

## ABSTRACT

STOUT, MARK A. Off Flavor Development and Vitamin and Carotenoid Degradation in Whey Protein Ingredients due to Processing and the Detection of Carotenoids and Vitamins in Dairy Matrices. (Under the direction of Dr. MaryAnne Drake).

Vitamin and carotenoid content in dairy matrices contribute to flavor, color, and antioxidant capacity in addition to nutritional quality. Carotenoids can also serve as markers for feed quality in bovine milk. Degradation of carotenoids and vitamins in whey protein concentrates (WPC) subjected to bleaching has not been previously addressed. Additionally, simultaneous extraction and measurement of carotenoids and vitamins in milk can be challenging due to the wide range of polarity of these compounds. Vitamin degradation in bleached WPC was evaluated and an optimized vitamin and carotenoid detection method was developed for fluid milk.

In the first study, the effects of bleaching whey by hydrogen peroxide (HP), benzoyl peroxide (BP), or native lactoperoxidase (LP) on vitamin and carotenoid degradation in WPC 80 were determined. An alternative colorant was also evaluated. Cheddar whey colored with annatto (15 mL/454 L milk) was manufactured, pasteurized, and fat separated and then assigned to 250 mg/kg HP, 25 mg/kg BP, or 15 mg/kg HP (LP system) at 50C for 1h. In addition to a control (Con) whey colored with annatto, whey from cheese milk colored with  $\beta$ -carotene an alternative colorant (AltC) was evaluated. Wheys were concentrated to 80% protein by ultrafiltration and spray dried. Samples were taken after initial milk pasteurization, initial whey formation, after fat separation, after whey pasteurization, after bleaching, and after spray drying for vitamin and carotenoid analyses. Concentrations of retinol,  $\alpha$ -tocopherol, water soluble vitamins, norbixin, and other carotenoids were determined by high performance liquid chromatography. Volatile compounds were measured by gas

chromatography mass spectrometry. Sensory attributes of the rehydrated WPC80 were documented by a trained panel. After chemical or enzymatic bleaching, WPC80 displayed a 7.0-33.3% reduction in retinol,  $\beta$ -carotene, ascorbic acid, thiamine,  $\alpha$ -carotene, and  $\alpha$ -tocopherol ( $p < 0.05$ ). WPC80 bleached with BP contained significantly less of these compounds than HP or LP WPC80 ( $p < 0.05$ ). Riboflavin, pantothenic acid, pyridoxine, nicotinic acid, and cobalamin concentrations in fluid whey were not impacted by bleaching ( $p > 0.05$ ). Concentrations of fat soluble vitamins were more than 90% lower in all samples following curd formation and fat separation. With the exception of cobalamin and ascorbic acid, water soluble vitamins were reduced by less than 20% throughout processing. WPC80 colored with AltC had a similar sensory profile, volatile compound profile, and vitamin concentration as Con WPC80 ( $p > 0.05$ ).

The objective of the second study was to develop a simple ultra-performance liquid chromatography (UPLC) method to optimize chromatographic sensitivity of lutein and zeaxanthin in bovine milk without decreasing sensitivity to other vitamins or carotenoids. Xanthophylls have different polarity, solubility, and degradation rates than carotenes or fat soluble vitamins, which may decrease percent recovery using current techniques. The developed method evaluates lutein, zeaxanthin,  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol simultaneously through reversed phase photodiode array (UPLC-PDA) detection. Solvents were evaluated for optimal xanthophyll extraction (diethyl ether, dichloromethane, hexane, and tetrahydrofuran). Common saponification temperatures (40-60°C) and concentrations of KOH in caustic solutions (10%-50% KOH w/v) were evaluated. All experiments were performed in triplicate. Milks solubilized in solutions containing diethyl ether had greater concentrations of lutein than hexane or tetrahydrofuran (THF) based solutions ( $p < 0.05$ ), with

peak areas above LoQ values ( $p < 0.05$ ). The solution containing diethyl ether solubilized similar concentrations of retinol,  $\alpha$ -tocopherol, and  $\beta$ -carotene when compared to other solutions ( $p > 0.05$ ). The optimal saponification procedure was a concentration of 25% KOH at either 40°C or 50°C. The proposed method allows for the simultaneous determination of carotenoids from milk with increased lutein and zeaxanthin sensitivity without sacrificing recovery of retinol,  $\alpha$ -tocopherol, and  $\beta$ -carotene.

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Off Flavor Development and Vitamin and Carotenoid Degradation in Whey Protein  
Ingredients due to Processing and the Detection of Carotenoids and Vitamins in Dairy  
Matrices.

by  
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## **DEDICATION**

To my wife, Emily Stout, for her brilliant example and unconditional love. Thank you for your amazing support, your dedication, and for dragging me out to North Carolina. None of this would have been possible without you. Also, to my parents, Rick and Shauna Stout, for teaching me to find creative solutions to problems and to think critically.

## **BIOGRAPHY**

Mark Stout grew up in Utah County, UT with his parents and four siblings. After graduating from Lone Peak High School in 2007, he had the honor of serving a two year service mission for his church in Frankfurt Germany, where he learned to love the German language and culture. Mark then attended Brigham Young University studying food science. During this time he worked in a research lab and had several internships in fields including quality assurance, product development, and marketing. He graduated from Brigham Young University in 2014 with a major in food science and a minor in chemistry. He joined Dr. MaryAnne Drake's lab in 2014 as research assistant and later as a graduate student in 2015. He plans on completing his Master's Degree in May of 2017.

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**CHAPTER 1: LITERATURE REVIEW. FLAVOR ASPECTS OF WHEY  
PROTEIN INGREDIENTS**



# **Flavor aspects of whey protein ingredients**

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## ABSTRACT

The demand for whey protein ingredients is increasing globally. Whey protein ingredients are typically used for their unique functionality and nutritional qualities; however flavor is a primary driver in whey protein acceptance that should not be overlooked. Sensory analysis techniques can be used to measure flavor intensity and variability in whey protein products. When combined with analytical chemistry techniques, these tests can be used to determine the origin of many flavors and off flavors common to whey protein. This chapter addresses current research on whey protein flavors and the influence of processing and handling on whey protein flavor and flavor stability.

## INTRODUCTION

Whey protein products are primarily added to foods as an ingredient for their functional and nutritional properties (Kosikowski and Mistry, 1997). However, flavor remains a primary driver of whey protein ingredient sales (Drake et al., 2009; Russell et al., 2006; Morr and Ha, 1991). Common off-flavors in whey protein ingredients may be noticeable and affect the flavor of final products (Drake, 2006; Wright et al., 2009; Carunchia Whetstine et al., 2005; Evans et al., 2010, Oltman et al., 2015). Whey protein ingredients should ideally have a bland flavor (Childs et al., 2007; Croissant et al., 2009). Pure undegraded protein should theoretically be flavorless, but no dried ingredients are pure protein. Fat, carbohydrate, ash, and other trace compounds as well as unit operations in the process of manufacturing can dramatically influence the flavor of whey ingredients through lipid oxidation and degradation of protein amino acid side groups (Drake et al., 2009). Lipid oxidation can cause volatile off flavors through aldehyde and ketone formation and oxidation

of sulfur containing amino acid side groups (Carunchia Whetstine et al., 2005; Wright, Whetstine et al., 2006). Carbohydrates in the presence of protein and heat can cause Maillard reaction compounds (Whitfield, 1992). Protein proteolysis or side group reactions can cause bitter and metallic tastes, as well as flavor active volatile compounds which further impact flavor (Wright et al., 2006; Campbell et al., 2011a; Leksrisompong et al., 2012).

Understanding the parameters that influence flavor generation in whey protein ingredients can help mitigate off flavors. Many of the reactions related to off flavors are encouraged by heat, oxygen exposure, and chemical oxidizing agents (Carunchia et al., 2003; Jervis et al., 2012). Many off flavors in whey protein ingredients largely originate from processing and handling of milk and whey (Singh et al., 2006; Park et al., 2016a). Processing variables such as pasteurization, separation, bleaching, spray drying, and storage all influence flavor development in dried whey protein ingredients (Liaw et al., 2010; Park and Drake, 2014; Jervis et al., 2012; Smith et al., 2015). The objective of this chapter is to address the current research on whey ingredient flavors and the influence of processing and handling of whey on flavor generation.

### *Sensory analysis*

Sensory science analyzes all aspects of food or other materials which are perceived by human senses (Lawless and Heymann, 1999; Drake, 2007). A full understanding of the flavor profile, as well as consumer perception of a product is critical for maintaining a competitive edge in food related markets (Drake, 2007). Many objective and subjective sensory tests exist which can be applied to dairy products (Drake et al., 2009; Meilgaard et al., 2007; Lawless and Heymann, 1999). These tests fall into two major categories: analytical

tests and affective tests (Lawless and Heymann, 1999). Analytical sensory tests provide objective results, utilizing trained or screened panelists. This category of tests includes descriptive analysis and discrimination testing. Affective tests use untrained consumers to provide subjective data (Lawless and Heymann, 1999). In these tests, participants often indicate preference or degree of liking (Meilgaard et al., 2007). Off-flavors are determined by consumers and are therefore determined by affective testing. The appropriate sensory test must be determined prior to data collection or meaningful conclusions cannot be made (Drake, 2007). Sensory attributes of fluid whey and dried whey proteins may be described using standardized terms and can be documented by trained panelists (Table 1). These attributes and their intensities are typically applied to rehydrated powders (10% w/v) or directly to fluid whey by a trained descriptive panel. More concentrated liquids are generally diluted to 10% solids (w/v). The application of a standardized and clearly defined sensory language provides the platform for understanding whey protein flavor, identifying flavor sources and relating to consumer perception.

#### *Origin of flavors in whey*

Flavors in fluid and dried whey ingredients can be grouped into two categories, dairy and non-dairy flavors (Carunchia Whetstine et al., 2005). Whey products are often described as having dairy like flavors such as sweet aromatic, buttery, and cooked/milky notes (Carunchia Whetstine et al., 2003; Singh et al., 2006; Smith et al., 2016b). These dairy flavors develop during heat processing of milk (Singh et al., 2006; Contarini et al., 1997). Buttery flavors can originate from starter cultures, as many cheese cultures naturally produce diacetyl (Campbell et al., 2011a; Carunchia Whetstine et al., 2005). However, other flavors

are present in whey and whey ingredients which are not typically associated with dairy products, such as cardboard, wet dog, grass, and cabbage (Carunchia Whetstine et al., 2005; Gallardo-Escamilla et al., 2005a; Evans et al., 2010; Karagül-Yüceer et al., 2003; Whitson et al., 2010). These non-dairy flavors are generally considered off-flavors (Carunchia Whetstine et al., 2003, Drake, 2006; Marsili, 2003). Although several chemical processes can be involved in the development of these off-flavors, lipid oxidation is the primary cause of off-flavors in fluid whey and dried milk and whey ingredients (Carunchia Whetstine et al., 2003; 2005; McClements and Decker, 2008; Smith et al., 2016c). Lipid oxidation produces volatile compounds such as aldehydes and ketones which remain in the whey during processing and are present in spray dried powders. Free radicals from lipid oxidation can also attack protein amino acid side groups and create other flavor active degradation products (Wright et al., 2006; Gallardo-Escamilla et al., 2005b). Thus lipid oxidation of the milk, liquid whey, or concentrated and fractionated whey during processing may cause off-flavors in the final ingredient and negatively influence consumer liking (Drake et al., 2009; Carunchia Whetstine et al., 2005; Wright et al., 2009; Whitson et al., 2011).

Volatile lipid oxidation compounds are present in fresh fluid milk and whey, but concentrations increase due to heat, storage, and other processing steps which increase oxidation (Park et al., 2016b; Kelly et al., 2002; Jervis et al., 2012; Gallardo-Escamilla et al., 2005b; Huffman, 1996). Understanding oxidation is essential in understanding off flavor formation in whey products (McClements and Decker, 2008). Peroxidation is a process by which molecular oxygen reacts directly with organic compounds, affecting confirmation and structure (Frankel, 1998). Free radicals, which are the most common reactive species in

autoxidation, are unpaired electrons which can cause hydrogen abstraction in lipids. As a biradical, oxygen contains two unbound electrons, which facilitates the transfer of free radicals onto an organic system causing oxidation through hydrogen abstraction (Frankel, 1998).

