk Based	Lactaid, Real Goodness
	Lactose Free Not Milk Based	Soy
Fat Content	Full Fat	
	Reduced Fat	
	Fat Free	
Sweetener	Sugar	White, Raw, Honey
	No Sweetener	
	No Calorie Sweetener	Splenda, Equal, Sweet n’ Low
	Natural No Calorie Sweetener	Stevia, Truvia
Additional Flavor	Flavor	
	No Flavor	

Table 2: Checklist of observations for ethnographical analysis

Observation
Gender
Approximate age
Frequency of coffee customers for each time of observation.
Did they smile when they received their coffee?
What kind of coffee did they order (size, cream, sugar, etc)?
Did they order more than one coffee?
Did they order any food/indulgent treat with the coffee?
Did they stay and drink their coffee? If so what are they doing while they stay? (e.g.
Approximate amount of time from ordering to receiving a beverage?
Is there a drive thru? If so can the drive thru window be observed?
How many people utilize the coffee condiment table? What are they using?
How many people come in with other people/friends? How large are the groups?
Did they meet someone at the location?
Do they make special requests/modifications & how are those requests received?
Frequency of parents bringing children.
For parents that come in with children, how many order coffee for themselves?
How many order a treat or a coffee-type treat for their children?
Overall atmosphere and other observations

Table 3: Respondent demographics from conjoint survey

		Total	Segment 1	Segment 2	Segment 3
		n = 721	n = 185	n = 200	n = 336
Gender	Male	25.7	23.5	24.1	28.9
	Female	74.3	76.5	75.9	71.1
Age	Under 18 years old	0.0	0.0	0.0	0.0
	18 - 24 years old	15.7	15.2	16.0	16.0
	25 - 29 years old	12.3	17.2	12.1	9.0
	30 - 34 years old	13.6	12.3	16.0	12.5
	35 - 39 years old	11.2	9.8	12.5	11.3
	40 - 44 years old	9.4	8.3	10.9	9.0
	45 - 49 years old	10.3	8.8	9.8	12.1
	50 - 54 years old	9.4	8.8	7.4	12.1
	55 - 59 years old	7.6	6.9	7.4	8.6
	60 - 64 years old	6.1	6.9	6.6	5.1
	65 - 69 years old	2.4	2.9	2.0	2.3
	70 years old and older	1.9	2.9	1.2	2.0
Annual Household	Under $20,000 per year	11.9	10.3	13.3	12.1
	$20,000-$34,999 per year	13.9	15.7	12.1	14.5
	$35,000-$49,999 per year	17.1	17.6	17.2	16.8
	$50,000-$64,999 per year	13.6	12.3	15.2	13.3
	$65,000-$79,999 per year	13.5	14.2	14.1	12.5
	$80,000-$94,000 per year	13.0	10.8	13.7	14.5
	More than $95,000 per year	17.1	19.1	16.4	16.4
Highest Level of	Middle School	0.0	0.0	0.0	0.0
	Some High School	0.6	0.5	0.4	0.8
	High School Diploma	4.9	4.9	3.9	5.9
	Some College	18.4	15.7	21.9	17.6
	Associates Degree	8.6	5.9	10.5	9.0
	B.A./B.S. Degree	32.3	36.3	29.3	32.8
	Some Post Graduate School	9.0	8.3	7.8	10.9
	M.S. Degree	18.7	21.1	19.1	16.8
	Doctorate or Professional School	7.5	7.4	9.0	6.3
Ethnicity	American Indian or Alaska	1.1	2.0	1.6	0.0
	Asian	6.8	5.9	7.0	7.4
	Black or African American	14.7	13.7	15.2	15.2
	Hispanic	2.6	2.0	3.5	2.3
	Latino	1.2	1.5	2.0	0.4
	Native Hawaiian or Other	0.3	0.0	0.4	0.4
	White	74.5	77.5	72.7	75.4
	Other	0.7	0.5	0.8	0.8

*Ethnicity was a check all that apply. Total percentages within a column will be greater than 100%