The lipid oxidation process is divided into three sections, initiation, propagation, and termination. During initiation, lipids lose a hydrogen radical, forming a lipid containing an alkyl radical. This free radical is stabilized across the double bond structure (McClements and Decker, 2008). During propagation, the fatty acid containing a free radical reacts with molecular oxygen forming peroxy radicals ( $\text{LOO} \bullet$ ). Because molecular oxygen contains two radicals, this excess electron density attracts a hydrogen atom forming a hydroperoxide ( $\text{LOOH}$ ) and passing a free radical on to another molecule (McClements and Decker, 2008). During termination, two hydroperoxides interact with each other forming an unstable tetroxide which quickly degrades into a non-radical product (Frankel, 1998).

In fluid whey, dried protein, and dried whey ingredients, compounds associated with off flavors are likely to develop from the oxidation of unsaturated oleic, linoleic, and linolenic acids (Frankel, 1998). Oleic, linoleic, and linolenic acids represent 32.5%, 2.5%, and 0.6% of the total fatty acids of liquid Cheddar whey, respectively (Tomaino et al., 2004). This oxidation process begins in fluid milk and can be encouraged through the cheese making process, Campbell et al. (2011a) observed that cheeses which used a starter culture encouraged further lipid oxidation in the whey stream. By the time fresh fluid whey is formed and drained from the cheese vat, lipid oxidation products are present (Carunchia-Whetstine et al., 2003).

### *Fluid milk*

The first factor that affects the flavor of whey ingredients is the flavor of milk (figure 2; Drake et al., 2009). As milk is stored, autoxidation, lipolysis, and proteolysis occur, resulting in the formation of volatile compounds that can be flavor active (Singh et al., 2006). Autoxidation of unsaturated fatty acids can cause the formation of the many of the same volatile compounds which cause off flavors in whey, such as aldehydes, alcohols, and esters (Croissant et al., 2007; Bendall, 2001). Lipolysis from microbial growth can result in free fatty acid release. Proteolysis from microbial growth can also introduce precursors of compounds which will also cause off-flavors in whey (Singh et al., 2006; Brennan et al., 1989). As such, microbial quality of raw milk influences milk flavor which impacts whey flavor. Although many of these off flavor compounds are present in fresh milk, they increase with storage time and temperature (Bassette et al., 1986; Lee et al., 2016).

Storage and microbial quality are not the only factors that influence flavor formation in fluid milk. The composition of a cow's diet also influences the flavor of the milk (Park et al., 1969; Bendall, 2001). There is a distinct difference in flavor between milk from pasture fed cows and from traditional total mixed ration (TMR) fed cows (Bendall, 2001; Croissant et al., 2007). Grassy and mothball flavors have been documented in milk, cheeses, dried milk, and whey protein ingredients from milk from cows fed a pasture based diet (Drake, 2004; Khanal et al., 2005; Drake et al., 2005; Kelly et al., 1998; Bugaud et al., 2001). Bendall (2001) demonstrated that most aroma active compounds present in milk were found in both pasture fed cows and TMR fed cows, but the concentration of specific volatile compounds accounted for the observed differences in the flavor of pasture fed milk and TMR milk. One

of the most dramatic differences between volatiles of pasture fed milk and TMR fed milk was increased skatole and indole concentrations, which are likely components of grassy/mothball flavor (Bendall, 2001; Croissant et al., 2007; Drake et al., 2005). Pasture based diets are less energy dense and contain more L-tryptophan than traditional mixed ration diets, which encourages gluconeogenesis of L-tryptophan. This process in turn increases the concentration of skatole in milk from pasture fed cows (Croissant et al., 2007; Bendall, 2001). These compounds and subsequent grassy and mothball flavors are present in pasture fed milk and will also be present in the resulting whey stream.

Heat treatments promote autoxidation of unsaturated fatty acids in milk (Contarini et al., 1997). Concentrations of many volatile aldehydes, ketones, and alcohols increase due to pasteurization time and temperature (Vazquez-Landaverde et al., 2006; Jousse et al., 2002). Heating can also encourage the release of sulfur compounds from protein, encouraging formation of volatile sulfhydryl compounds such as methanethiol and dimethyl trisulfide, causing intense cooked, sulfurous, and eggy flavors (Contarini et al., 1997; Lee et al., 1996; Friedman, 1996; Lee et al., 2017). Caramelized flavors develop in fluid milk subjected to heat treatments greater than traditional HTST pasteurization, due to sugar degradation through Maillard reactions (Calvo and de la Hoz, 1992; Lee et al., 1996). This process leads to the formation of pyrazines, furans, and other Maillard products which impart cooked, malty and brown flavors (Singh et al., 2006). These volatile compounds will also be present in fluid whey and dried whey ingredients from heat treated milk.



## Flavor aspects of liquid whey

### *Liquid whey*

During cheese production, many carbohydrates, fat globules, proteases, minerals, and organic acids are not incorporated into the curd. These unincorporated compounds stay in the aqueous phase with the fluid whey where they can impact flavor and be flavor precursors (Carunchia Whetstine et al., 2003; Smith et al., 2016a). Depending on the cheese, fluid whey is either classified as sweet whey or acid whey (Varnam and Sutherland, 2001; Smith et al., 2016b; Drake et al., 2009). Sweet whey is the byproduct of cheese produced from rennet coagulation and generally has a pH of 6.00-6.40, while acid whey is the byproduct of acid coagulated cheese and thus has a much lower pH of 4.30-4.60 (Morr and Ha, 1993). Cheese type will also impact the flavor of sweet liquid whey, as will be discussed later in this chapter (Smith et al., 2016b; Liaw et al., 2011). Generally, sweet fluid whey is described as having milky and sweet aromatic flavors as well as low cardboard and metallic flavors (Carunchia Whetstine et al., 2003; Gallardo-Escamilla et al., 2005a; Lubran et al., 2005). Acid whey generally contains higher concentrations of organic acids, and increased calcium, potassium, and iron compared to other fluid wheys (Durham et al., 1997; Gallardo-Escamilla et al., 2005a). The higher organic acids and minerals cause acid whey to have increased lipid oxidation and sour aromatic and cardboard flavors, as well as increased sour taste compared to sweet wheys (Gallardo-Escamilla et al., 2005a; Smith et al., 2016b).

Fluid whey contains  $\leq 0.5\%$  fat by weight, however this is enough for lipid oxidation to be a primary source of off flavors (Carunchia Whetstine et al., 2003; Smith et al., 2016a; Drake et al., 2009). Many of the same aldehydes, methyl ketones, and free fatty acids that

contribute to off flavors in stored milk are also found in fluid whey (Carunchia Whetstine et al., 2003; Cadwallader and Singh, 2009). As fluid whey is stored, more lipid oxidation occurs than in powdered whey due to increased water activity (McDonough et al., 1968; Liaw et al., 2010; 2011; Park et al., 2016b). These reactions are sensitive to heat and oxygen exposure, thus autoxidation of fat must be considered at every stage of fluid whey processing and storage.

#### *Chymosin and proteolysis*

One critical factor in proteolysis related off-flavor in fluid whey is the presence of chymosin and other proteases (Carunchia Whetstine et al., 2003). Chymosin is used to coagulate the curd during cheese production and can be present at residual levels in cheese whey along with any proteases produced by the starter culture that carry over into the whey (Holmes et al., 1977; Campbell and Drake 2013b). These enzymes encourage proteolytic degradation which in turn can cause increased astringency as well as bitter and metallic tastes (Harwalker et al., 1993). Stevenson and Chen (1996) stated that proteins may also hydrophobically bind to volatile flavor compounds in fluid whey which may then release when spray dried. Thus proteolysis during fluid whey processing may influence flavor of powdered products through volatile compound/peptide binding.

#### *Cheese type and influence of culture on whey flavor*

The type of starter culture used to make the whey influences fluid whey flavor. Whey from cheese produced without culture using rennet (rennet whey) is characterized by sweet aromatic and cooked milky flavors as well as sweet taste (Campbell et al., 2011b; Smith et al. 2016c). Rennet wheys also have distinct cooked notes and will develop cardboard flavors

when stored (Campbell et al., 2011a; Smith et al., 2016c). The simple flavor profile of whey can be complicated by cheese cultures which encourage oxidation, proteolysis, and lipolysis (Liaw et al., 2010; Campbell et al., 2011a; 2011b; El Soda et al., 1995). Fatty acids found in milk are released from triglyceride form by lipolytic enzymes from starter cultures. These free fatty acids are more easily oxidized, contributing to off-flavor development in the whey ingredients (Cadwallader and Singh, 2009). Although free fatty acid degradation occurs naturally, starter cultures can increase this degradation rate in milk and whey (Drake et al., 2009; Gallardo-Escamilla et al., 2005a; 2005b). Campbell et al., (2011a) confirmed that the increased lipid oxidation compounds in fluid whey were due to cheese culture. Fluid wheys produced from cultured cheeses are characterized by higher rates of free fatty acid, as well as lipid oxidation compounds due to increased enzymatic lipolysis compared to rennet wheys (Campbell et al., 2011a; Smith et al., 2016d; Drake et al., 2009).

Gallardo-Escamilla et al. (2005b) compared the volatile composition of fluid whey produced with Cheddar cheese cultures to rennet whey. The whey produced with Cheddar cheese culture had elevated volatile lipid oxidation compounds as well as increased proteolysis and lipolysis compared to the rennet whey (Gallardo-Escamilla et al., 2005b). Campbell et al. (2011a) demonstrated that after 48 h storage at 4°C, fluid whey produced with Cheddar cheese cultures had increased cardboard and sour aromatic flavors compared to fresh fluid Cheddar wheys and fluid rennet wheys. Not all flavors related to cheese cultures are off-flavors. Many cheese cultures produce diacetyl, which contributes a buttery flavor to fluid whey (Carunchia et al., 2003; Campbell et al., 2011a).

Campbell et al. (2011a) demonstrated that different starter cultures also influenced fluid whey flavor in a different manner. Mesophilic starter cultures used for Cheddar whey caused greater lipid oxidation than thermophilic starter cultures used in Mozzarella whey (Campbell et al., 2011b; Liaw et al., 2011). As such, fresh Cheddar whey had increased cardboard flavor and decreased cooked/milky flavor compared to fresh Mozzarella whey (Campbell et al., 2011a). Although Mozzarella whey contained less lipid oxidation than Cheddar whey, lipid oxidation was still present. Mozzarella whey also had more oxidation compared to rennet whey, and storage of fluid Mozzarella whey reduced flavor quality (Whitson et al., 2011; Campbell et al., 2011a). Liaw et al. (2010) demonstrated that optimal fat separation and possibly the addition of an antioxidant reduced lipid oxidation in both Cheddar and Mozzarella wheys during storage.

Cottage cheese curds are formed through acidification, meaning the product ferments until the pH of the milk is below the isoelectric point of casein (around pH 4.6). Fluid Cottage cheese whey is therefore an acid whey. Increased organic acid concentration and prolonged heat exposure cause fluid Cottage whey to be characterized by acidic, sweaty, potato, oxidized, and stale flavors (Gallardo-Escamilla et al., 2005a; Smith et al., 2016b). Smith et al. (2016b) characterized fluid Cottage cheese whey by higher sour aromatic flavor and distinct potato/brothy flavor compared to Cheddar or rennet fluid wheys. Cottage cheese fluid whey also had much higher concentrations of diacetyl, which was likely the result of extended fermentation (Smith et al., 2016d; Gallardo-Escamilla et al., 2005a; Durham et al., 1997). The higher diacetyl concentration contributes to intense buttery flavors associated with Cottage cheese liquid whey (Smith et al., 2016d). Many of the flavor characteristics of

fluid Cottage whey carry over into dried protein ingredients from Cottage cheese whey. Smith et al. (2016d) reported that Cottage cheese WPI was higher in sour aromatic and potato/brothy flavors and lower in sweet aromatic flavor than Cheddar, Mozzarella, or rennet whey WPI. Cottage cheese acid whey is often acidified at least in part by microbial growth, acid whey can also be produced by direct acid addition, known as acid casein whey. Acid casein whey does not have the distinct buttery notes of Cottage cheese whey, but contains similar sour aromatic and stale flavors as well as rancid and chemical flavors and bitter taste (Gallardo-Escamilla et al., 2005a).

## Fluid whey processing

### *Pasteurization*

When fluid whey is drained from the cheese vat, cheese cultures will continue to ferment and reduce the pH. Pasteurization is conducted to inactivate the starter culture, but the lactose content of fluid whey can encourage Maillard reaction products to form due to the heat (Whitfield, 1992; Mahajan et al., 2004). Although some of these Maillard reaction products contribute to cooked/milky flavors, many contribute to Strecker degradation volatile compounds (Whitfield, 1992). Strecker degradation is the process of heat induced oxidative deamination, which forms odorous volatile compounds as well as potent intermediates in lipid oxidation (Whitfield, 1992). Lipid oxidation compounds such as methyl ketones and aldehydes also react with Maillard reaction products encouraging further development of oxidation related flavors in fluid whey (Newton et al., 2012). These factors are temperature and time dependent, thus control over pasteurization parameters is critical in mitigating off flavor.

### *Fat separation*

As might be expected, the concentration of lipids in the whey dramatically impact lipid oxidation in both fluid and powdered products. To mitigate these effects, fluid whey is subject to centrifugal fat separation, where fat concentrations reach  $\leq 0.5\%$  of the total weight (Bylund et al., 2003). Liaw et al. (2011) demonstrated that lipid oxidation was reduced by fat separation in both Cheddar and Mozzarella wheys. After three days of storage at 4°C, fat separated wheys were compared to non-separated wheys. Aldehydes and cardboard flavor were reduced in fluid fat separated Cheddar and Mozzarella wheys compared to those which were not fat separated (Liaw et al., 2011).

### Composition of whey ingredients

#### *Whey protein concentrates and isolates*

The composition of a dried whey ingredient has a direct impact on its flavor. Higher protein content products are distinct in flavor compared to products with lower protein content. Sweet fluid whey is approximately 93.7% water, 4.8% lactose and 0.8% protein (Kosikowski and Mistry, 1997). Water can be removed to concentrate the total solids in fluid whey. By removing water through reverse osmosis (RO) or evaporation, fluid whey can be concentrated to a product that when dried is sweet whey powder (SWP), which contains 75% lactose and 13% protein (Mahajan et al., 2004; figure 6). The flavor of SWP is characterized by cooked/milky and oxidized flavors and sweet taste (Sithole et al., 2006). The high concentration of lactose in SWP increases sugar caramelization and Maillard reaction products in SWP. These reactions encourage Strecker degradation compounds, furans, and

pyrroles, which contribute to the predominant caramelized and cooked flavors in SWP as well as many oxidized flavors (Sithole et al., 2006).

Often an ingredient is needed with a higher ratio of protein to lactose than SWP. By utilizing ultra-filtration (UF), water and lactose can be separated from the protein through size exclusion (figure 6). By this method, lactose can be separated out until 35% of the total solids is protein, creating 34% whey protein concentrate, or WPC34 (Onwulata, 2008).

Additional concentration is performed by diafiltration (or the addition of deionized water to facilitate further filtration), which allows protein concentration to increase further, allowing for the creation of WPC80 (Onwulata, 2008; Rosenberg, 1995). Concentration of protein above 90% of the total solids is referred to as whey protein isolate (WPI). For WPI, fat must be removed through either microfiltration or anion exchange (Smith et al., 2016a; Huffman, 1996).

The lactose and fat concentration play a critical role in the sensory attributes of dried whey products. WPC is generally characterized by sweet aromatic, milky, cardboard, and fatty flavors although these flavors vary based on protein concentration. The lower protein content and higher lactose content of WPC34 result in a product that has distinct cooked/milky and sweet aromatic flavors and sweet taste compared to higher protein whey ingredients (Listiyani et al., 2011; Evans et al., 2009). As protein content increases, cooked/milky and sweet aromatic flavors decrease. During diafiltration for WPC80 production, lactose is removed and protein and fat are both concentrated. WPC80 contains between 4.6-6.5% fat (on a dry weight basis). Lipid oxidation and off-flavor development occur in this product due to bleaching, storage, and drying (Carunchia Whetstine et al., 2005;

Evans et al., 2010; Campbell et al., 2011b). WPI has much lower concentrations of fat (between 0.15-0.60% on a dry weight basis) as fat removal is a necessary step in concentrating protein above 90% of the total solids (Carunchia Whetstine et al., 2005; Whitson et al., 2011; Smith et al., 2016d). The reduced fat content in WPI can reduce lipid oxidation and off flavors. Carunchia Whetstine et al. (2005) demonstrated that WPI powders contained fewer aroma-active lipid oxidation products than WPC80 powders. Although lipid content influences off-flavor formation, the binding capacity of volatile compounds to protein in WPI may also decrease the perception of off flavor in WPI compared to WPC80 (Stevenson and Chen 1996).

### *Serum protein*

Serum proteins refer to whey proteins that are removed from milk before cheese manufacture (Nelson and Barbano, 2005; Evans et al., 2009; 2010; Drake et al., 2009). Serum proteins are not exposed to the cheese making process which reduces lipid oxidation and proteolysis in these products compared to traditionally produced whey protein (Nelson and Barbano, 2005; Smith et al., 2016a; Campbell et al., 2011b). As a result, serum proteins tend to have a mild flavor compared to traditional whey protein. To create serum protein, skim milk is first subject to micro-filtration, where the serum protein and lactose are separated from the casein and fat of the milk (Saboya and Maubois, 2000; Nelson and Barbano, 2005). At this point, lactose is removed through ultra-filtration, the retentate produced is serum protein concentrate (SPC) with concentrations ranging from 34% protein (SPC34) to 80% protein (SPC80) or serum protein isolate (SPI) with protein concentration above 90% (Saboya and Maubois, 2000; Nelson and Barbano, 2005; Evans et al., 2009;



2010). The micro-filtration process used to remove serum protein from skim milk also removes fat (Evans et al., 2009; Nelson and Barbano, 2005). As such, serum protein concentrates (SPC) contain less fat than WPC at the same protein concentration (0.53% vs. 4.67% on a dry weight basis), which reduces

lipid oxidation and oxidation related off flavors (Evans et al., 2009; 2010). Evans et al. (2009) reported that SPC34 had lower diacetyl and cardboard flavors compared to WPC34. Evans et al. (2010) compared the flavor profile of SPC80 to WPC80, and demonstrated that SPC80 had lower oxidation and culture related flavors than WPC80, including cardboard and diacetyl flavors. Serum protein isolate (SPI), which contains greater than 90% protein on a dry weight basis, exhibited lower flavor intensities compared to whey protein isolate and like other serum proteins, lower oxidation.

#### *Whey protein hydrolysates*

Whey protein hydrolysates (WPH) are filtered whey protein products (WPC or WPI) which have undergone enzymatic hydrolysis of peptide bonds and an aggressive heat step to inactivate the added enzymes (Drake et al., 2009; Smith et al., 2016a; Leksrisonpong et al., 2010). Hydrolysis improves digestibility of proteins as well as altering functional properties including solubility and heat stability (Nongonierma and FitzGerald, 2015; Nnanna and Wu, 2006; Tang et al., 2009; Pedrosa et al., 2006). Despite the health benefits, their use in food applications is limited by strong negative flavor attributes. WPH differ in flavor from WPC and WPI due to their enzymatic treatment and heating step (Leksrisonpong et al., 2010). The degree of hydrolysis, enzyme used, and hydrolysis conditions all influence WPH flavor

(Ziajka et al., 1994; Leksrisonpong et al., 2010). WPH generally have distinct bitter taste due to proteolysis and the bitter taste intensity generally is associated with the degree of hydrolysis (Leksrisonpong et al., 2010; 2012; Harwalker et al., 1993).

Leksrisonpong et al. (2010) demonstrated that bitterness in WPH was correlated with smaller peptides (<600 – 4142 Da) which contained hydrophobic residues at a C terminal. Larger peptides (3000-6000 Da) caused much less bitter taste, likely due to the ability of these peptides to bind their own C terminal hydrophobic sites, blocking hydrophobic interactions (Leksrisonpong et al., 2012; Pedrosa et al., 2006). Peptides between 3 and 6 amino acids in length contributed to bitterness, as did many amino acids with L-confirmation and hydrophobic side chains (Leksrisonpong et al., 2010). Thus the degree to which a product is hydrolyzed is less important to the bitterness of WPH than the concentration of peptides (Newman et al., 2014a).

WPH have many other sensory challenges than just bitter taste. Leksrisonpong et al. (2010) demonstrated that WPH contained high levels of cooked/sulfer, potato/brothy, tortilla, and animal flavors due to the extended heating and proteolysis needed to produce WPH. Newman et al., (2014b) confirmed these findings, and developed a lexicon for WPH and casein based hydrolysates, which contained metallic, cabbage, cereal, and burnt flavors. This sensory language was similar to the one independently developed and used by Leksrisonpong et al. (2010). Unsurprisingly, protein degradation compounds play a pivotal role in the volatile composition of WPH. These compounds include sulfur compounds such as dimethyl trisulfide which imparts cabbage flavor and methional which imparts potato flavor in WPH and other whey protein ingredients (Wright et al., 2006; Leksrisonpong et al., 2010).

## Bleaching

### *Impact of bleaching on whey*

Annatto, the colorant typically used to color Cheddar cheese, contributes no flavor directly to whey or whey ingredients but provides unique flavor challenges for whey ingredients (Kang et al., 2010; Campbell et al., 2011a; Jervis and Drake, 2013). Once separated from the cheese curd, fluid whey retains approximately 10% of the total added annatto (Smith et al., 2014; Kang et al., 2010). Annatto is composed of two carotenoids, water-soluble norbixin and fat-soluble bixin, but norbixin is the primary colorant used in cheese production due to its solubility (Giuliano et al., 2003; Smith et al., 2014). The residual annatto causes an unfavorable color in dried whey products, which necessitates bleaching (McDonough et al., 1968; Croissant et al., 2009; Kang et al., 2010; figure 1).

### *General bleaching*

Due to their long carbon chains and double bond configuration, norbixin is susceptible to oxidation (Scotter, 2009; Kang et al., 2010; Jervis et al., 2012). Oxidative cleavage along the carbon backbone of norbixin will cause a disruption of the chromophore and render the compound colorless (Guiliano et al., 2003). Oxidative bleaching is the industry standard to remove color from fluid whey (Kang et al., 2010). Bleaching is traditionally accomplished chemically or enzymatically (Kang et al., 2010; Kang et al., 2012; Campbell et al., 2012). Both of these approaches are general oxidative bleaching methods and have adverse effects on the flavor of whey ingredients since the process is not specific to annatto (Listiyani et al., 2012; Jervis et al., 2012; Jervis et al., 2015; Campbell and Drake, 2014; Smith et al., 2015). However, different bleaching agents impact whey ingredient flavor

differently and can be optimized to mitigate off flavor (Jervis et al., 2012; Campbell et al., 2012; Campbell and Drake, 2014).