Table 4: Purchase behavior and coffee consumption demographics by segment

		Total Population	Segment 1	Segment 2	Segment 3
		n = 721	n = 185	n = 200	n = 336
How often would you say you drink coffee/espresso or a drink made with coffee/espresso?	More than once a day	40.6	39.7	40.2	41.8
	Once a day	31.5	28.9	33	31.6
	A few times per week (2 - 6)	19.1	22.1	19.9	16
	A few times per month (1 - 4)	8.7	9.3	6.5	10.5
	At least once in the last 3 months	0	0	0	0
	At least once in the last 6 months	0	0	0	0
	I rarely/never drink coffee	0	0	0	0
*What kind of lightener do you use in your coffee beverages?	Whole Milk	23.2	21.6	24.9	22.7
	1 or 2% Milk	35.1	34.8	37.2	33.2
	Skim Milk	24.4	25.5	22.6	25.4
	Cream	33	34.8	35.6	28.9
	Half & Half	45.2	46.6	43.7	45.7
	Instant/powdered creamer	23.6	26.5	23	21.9
	Lactose free milk	3.6	3.9	3.1	3.9
	Soy Milk	10.7	11.3	9.6	11.3
	Rice Milk	1.4	2.5	1.1	0.8
	Almond Milk	6.5	6.4	4.6	8.6
	Whipped Cream	12.6	16.7	11.9	10.2
	Other	7.6	7.8	8	7
	I do not use any lightener (I drink black coffee)	15.3	15.2	16.1	14.5
If all options were available to you, which would be your first choice to use?	Whole Milk	5	3.9	5.4	5.5
	1 or 2% Milk	10	10.3	9.6	10.2
	Skim Milk	9.4	8.8	8.4	10.9
	Cream	16	17.2	15.7	15.2
	Half & Half	29.1	31.4	28.7	27.7
	Instant/powdered creamer	5.7	4.9	7.3	5.1
	Lactose free milk	1.5	2	1.1	1.6
	Soy Milk	4.3	3.4	3.8	5.5
	Rice Milk	0.1	0	0	0.4
	Almond Milk	2.6	2.9	1.5	3.5
	Whipped Cream	1.5	2.5	0.8	1.6
	Other	3.9	3.4	5.4	2.7
	I do not use any lightener (I drink black coffee)	10.7	9.3	11.9	10.2

Table 4: Continued

		Total Population	Segment 1	Segment 2	Segment 3
		n = 721	n = 185	n = 200	n = 336
*What kind of sweetener do you use in your coffee beverages?	Regular white sugar	40.5	38.7	40.2	42.2
	No calorie sweetener (e.g. Sweet n' Low, Equal, Splenda)	33.4	38.2	30.7	32.4
	Natural no calorie sweetener (e.g. Truvia, Stevia)	11.2	11.8	10	12.1
	Natural sweetener (honey, agave)	11.2	12.7	10	11.3
	Other	7.1	5.4	9.6	5.9
	I do not use any sweeteners	29.5	30.9	29.9	28.1
Where do you primarily go to buy a coffee drink?	Coffee Houses (Starbucks, Caribou, Port City Java, etc)	58.5	56.4	60.5	58.2
	Fast Food Chains (McDonalds, Dunkin' Donuts, etc)	21.9	22.5	21.5	21.9
	Sit-down Restaurant (Panera, IHOP, etc.)	3.2	2.9	4.2	2.3
	Convenience Stores (7-11, Sheets, Gas Station, etc)	4.6	4.4	3.4	5.9
	Grocery Store (Pre-made/pre-packaged drinks)	6.4	6.4	6.1	6.3
	Other	5.4	7.4	3.8	5.5
How often do you purchase coffee drinks?	Two or more times per week	31.5	32.8	29.9	32
	At least once per week	25.2	26	23.8	26.2
	At least 2 – 3 times per month	18.4	19.6	20.7	15.2
	At least once per month	11.7	10.3	11.5	12.9
	At least once in the last six months	5	2.9	5.7	5.5
	Almost never, I primarily make coffee drinks at home	8.2	8.3	8	8.2
When is the most frequent time for you to drink coffee?	Early morning (5:00am - 8:00am)	32.3	27.5	33.7	34.8
	In the morning (8:00am - 10:00am)	50.6	56.4	49.4	46.9
	Mid morning (10:00am - 12:00pm)	6.7	7.4	6.5	6.3
	Lunch time (12:00pm - 2:00pm)	1.7	1	2.3	1.6
	Mid afternoon (2:00pm - 5:00pm)	5.1	5.4	3.8	6.3
	Evening (5:00pm - 7:00pm)	1.5	2	1.9	0.8
	Late evening (7:00pm - 9:00pm)	1.4	0	1.1	2.7
	Late Night (9:00pm - Midnight or later)	0.7	0.5	0.8	0.8
Where are you during that time?	At home	41.6	40.2	41	43.4
	At work	30.7	31.9	31.4	28.5
	In the car	17.9	16.2	18.8	18.4
	At a restaurant	0.7	0	1.1	0.8
	At a coffee shop	4.4	5.4	4.6	3.5
	Out shopping/running errands	2.8	4.9	0.8	3.1
	Other	1.9	1.5	1.9	2.3