### *Chemical bleaching*

The two prominent chemical bleaching agents used for fluid whey are hydrogen peroxide and benzoyl peroxide. Both have been in use for more than 20 years. Hydrogen peroxide is a water soluble bleaching agent that causes a 37-44% reduction in norbixin when applied at concentrations up to 500 ppm followed by deactivation by the addition of catalase (Jervis et al., 2012; Fox et al., 2013; Listiyani et al., 2012; Gilliland, 1969; Teply et al., 1957; Kang et al., 2010; Croissant et al., 2009). Benzoyl peroxide is a fat-soluble bleaching agent that can result in >95% norbixin removal at concentrations between 20 and 50 ppm, but leaves a benzoic acid residue when used, which is prohibited in some countries (Smith et al., 2015; Listiyani et al., 2011; Jervis et al., 2012). Croissant et al. (2009) first documented that flavor differences existed between wheys bleached with hydrogen peroxide or benzoyl peroxide. Bleaching with hydrogen peroxide (500 mg/kg) resulted in more lipid oxidation compared to benzoyl peroxide (20 mg/kg). Consequently, increased cardboard flavor was observed. Listiyani et al. (2012) demonstrated that higher bleaching temperature increased lipid oxidation in fluid whey when bleached by hydrogen peroxide. The effect of temperature on benzoyl peroxide bleaching of fluid whey, in contrast, was less pronounced than hydrogen peroxide (Listiyani et al., 2012; Smith et al., 2015; Fox et al., 2013). Off flavor production is a barrier to use of hydrogen peroxide, as hydrogen peroxide bleaching efficiency in fluid whey is reduced at lower temperatures. The balance between efficient norbixin bleaching and

reduction of off-flavor must be considered when using hydrogen peroxide (Fox et al., 2013; Listiyani et al., 2012).

As bleached fluid whey is concentrated and dried, these differences in flavor profile due to bleaching agents remain noticeable. WPC80 from hydrogen peroxide bleached whey contained distinct potato, cardboard, and fatty flavors compared to WPC80 from whey bleached with benzoyl peroxide (Jervis et al., 2012). In SWP and WPC34, hydrogen peroxide bleaching also encouraged lipid and protein oxidation as well as cardboard and fatty flavors when compared to benzoyl peroxide bleaching (Listiyani et al., 2011; Jervis et al., 2015). Bleaching can either be applied before or after filtration. However, bleaching whey retentate (whey protein after concentration) with hydrogen peroxide or benzoyl peroxide increased cardboard and fatty flavors compared to samples bleached before filtration (Fox et al., 2013).

#### *Enzymatic bleaching*

Enzymatic bleaching involves the use of either the native lactoperoxidase system or an exogenous peroxidase to bleach whey (Campbell et al., 2012; Campbell and Drake, 2013b; 2014; Kang et al., 2010). Lactoperoxidase is a native enzyme to milk which, in the presence of low levels of hydrogen peroxide, converts thiocyanate to the active peroxide hypothiocyanate. The resulting radical causes oxidation and can be used to bleach whey (Campbell et al., 2012; Campbell and Drake, 2013b; 2014; Reiter and Harnulv, 1982). The lactoperoxidase system has a strong oxidizing capacity and is self-perpetuating until terminated by catalase, thus this system is extremely efficient at bleaching norbixin, causing >90% destruction compared to 37-44% norbixin destruction from hydrogen peroxide bleaching (Campbell and Drake, 2014; Reiter and Harnulv, 1982). Unfortunately, this

enzymatic system is also nonspecific, and also encourages lipid and protein oxidation (Kang et al., 2010). Campbell et al. (2012) demonstrated that bleaching fluid whey with lactoperoxidase had higher norbixin destruction but also increased total aldehydes and cabbage flavors in WPC80 compared to WPC80 from fluid whey bleached with hydrogen peroxide. The lactoperoxidase system can also be variable, as enzyme concentration varies depending on the lactation cycle of the cow, season, feeding regime, and breed (Kussendrager and Hooijdonk, 2000; Campbell and Drake, 2014). A solution to lactoperoxidase variability is the addition of an exogenous peroxidase. Exogenous peroxidases are peroxidases isolated from a non-dairy source, and do not require thiocyanate in milk to activate (Kang et al., 2010; Campbell and Drake, 2014). Exogenous peroxidase bleached WPC80 had similar flavor to lactoperoxidase bleached WPC80 (Campbell and Drake, 2013a). Some off flavor can be mitigated by enzymatic bleaching at 4°C, particularly with exogenous peroxide, which resulted in faster bleaching than lactoperoxidase at lower temperatures (Campbell and Drake, 2013a).

The process of bleaching, regardless of the agent, generally has a negative impact on whey ingredient flavor (Croissant et al., 2009; Jervis et al., 2012; Campbell et al., 2013). A decrease in sweet aromatic flavor and increased cardboard flavor are ubiquitous among bleached whey ingredients compared to unbleached counterparts (Jervis et al., 2012). Both hydrogen peroxide and enzymatic bleaching may cause fatty, cabbage, and potato flavors (Campbell et al., 2012; Campbell and Drake, 2013b). Benzoyl peroxide has the bleaching efficiency of enzymatic bleaching with lower negative impacts on flavor, but the issues of benzoic acid residues remain (Smith et al., 2015; Campbell et al., 2012; Jervis et al., 2012).

### *Storage*

Storage of fluid whey prior to spray drying increases lipid oxidation and has a negative impact on sensory attributes of liquid whey and dried ingredients manufactured from stored fluid whey. Commercially, fluid or concentrated whey may be stored before drying (figure 2). Lipid oxidation still occurs during storage at 3°C (Whitson et al., 2011; Park et al., 2016; Liaw et al., 2011). Storage of liquid WPC80 or WPI retentate for 48 h at 3°C prior to drying increased lipid oxidation and resulted in increased cardboard and decreased sweet aromatic flavors in spray dried protein powders (Whitson et al., 2011). Fluid storage for more than 6 h was detrimental to spray dried protein flavor. However, steps can be taken to minimize storage effects. Park et al. (2016b) compared storage of liquid whey to storage of liquid WPC80 and determined that lipid oxidation was mitigated in unbleached whey by storing the whey as liquid WPC80 instead of as fluid whey. However, in bleached whey, storage as fluid WPC80 increased lipid oxidation compared to storage at lower solids as fluid whey.

### *Spray drying and dried storage*

Drying fluid dairy products can be an efficient method to slow lipid oxidation and facilitate transport and storage (figure 1). As one of the most common drying methods in the dairy industry, spray drying removes moisture by atomizing liquid and exposing the product to brief but intense heat. Due to rapid heating and evaporative cooling, the interior temperature of the whey particles generally do not exceed 60°C (Park et al., 2014a). This system is minimally invasive, however the heating step will introduce other opportunities for heat related off-flavors to develop. As whey products are exposed to the intense inlet

temperatures (often above 180°C), Maillard reactions, oxidation, and sugar caramelization can encourage flavor development (Park and Drake, 2014).

Products high in lactose such as SWP, are particularly susceptible to Maillard reactions during the drying stage, which encourages the formation of sweet aromatic and milky flavors, as well as oxidized flavors (Sithole et al., 2006). This process can be avoided through freeze drying, which does not cause Maillard reaction products (Evans et al., 2009). However, freeze drying increased lipid oxidation compounds compared to spray dried samples, possibly due to the longer processing time (Evans et al., 2009). Spray dried WPC34 had sweet aromatic and cooked/milky flavors, indicative of Maillard reactions, and lower cardboard flavor compared to freeze dried product.

In dried high protein whey products (WPC and WPI), lipid oxidation occurs during storage at ambient temperatures which is responsible for off flavors at the end of shelf life (Wright et al., 2009). Wright et al. (2009) demonstrated that dried WPC and WPI stored at 21°C for 6-24 months had higher levels of cardboard, cucumber, and fatty flavors than fresh dried WPC and WPI. They concluded that shelf life of regular nonagglomerated WPC80 and WPI was 12 to 15 mo. Javidipour and Qian (2008) found that powdered WPC80 stored at temperatures between 35-45°C had elevated volatile lipid oxidation compounds after 15 weeks. Shelf life of dried WPC and WPI can be influenced by processing parameters such as agglomeration and instantization with lecithin (Javidipour and Qian, 2008; Wright et al., 2009). Agglomeration of powdered whey ingredients is a process of creating small clumps of particles which allow for increased dispersability and reduced dispersion time in fluids (Turchiuli et al., 2005). Instantization of whey protein is the process of agglomerating



product with lecithin to aid in dispersion (Wright et al., 2009). These processes both encourage volatile lipid oxidation compound formation and detectable off flavor development in WPC and WPI more quickly than non-agglomerated WPC and WPI stored under the same conditions (Wright et al., 2009).

Park et al. (2014a) demonstrated that spray drying parameters influenced lipid oxidation and subsequently flavor, by exposure of surface free fat (Park and Drake, 2014; Park et al., 2014a). During spray drying, the sample is atomized and sprayed into small droplets, which are evaporated under hot air, leaving dry particles. During drying, fat can migrate out of the globular membrane becoming surface free fat, where it is more susceptible to lipid oxidation (Park and Drake, 2014). The inlet temperature of the spray dryer and the feed solids concentration will determine the extent to which fluid migration can occur and thus influence lipid oxidation rates (Kelly et al., 2002; Park et al., 2014a). Increasing the inlet temperature during spray drying increased particle drying rates and decreased surface free fat (Park et al., 2014a). Park et al. (2014a) demonstrated that WPC80 spray dried with an inlet temperature of 220°C had decreased cardboard flavor intensity compared to WPC80 spray dried with an inlet temperature of 180°C. The rate of fat migration within the globular membrane was also influenced by solids concentration, with higher solids decreasing lipid oxidation by decreasing surface free fat. Park et al. (2014a) demonstrated that WPC80 spray dried at 10% solids had higher surface free fat, smaller particle size, and increased cardboard and cabbage flavors compared to WPC80 spray dried at 18 or 25% solids.

The pH of fluid product will also influence lipid oxidation and off flavors in spray dried whey proteins. WPC and WPI used for acidic beverages are often acidified prior to

spray drying, which can reduce turbidity in the beverage as well as improve flavor (White et al., 2013). Park et al. (2014b) demonstrated that liquid WPC80 acidified to pH 3.5 prior to spray drying had reduced lipid oxidation compounds and decreased off-flavors compared to WPC80 adjusted to pH 5.5 or 6.5. At a pH of 3.5, the whey protein  $\beta$ -lactoglobulin has a conformation that encourages more lipid oxidation compounds to bind to hydrophobic regions (Park et al., 2014b). Park et al. (2014b) hypothesized that lipid oxidation compounds were bound to protein at pH 3.5 and they were less able volatilize and impact flavor.

## CONCLUSIONS

The functional and nutritional aspects of whey proteins are valuable as ingredients, but flavor is a major driver in the acceptance of whey ingredients. Understanding the flavor attributes of whey products is essential for the continued success of the whey industry. Ideally whey ingredients should be flavorless, as this allows for the greatest versatility in ingredient applications. However, off flavors easily develop in whey products due to lipid oxidation, proteolysis, and Maillard reactions. These flavors begin to develop in fluid milk, with bovine diet and milk handling. They also develop in whey during unit operations in whey processing, ingredient manufacture, and storage. There has been a significant amount of research regarding the flavor of whey ingredients, however the full potential of whey ingredients will only be realized through continued research on flavor reduction.

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## TABLES AND FIGURES

Table 1. Sensory language for descriptive analysis of fluid whey and whey proteins (adapted from Carunchia Whetstine et al., 2003; Drake et al., 2003; Drake et al., 2009; Smith et al., 2016a; Kussy et al., 2009).

<b>Term</b>	<b>Definition</b>	<b>Reference</b>	<b>Example/preparation</b>
Overall aroma intensity	The total orthonasal aroma impact		Evaluated as the lid is removed from the cupped sample
Sweet aromatic	Sweet aromatics associated with dairy products	Vanillin in milk	Vanilla cake mix or 20 ppm vanillin in milk
Sour aromatic	Sour aromatics associated with dairy fermentation	Cultured sour cream	
Buttery/diacetyl	Aromatic associated with diacetyl	Diacetyl	1 ppm diacetyl onto filter paper strips in 125 ml sniff jar
Cooked/milky	Aromatics associated with cooked milk	Cooked skim milk	Heating skim milk to 85 °C for 30 min
Cardboard/wet paper	Aromatics associated with wet cardboard or paper	Cardboard paper	Brown paper bag cut into strips and soaked in water
Cabbage brothy	Aromatics associated with boiled cabbage	Boiled cabbage	Cabbage leaf boiled in 500 mL water for 5 min, 1 ppb dimethyl trisulfide
Potato brothy	Aromatics associated with either broth or boiled potatoes	Methional	1 ppm methional in water or canned potatoes
Cucumber	Aromatics associated with freshly sliced cucumber	(E)-2-nonenal	1 ppm (E)-2-nonenal or freshly sliced cucumbers
Grassy/hay	Aromatics associated with dried grasses	Alfalfa or grass hay	
Doughy	Aromatics associated with canned biscuit dough	(Z)-4-heptenal	1 ppm (Z)-4-heptenal, canned biscuit dough, or cooked pasta water
Fried fatty/painty	Aromatics associated with old frying oil and lipid oxidation products	2,4-decadienal	Old (stored) vegetable oil
Pasta water/cereal	Aromatics associated with water after pasta has been boiled in it or oatmeal	Boiled pasta or plain boiled oats	Pasta boiled in water for 30 min
Metallic/serumy	Aromatic associated with metals or with juices of raw or rare beef	Juices from seared beef	Aroma of fresh raw beef steak or juices from seared beef steak
Animal/wet dog	Aromatics associated with wet dog hair	Knox brand gelatin	One bag of gelatin (28 g) dissolved in two cups of distilled water
Cowy/barny	Aromatics associated with cow feces and urine	p-cresol	20 ppm p-cresol in skim milk
Soapy	Aromatics associated with soap	Lauric acid	1 ppm lauric acid or shaved bar soap

Table 2. Trained sensory panel profiles of fluid Cheddar wheys (Liaw et al., 2011; Campbell et al., 2011a; Carunchia Whetstine et al., 2003).

Descriptor	Mean intensities			
	W1	W2	W3	W4
Aroma intensity	2.0	2.2	2.1	2.5
Cooked/ milky	3.0	3.3	3.0	3.0
Sweet aromatic	1.5	ND	1.0	2.5
Buttery	1.0	ND	ND	1.4
Cardboard	1.4	1.0	0.8	ND
Cooked/ milky	3.0	3.3	3.0	3.0
Sweet	2.0	2.2	1.7	2.0
Astringent	1.5	1.5	1.9	1.5

W1-W4 = fluid wheys

Scores based on a universal 0 to 15 point intensity scale

ND = Not detected

Table 3. Trained sensory panel profiles of rehydrated WPC34, WPC80, and WPI (adapted from Evans et al., 2009; Evans et al., 2010; Carunchia et al., 2005).

Attributes	Mean scores		
	WPC34	WPC80	WPI
Aroma Intensity	2.3a	2.5a	2.0b
Sweet aromatic	2.6a	1.4b	ND
Cardboard	1.5c	2.0b	2.3a
Cooked/milky	3.6	ND	ND
Sweet taste	2.3	ND	ND

Scores based on a universal 0 to 15 point intensity scale

ND = Not detected

<sup>a,b,c</sup> Means in a row followed by different letters are different ( $p < 0.05$ )

Table 4. Trained sensory panel profiles of rehydrated SPC34, SPC80, and SPI (adapted from Evans et al., 2009; 2010).

Descriptor	Mean scores		
	SPC34	SPC80	SPI
Aroma intensity	1.7	1.2	1.3
Sweet aromatic	1.8	1.2	1.5
Cardboard	ND	1.0	1.6
Cooked milky	2.0	1.1	ND
Sweet taste	2.0	ND	ND

Scores based on a universal 0 to 15 point intensity scale  
 ND = Not detected



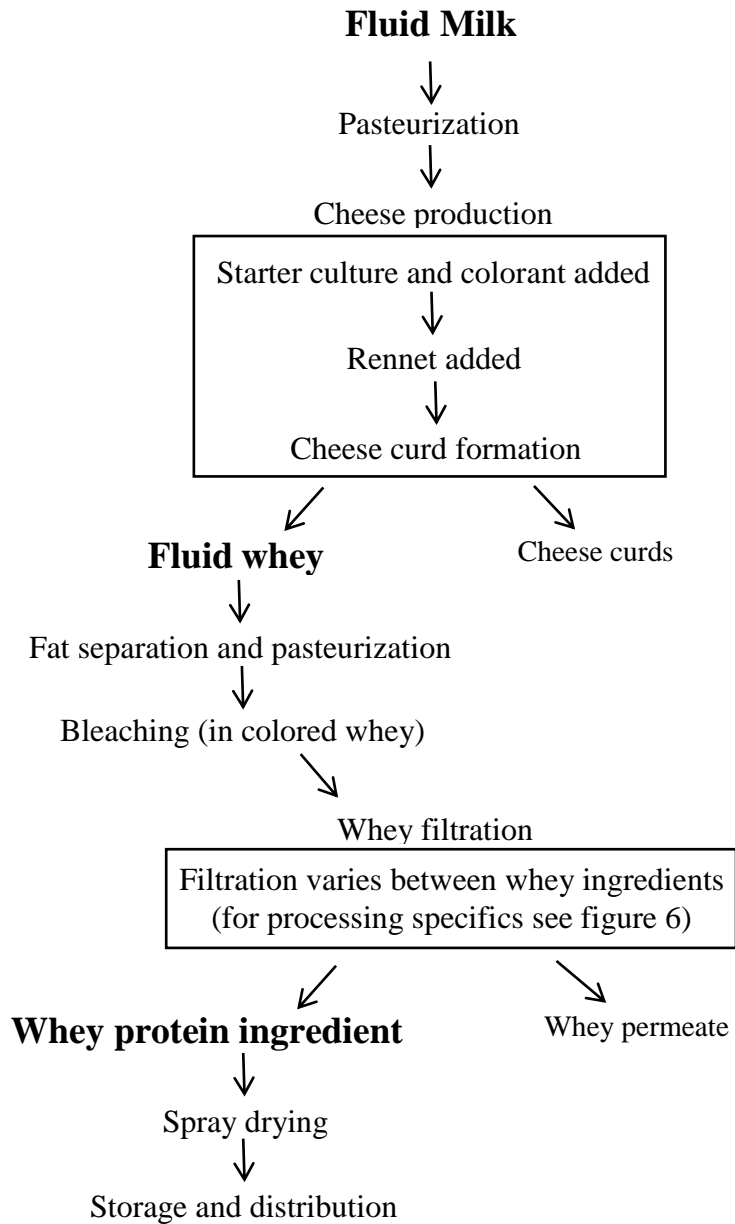


Figure 1. Flow diagram of general whey processing from raw milk to powdered whey ingredients (adapted from Bylund, 2003).

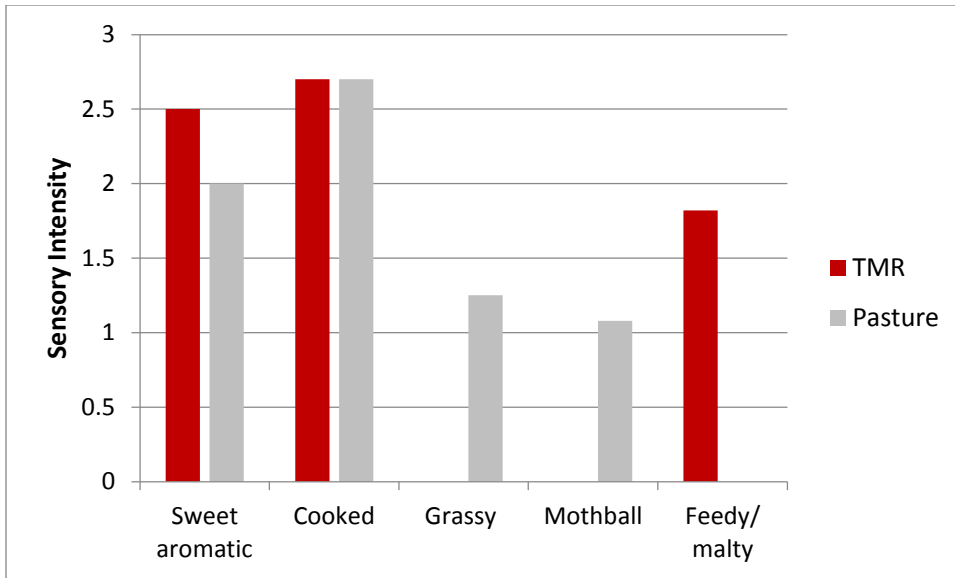


Figure 1. Flow diagram of general whey processing from raw milk to powdered whey ingredients (adapted from Bylund, 2003). Attributes were scored using a universal 0-15 point intensity scale.

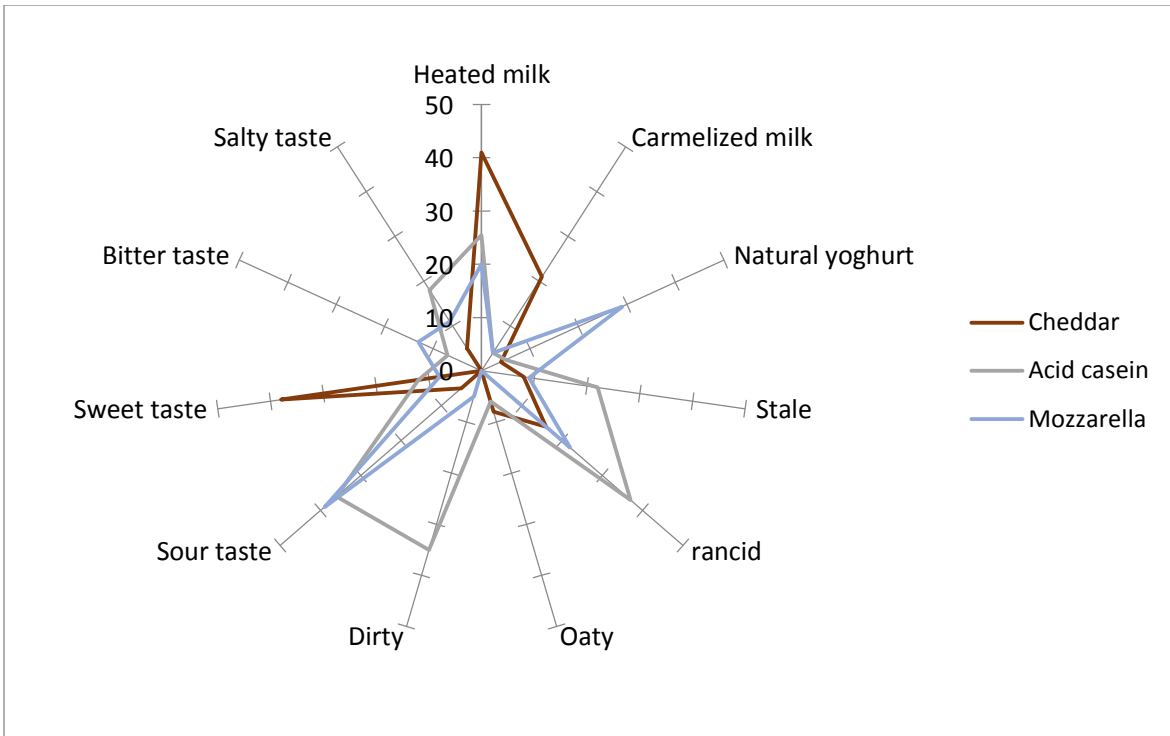


Figure 3. Trained panel flavor profiles of fluid whey from Mozzarella cheese, Cheddar cheese or acid casein manufacture (adapted from Gallardo Escamilla et al., 2005a).