Table 4: Continued

		Total Population	Segment 1	Segment 2	Segment 3
		n = 721	n = 185	n = 200	n = 336
**When you are choosing a coffee beverage, how important are each of the following	Price	3	2.9	2.9	3.1
	Flavor	4.3	4.3	4.3	4.4
	Lightener type	2.7	2.8	2.6	2.8
	Sweetener type	2.6	2.6	2.5	2.8
	Nutritional value	2	2	2	2
	Convenience	3.4	3.4	3.3	3.4
	Availability	3.6	3.5	3.6	3.6
	Texture/Mouthfeel	3.4	3.4	3.4	3.4
	Appearance	3	3	3	3.1
	Place where I'm getting the beverage from	2.7	2.7	2.7	2.8
**How important are each of the following to you when choosing a coffee beverage?	Fat content	2.4	2.4	2.4	2.5
	Calorie content	2.5	2.5	2.4	2.6
	Lactose free lightener availability	1.5	1.5	1.4	1.5
	Non-white sugar sweetener availability	1.8	2	1.7	1.9
How would you describe yourself?	Highly lactose intolerant	1.9	1.5	1.9	2.3
	Moderately lactose intolerant	4.9	5.4	4.2	5.1
	Mildly lactose intolerant	9.4	9.8	10.7	7.4
	Occasional intolerance	16.8	18.1	16.5	16
	Not lactose intolerant at all	67	65.2	66.3	69.1
How sweet do you like your coffee beverages?	Extremely sweet (5 or more packets/spoonfuls of	4.7	3.4	5.4	5.1
	Very sweet (3 - 4 packets/spoonfuls of sweetener)	13.9	13.2	13.8	14.5
	Sweet (1- 2 packets/spoonfuls of sweetener)	37	39.2	34.5	37.9
	Mildly sweet (less than 1 packet/spoonful of sweetener)	19.6	18.1	21.8	18.4
	Not sweet at all (no sweetener)	24.8	26	24.1	24.2
***"I drink coffee…"	to wake up in the morning	3.6	3.6	3.6	3.7
	to stay awake	3.3	3.3	3.3	3.4
	to stay focused	3.1	3.1	3	3.1
	to de-stress	2.8	2.8	2.7	2.7
	because I like the taste	4.3	4.3	4.2	4.2
	to socialize	3.2	3.3	3.3	3.1
	to give a boost of energy during the day	3.5	3.6	3.5	3.5
	As a meal	1.8	1.7	1.8	1.8

*Check all that apply. Totals within a column may be greater than 100%

**Questions answered on a five point scale where 1 = not important at all and 5 = very important

***Questions answered on a five point scale where 1 = strongly disagree and 5 = strongly agree

Table 5: Zero centered utility values for attributes and levels of each segment. Letters indicate significant differences ($P < 0.05$) within each attribute for each segment.

	Segment 1 n = 185	Segment 2 n = 200	Segment 3 n = 336
Coffee House	63.96 a	119.30 a	78.37 a
Fast Food Restaurant	2.46 b	-0.05 c	18.81 b
Sit Down Restaurant	-0.67 c	32.35 b	-10.78 c
Convenience Store	-29.98 d	-55.10 d	-31.75 d
Grocery Store	-35.77 e	-96.49 e	-54.64 e
Milk based	98.59 a	82.08 a	5.70 a
Lactose free milk based	-40.65 b	-23.60 b	4.09 a
Lactose free non-milk based	-57.94 c	-58.48 c	-9.79 b
Full fat	12.24 a	-34.60 c	-66.75 b
Reduced fat	4.31 a	27.19 a	33.31 a
Fat free	-16.55 b	7.42 b	33.44 a
Sugar	96.12 a	-4.67 b	-35.43 c
No sweetener	22.76 b	-20.56 c	-62.90 d
No calorie sweetener	-81.19 d	3.50 b	30.84 b
Natural no calorie sweetener	-37.69 c	21.74 a	67.50 a
No additional flavor	18.82 a	-19.79 b	-60.46 b
Flavor	-18.82 b	19.79 a	60.46 a

*Different letters within a column within an attribute are different ($p<0.05$)