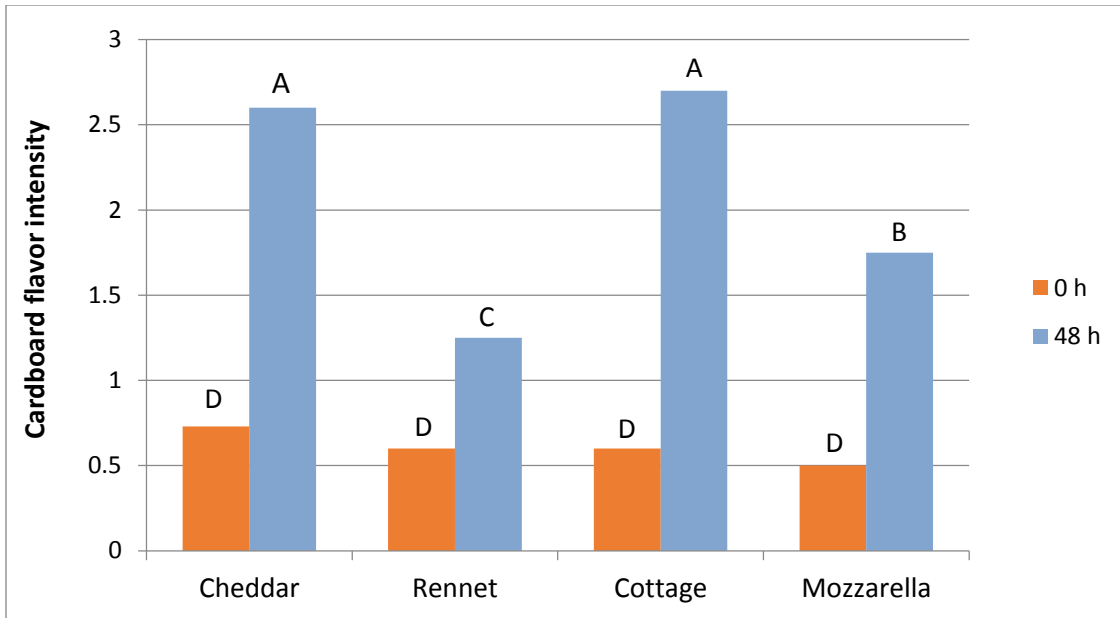


Figure 4. Trained panel cardboard flavor intensities of fluid wheys before and after storage at 4°C (adapted from Smith et al., 2016b; Liaw et al., 2010). Scores based on a universal 15-point intensity scale. <sup>a,b,c</sup>Means within rows with different letters are statistically different (P<0.05)

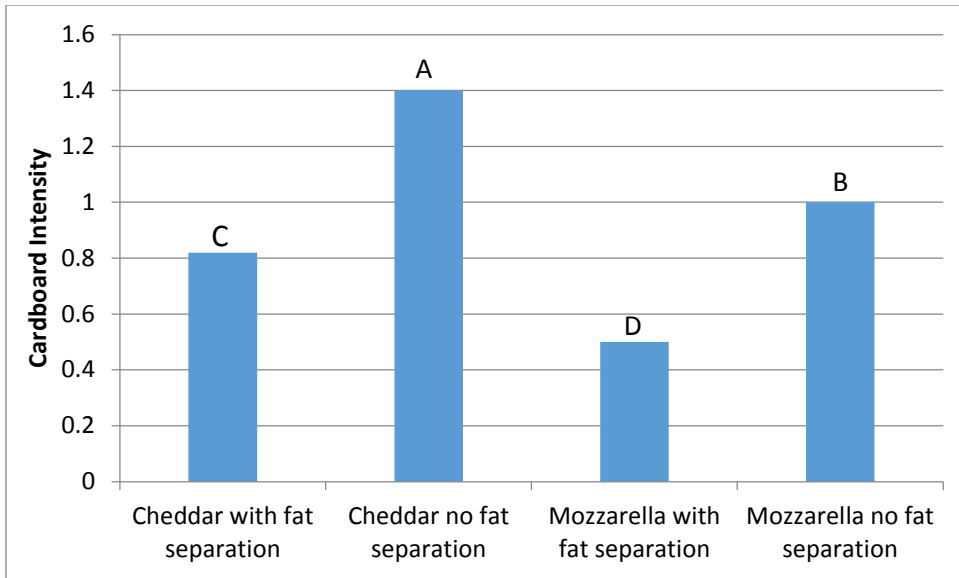


Figure 5. Trained panel cardboard flavor intensities of Cheddar and Mozzarella liquid wheys after 72h of storage at 3°C (adapted from Liaw et al., 2011). Scores based on a universal 15-point intensity scale. <sup>a,b,c</sup>Means within rows with different letters are statistically different (P<0.05).

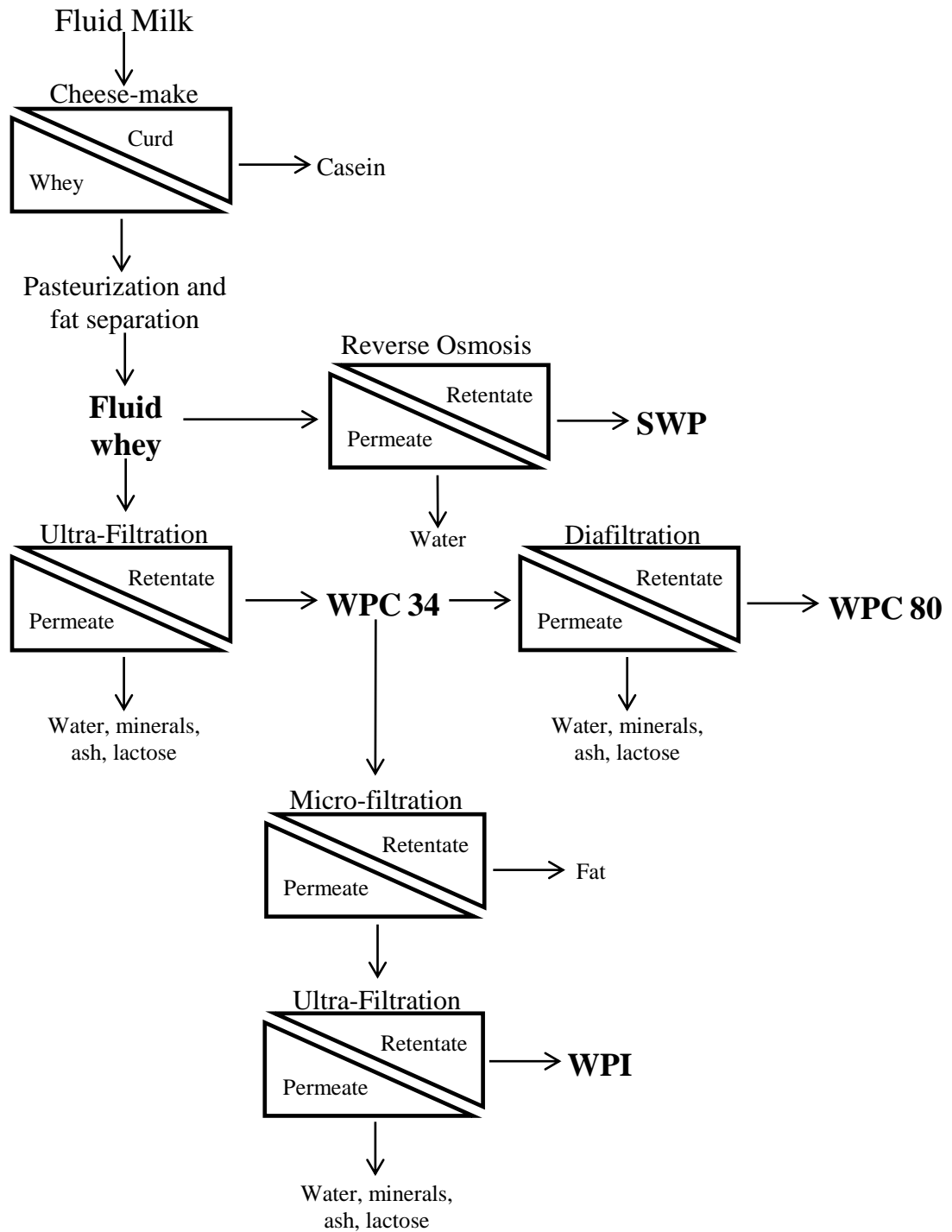


Figure 6. Flow diagram of whey process from cheese vat to whey ingredients (adapted from Bylund, 2003). SWP=Sweet whey protein, WPC= Whey protein concentrate, WPI = Whey protein isolate.

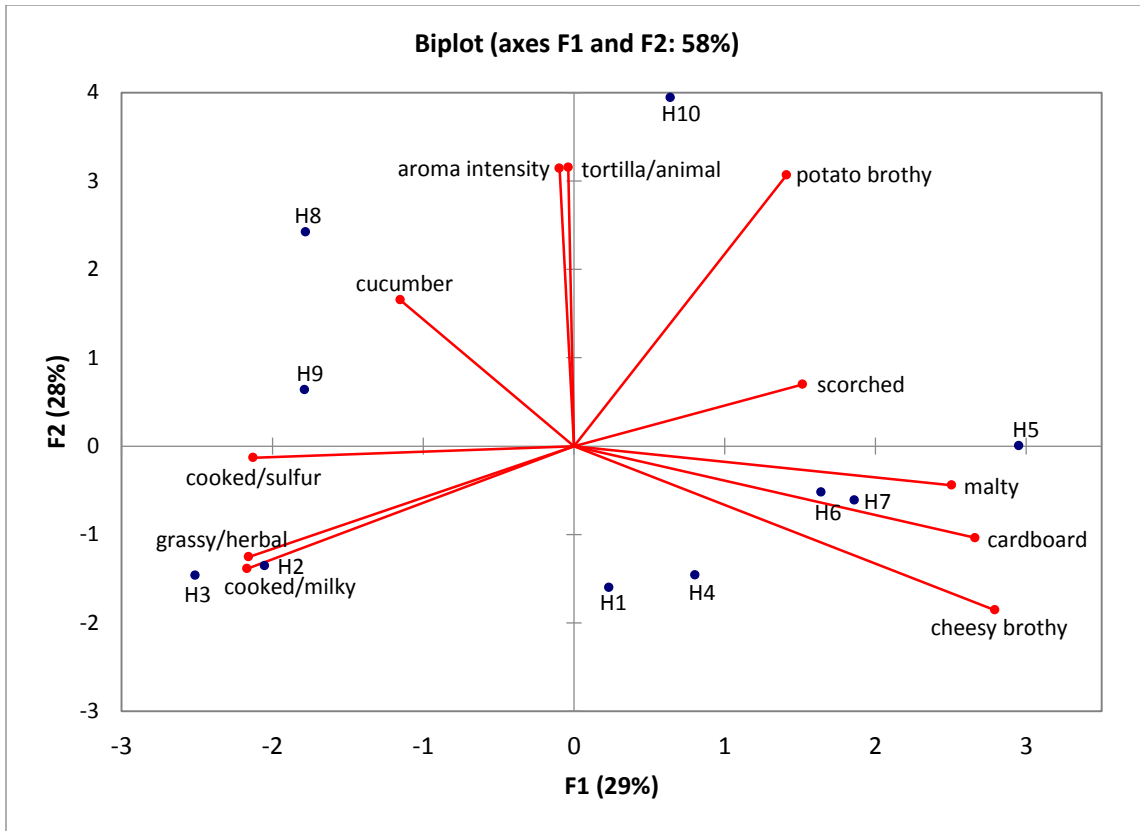


Figure 7. Principal component analysis biplot of trained panel sensory profiles of selected rehydrated whey protein hydrolysates (WPH) (adapted from Leksrisompong et al., 2010). H1-H10 = WPH samples.

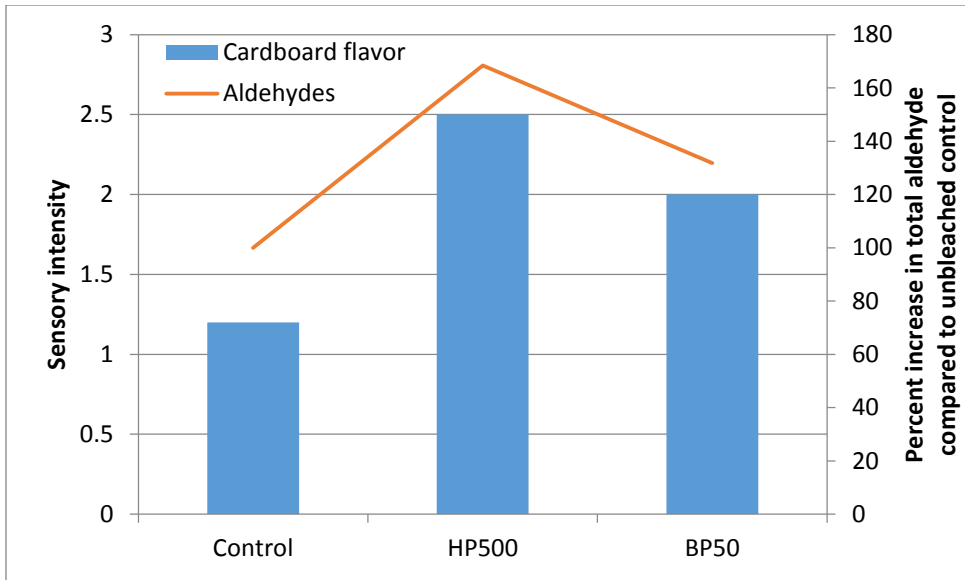


Figure 8. Comparison of trained panel cardboard flavor intensity and percent increase in total aldehydes compared to control in rehydrated WPC80 bleached with 500 ppm hydrogen peroxide (HP250) or 50 ppm benzoyl peroxide (BP50) (adapted from Jervis et al., 2012).



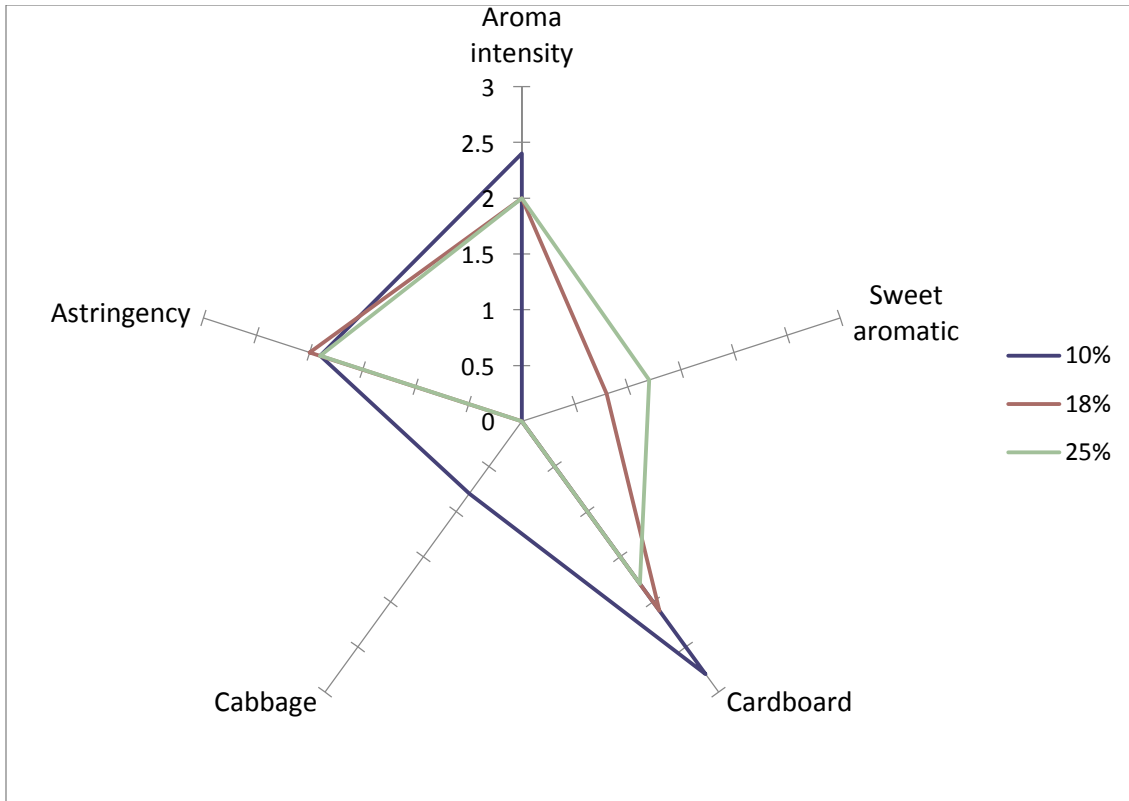


Figure 9. Trained sensory panel profiles of rehydrated WPC80 spray dried at different solids concentrations. All samples were spray dried at an inlet temperature of 200 °C (Adapted from Park et al., 2014a).

**CHAPTER 2: LITERATURE REVIEW. FLAVOR ASPECTS OF WHEY  
PROTEIN INGREDIENTS**

**The effect of bleaching agents on the degradation of vitamins and carotenoids  
in spray dried whey protein concentrate**

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## INTERPRATIVE SUMMARY

Whey from colored cheese is bleached to remove residual annatto (norbixin). Different bleaching agents to remove annatto impact whey protein functionality and flavor. The role of bleaching agents on vitamin and carotenoid degradation in whey is unknown. This paper demonstrated the effects of bleaching agents (hydrogen peroxide (HP), benzoyl peroxide (BP), or native lactoperoxidase (LP)) on vitamin and carotenoid degradation in liquid whey. These findings provide insight to whey manufacturers and unit operations specialists in selection of processing agents and unit operations.

## ABSTRACT

Previous research has shown that bleaching impacts flavor and functionality of whey proteins. The role of different bleaching agents on vitamin and carotenoid degradation is unknown. The objective of this study was to determine the effects of bleaching whey containing traditional annatto (norbixin) by hydrogen peroxide (HP), benzoyl peroxide (BP), or native lactoperoxidase (LP) as well as the addition of an alternative colorant on vitamin and carotenoid degradation in spray dried whey protein concentrate 80% protein (WPC 80). Cheddar whey colored with annatto (15 mL/454 L milk) was manufactured, pasteurized, and fat separated and then bleached with 250 mg/kg HP, 25 mg/kg BP, or 15 mg/kg HP (LP system) at 50°C for 1h. In addition to a control (Con) whey with norbixin, whey from cheese milk with an alternative colorant (AltC) was evaluated. Con and AltC wheys were also heated to 50 °C for 1h. Wheys were concentrated to 80% protein by ultrafiltration and spray dried. The experiment was replicated in triplicate. Samples were taken after initial milk pasteurization, initial whey formation, after fat separation, after whey pasteurization, after

bleaching, and after spray drying for vitamin and carotenoid analyses. Concentrations of retinol,  $\alpha$ -tocopherol, water soluble vitamins, norbixin, and other carotenoids were determined by high performance liquid chromatography (HPLC). Volatile compounds were measured by gas chromatography mass spectrometry. Sensory attributes of the rehydrated WPC80 were documented by a trained panel. Fat soluble vitamins were reduced in all wheys by more than 90% following curd formation and fat separation. After chemical or enzymatic bleaching, WPC80 displayed a 7.0-33.3% reduction in retinol,  $\beta$ -carotene, ascorbic acid, thiamine,  $\alpha$ -carotene, and  $\alpha$ -tocopherol ( $p < 0.05$ ). WPC80 bleached with BP contained significantly less of these compounds than HP or LP WPC80 ( $p < 0.05$ ). Riboflavin, pantothenic acid, pyridoxine, nicotinic acid, and cobalamin concentrations in fluid whey were not impacted by bleaching ( $p > 0.05$ ). With the exception of cobalamin and ascorbic acid, water soluble vitamins were reduced by less than 20% throughout processing. Norbixin destruction, volatile compound, and sensory results were consistent with previous studies on bleached WPC80. WPC80 colored with AltC had a similar sensory profile, volatile compound profile, and vitamin concentration as Con WPC80 ( $p > 0.05$ ).

## INTRODUCTION

The production of whey protein concentrate (WPC; 25 to 89.9% protein) has grown considerably in recent years. In 2014, approximately 244 million kilograms of WPC were produced in the United States (USDA, 2014) representing a 29.6% increase in WPC production from 2009 (USDA, 2009). As an ingredient, WPC is often utilized for its unique functional properties (Davis and Foegeding, 2007; Gildas et al., 2009; Foegeding et al., 2002) and for its high quality amino acid profile (Coker et al., 2012; Yang et al., 2012; Candow et

al., 2006). These characteristics have made WPC a useful ingredient as a protein additive (Sonja et al., 2011; Varnam and Sutherland, 1994), an emulsifier (Foegeding et al., 2002), and a thickening agent (Foegeding et al., 2006). However, WPC produced from colored Cheddar cheese retains approximately 10% of the added annatto colorant which, unless removed, limits its application as a food ingredient (Jervis et al., 2012; Smith et al., 2014). The primary colorant utilized for Cheddar cheese is annatto (Scotter, 2009), a naturally occurring substance extracted from achiote tree seeds which has a dark orange color and is comprised of the carotenoid norbixin (Kang et al., 2010). Because norbixin is soluble in polar solutions, approximately 10% of this compound is not retained in the cheese curd and is present in the whey (Smith et al., 2014).

Oxidizing bleaching agents effectively degrade norbixin by disrupting the double bonds that compose the chromophore of norbixin (Kang et al., 2010; Winter et al., 2008). Although effective at removing color, these chemical bleaches are nonspecific and also promote lipid oxidation, causing off flavors in spray dried whey ingredients (Jervis et al., 2012; Jervis and Drake, 2015; Smith et al., 2015; Campbell et al., 2012). Both chemical (hydrogen peroxide and benzoyl peroxide) and enzymatic (lactoperoxidase) bleaching agents can be applied to remove norbixin with variable results dependent on bleaching agent and bleaching conditions, but all function as oxidizing agents and can contribute lipid oxidation and off flavors (Jervis et al., 2012; Campbell et al., 2012, 2014; Smith et al., 2015).

One question that has not been addressed is how bleaching agents affect degradation of vitamins. Chemical bleaching reduces vitamin and carotenoid contents in white flour (Guo et al., 2016, McCay 1985). Native peroxidase in vegetables and fruit can degrade ascorbic

acid, anthocyanins, fatty acids, and carotenoids (Sivasankar 2002). However, little work has been done to address how bleaching agents affect vitamins in whey protein ingredients. Although flavor is the primary driver in liking of protein beverages, nutrient composition is an important indicator of consumer acceptance of these products(Oltman et al., 2015; Childs et al., 2008). Although the contents of vitamin content is below labeling ranges for a typical serving size, people that consume several servings of whey protein a day will get a significant amount of several vitamins from whey protein. It is therefore important to understand how to minimize the degradation of key nutrients during whey processing. Due to the flavor and functional effects of bleaching, alternative colorants for Cheddar cheese that do not partition into the whey have been proposed (Kang et al., 2012; Smith et al., 2014). The objective of this study was to determine the effects of bleaching whey containing traditional annatto colorant with hydrogen peroxide (HP), benzoyl peroxide (BP), or native lactoperoxidase (LP) on vitamin and carotenoid degradation in WPC 80. A control unbleached colored whey and a whey from cheese colored with an alternative  $\beta$ -carotene colorant were also included.

## MATERIALS AND METHODS

### *Experimental Design*

For each experimental replication, whey was manufactured in the NCSU dairy pilot plant from one lot of whole fat, raw bovine milk from the NCSU dairy enterprise system (DES). Each lot of milk was processed on the same day it was received. The milk was divided into two sections; one portion was used to produce Cheddar cheese colored with norbixin, while the other portion was used to make Cheddar cheese colored with an

alternative colorant (AltC) at the recommended dosage of 23 mL/454 kg milk (DairyMax™ Orange Red 002, a  $\beta$ -carotene based colorant, Chr Hansen, Copenhagen, Denmark). The whey from both vats was processed into powdered WPC80. Approximately 120 mL of sample were collected at nine points throughout processing (Table 1). Liquid samples and powdered WPC80 for vitamin and carotenoid testing were stored at -80C until evaluation (<30d). Descriptive sensory analysis and volatile compound analysis were also performed on powdered WPC80. Manufacturing procedures were repeated in triplicate, from three different batches of milk.