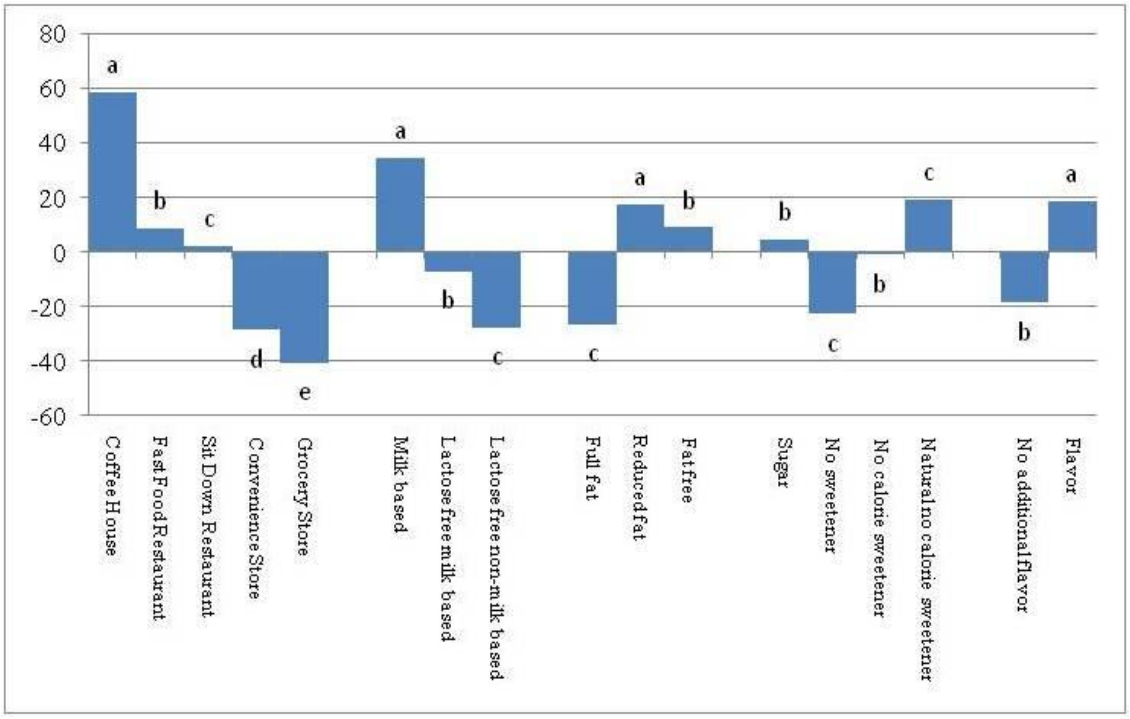

Figure 1: Zero centered utility values for attributes and levels. Letters indicate significant differences ($P<0.05$) within each attribute for total population ($n = 721$).

*Different letters within an attribute are different ($p<0.05$).

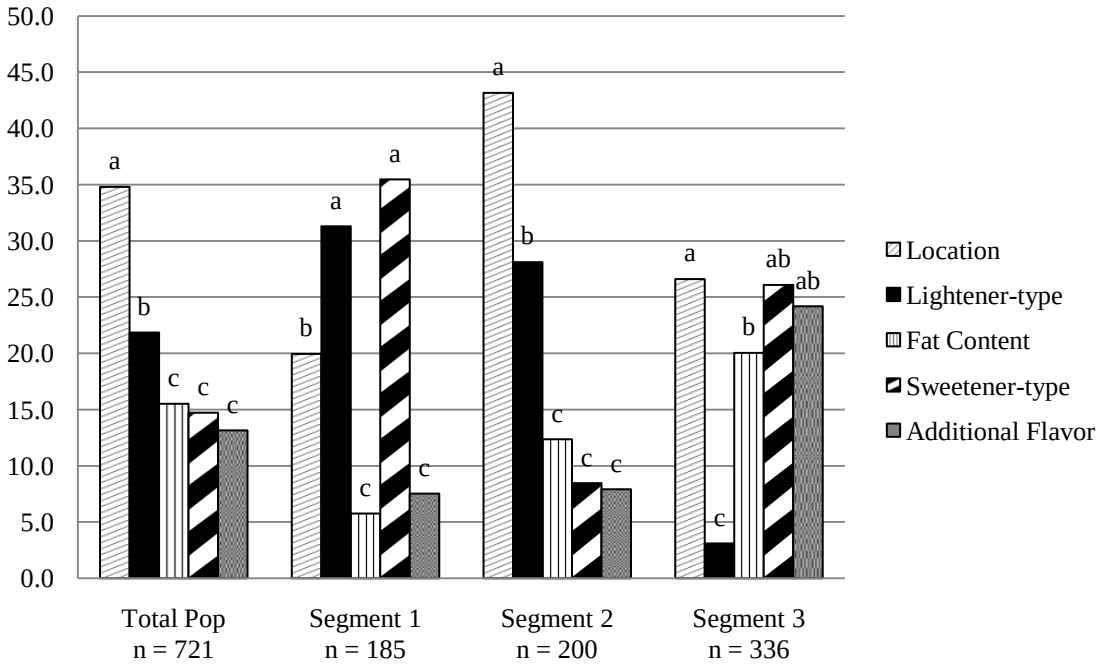

Figure 2: Attribute importance (%) scores for total population and segmented groups.

*Different letters within a population or segment indicate a significant difference ($p<0.05$)

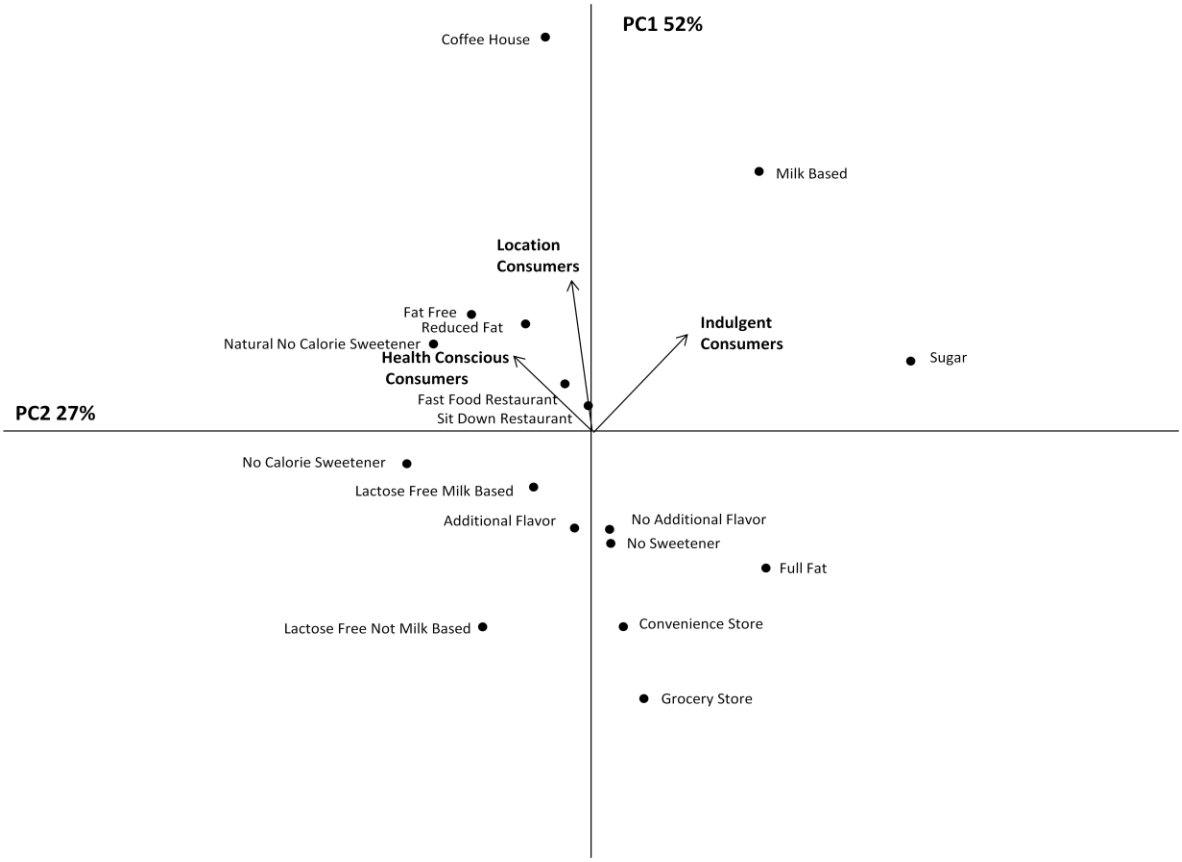

Figure 3: Principal component biplot of clusters from conjoint survey with attributes and levels