#### *WPC Manufacture*

WPC 80 was manufactured as described by Park et al. (2014), with raw whole milk received from the North Carolina State University DES. Approximately 68 kg of raw whole milk was High-Temperature-Short-Time (HTST) pasteurized at 73°C for 17 sec (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC). Milk was separated into two cheese vats, heated to 31°C, and inoculated with a mesophilic starter culture (Choozit™ MA 11, Danisco, New Century, NJ) at a concentration of 50 DCU/454 kg milk. Calcium chloride (50% w/v, Dairy Connection Inc., Madison, Wis., U.S.A.) was then added to both vats at 0.39 mL/kg. The milk was ripened for 30 min under constant agitation before colorants were added based on treatment. One received double-strength annatto (Cheese Color DS Double Strength, Dairy Connection Inc., Madison, WI) at a rate of 15 mL/454 Kg and the other received AltC (DairyMax™ Orange Red 002, Chr Hansen) at a rate of 23 mL/ 454 kg, before being allowed to ripen for an additional 30 min. Double strength recombinant rennet (Dairy Connection Inc.) was then added at a rate of 0.09 mL/kg



and allowed to set for 30 min. The coagulum was cut into cubes approximately 2.54 cm in length and allowed to rest for 5 min, followed by gradual heating to 39°C over the course of 30 min with gentle agitation. Once a pH of 6.35 was achieved, the whey was drained through a sieve, separated, and pasteurized at 73°C for 17 sec (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC), at which point the sweet whey was ready for individual treatments.

All samples that received a bleaching treatment were drawn from the annatto colored whey. All treatments were held at  $50.0 \pm 0.5$  °C and maintained at that temperature throughout the duration of this step. Bleaching treatments included HP with 250 ppm hydrogen peroxide (Sigma-Aldrich, St. Louis, MO), BP with 50 ppm benzoyl peroxide (Luperox A40FP-EZ9, Arkema Inc., King of Prussia, PA) (BP at 50 ppm), and LP with 20 ppm hydrogen peroxide used to activate the native lactoperoxidase (Campbell and Drake, 2013a, 2014), and a control with no added bleaching treatment. The AltC treated whey did not receive a bleaching treatment but was held at the same temperature for the same time as the other treatments. After 1 h, wheys containing hydrogen peroxide (HP and LP) were treated with catalase (FoodPro CAT; Danisco, New Century, NJ) at a rate of 20 mg/kg to consume any remaining HP. Wheys were then filtered through an ultrafiltration (UF) unit (model Pellicon 2, Millipore Inc., Darmstadt, Germany) containing 5 polyethersulfone cartridge membrane filters (model P2B010V05; 10-kDa nominal separation cutoffs, 0.5m<sup>2</sup> surface area; Millipore Inc.). Following primary filtration, samples were diluted with deionized water and diafiltered until protein content of the retentate reached 80% as determined by a Sprint rapid protein analyzer (CEM Corporation, Matthews, NC) and a

Smart System 5 moisture/solids analyzer (CEM Corporation). Samples were then spray dried (model Lab 1; Anhydro Inc., Søborg, Denmark) with an inlet temperature of 200°C and an outlet temperature of 90°C. Following the drying step, samples were placed in Mylar bags and stored at -80°C.

### *Proximate Analysis*

Samples for vitamin and carotenoid analysis were drawn at seven different points throughout processing (Table 1). For each sample, approximately 120 mL of product was taken. The total solids of each liquid sample were determined by forced draft oven (AOAC, 2012; method number 990.20: 33.2.44). The total fat of each sample was determined by ether extraction using a modified Mojonnier method (AOAC, 2012; method number 932.06;33.5.08).

Powdered WPC80 samples were measured for total solids, fat content, and total protein. Percent moisture was measured using a vacuum oven (AOAC, 2012; method number 990.20: 33.2.44). The total fat content was measured by ether extraction using a modified Mojonnier method (AOAC, 2012; method number 932.06; 33.5.08). Total protein was measured using the Kjeldahl method (AOAC, 2012; method number 991.20; 33.2.11) by multiplying total nitrogen by a factor of 6.38.

### *Vitamin and carotenoid analyses*

All extractions were performed under filtered lighting designed to limit UVA and UVB radiation below 520 nm (Ergomart, Dallas, TX). Powdered WPC80 samples were rehydrated to 5.0± 0.2% solids with deionized water prior to testing. Liquid samples were diluted to 5.0± 0.2% solids with deionized water prior to measurement. Frozen liquid

samples were first thawed at 37°C (Giuliano et al., 1992) prior to being vortexed. All samples were measured in triplicate.

#### *Vitamin and carotenoid analyses*

Retinol,  $\beta$ -carotene,  $\alpha$ -carotene, and lutein concentrations were determined by high-performance liquid chromatography as outlined by Giuliano et al. (1992) with minor adjustments. Twelve milliliters of liquid or rehydrated sample (5.0% solids) were added to a container with 5 mL of ethyl alcohol (Sigma-Aldrich) and 3 mL of 50% (w/v) KOH (VWR, Randor, PA.) dissolved in deionized water. Samples were flushed with nitrogen, capped with a Teflon-lined phenolic cap, and sonicated for 5 min. Samples for retinol and  $\beta$ -carotene determination were allowed to mix for 16 h on an oscillating plate mixer (RotoMix Type 50800) at 180 rotations/min, while samples for  $\alpha$ -carotene and lutein analysis were saponified for 30 min on an oscillating plate mixer under the same conditions. After the saponification step, 6 mL of hexane (Sigma-Aldrich) was poured into each vial, vortexed, sonicated for 5 min, and centrifuged at 800 x g for 10 min. The hexane layer was then extracted and the aqueous phase was treated with an additional 3 mL of hexane. This complex was then vortexed, sonicated for 5 min, and centrifuged again. The resulting hexane phase was extracted and added to the original hexane component and evaporated under nitrogen until 1-2 mL of hexane remained.

The hexane phase was then treated with 2.5 mL of ethanol and 3.5 mL of HPLC grade water (Fluka, Seelze, Germany). The complex was vortexed, sonicated for 5 min, and centrifuged for 10 min at 800 x g. The hexane layer was transferred into another container and the aqueous phase was treated with 3 mL of hexane before being sonicated and

centrifuged as before. Both hexane layers were combined and evaporated to dryness with nitrogen before being dissolved in 600  $\mu\text{L}$  of a HPLC grade tetrahydrofuran-methanol (20:80 v/v) complex (Sigma-Aldrich, Fluka, respectively). Samples were then filtered with nylon 0.20  $\mu\text{m}$  (VWR, Randor, PA) and 20.0  $\mu\text{L}$  were injected onto a high performance liquid chromatography unit with a C18 reversed phase column (Kinelex 5u XB-C18 100 x 4.60 mm, Phenomenex) with a flow rate of 0.7 mL/min. Aliquots of each sample were then eluted with a mobile phase composed of 10% THF, 90% methanol and 0.5 g/liter butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO). Column temperature was maintained at  $28^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$  and flow rate was maintained at 0.25 mL/min. Peaks were analyzed using a Waters 2998 photodiode array (PDA) detector set at 448 nm for  $\beta$ -carotene, 325 nm for retinol, and 452 nm for all other carotenoids. Compound concentrations were determined based on a standard curves composed of 6 dilutions of  $\geq 99.0\%$  crystalline retinol (Sigma-Aldrich),  $\geq 99.5\%$   $\beta$ -carotene (Sigma-Aldrich),  $\geq 99.5\%$   $\alpha$ -carotene (Sigma-Aldrich),  $\geq 99.5\%$  lutein (Sigma-Aldrich). Each sample was extracted in triplicate. Each extraction was injected and measured chromatographically twice. Norbixin was extracted from fluid wheys and powders and quantified as described by Campbell et al. (2014).

#### *Ascorbic acid determination*

Ascorbic acid concentration was determined using the high-performance liquid chromatographic method outlined by Romeu-Nadal et al. (2006). Three hundred  $\mu\text{L}$  of fluid or rehydrated whey ( $5.0 \pm 0.2\%$  solids) was mixed with 300  $\mu\text{l}$  of 0.56% (w/v) meta-phosphoric acid (33.5-36.5%, Sigma-Aldrich) and HPLC grade water (Fluka) was then added. Samples were shaken for 30 sec and centrifuged in a microcentrifuge at 12,000 x g for

10 min to separate fat and protein from the aqueous phase. All samples were then filtered through a 0.45  $\mu\text{m}$  cellulose filter (VWR) and analyzed by high performance liquid chromatography (Breeze 2 system, Waters Corporation, Milford, MA) with a reversed phase column (Kinelex 5u XB-C18 100 x 4.60 mm, Phenomenex). 30  $\mu\text{l}$  of sample was injected with a flow rate of 0.7 mL/min, with a mobile phase composed of HPLC grade water with 0.1% acetic acid (Sigma-Aldrich) and methanol (HPLC grade, Fluka) at a concentration of (95:5 v/v). Samples were analyzed using a Waters 2998 photodiode array detector with a wavelength of 254 nm. Ascorbic acid concentration was determined based on a seven point standard curve derived from an L-ascorbic acid standard ( $\geq 99.0\%$  purity, Sigma-Aldrich). All whey samples were extracted in triplicate. Each extraction was injected in duplicate.

#### *$\alpha$ -Tocopherol determination*

$\alpha$ -Tocopherol was quantified by high-performance liquid chromatography as outlined by Salo-Väänänen et al. (2000) with minor modifications. First, liquid and rehydrated samples ( $5.0 \pm 0.2\%$  solids) were thermostated at 40° C to stabilize fat distribution for 30 min. One gram of each sample was then added to a container with 0.5 g of 98.5% pyrogallol (Sigma-Aldrich) and 0.5 g of 95% ascorbic acid (Sigma-Aldrich) before being capped, vortexed, and allowed to stand for 10 min. The container was nitrogen flushed after the addition of 0.5 mL of 50% KOH. The tube was capped and transferred to a boiling water bath for 20 min and shaken once after 10 min. The tube was then cooled in an ice-water bath for 10 min and 6 mL of a (8:1:1, v/v) solution of water, hexane, and ethyl acetate (Sigma-Aldrich) were added. The tubes were then shaken and centrifuged for 10 min at 800 x g. This extraction was repeated two more times with 2 mL of the water-hexane-ethyl acetate solution

and the combined organic phase was evaporated to dryness under nitrogen before being reconstituted in 1 mL of a tetrahydrofuran (THF)- methanol complex (20:80 v/v). Samples were then filtered through a nylon 0.20 µm filter (VWR) and 30 µL were injected onto a high performance liquid chromatography unit with a reversed phase column (Kinelex 5u XB-C18 100 x 4.60 mm, Phenomenex). Aliquots of each sample were then quantified with a mobile phase composed of 10% THF, 90% methanol and 0.5 g/liter butylated hydroxytoluene. Column temperature was maintained at 30°C ± 0.4°C and flow rate was maintained at 0.7 mL/min. Peaks were analyzed using a Waters 2998 pathodiode array detector set at 265 nm for Vit D and 292 nm for α tocopherol. Vitamin concentration was determined based on standard curves composed of 6 concentrations of ≥99.0% α-tocopherol (Sigma-Aldrich), and ≥99.0% cholecalciferol (Sigma-Aldrich).

*Thiamine, riboflavin, nicotinic acid, pantothenic acid, and cobalamin determination*

Thiamine, riboflavin, nicotinic acid, pantothenic acid, and cobalamin were determined chromatographically in triplicate as outlined by Agostini-Costa et al. (2007). A 5-8 mL portion of liquid or rehydrated sample (5.0 ± 0.2% solids) was placed into a flask containing 45 mL of 0.1 N sulfuric acid (Fluka). The solution was mixed on an oscillating plate mixer at 180 rotations/min for 1 h. This complex was then transferred to a 100 mL volumetric flask and brought to volume with methanol before being filtered through a 0.45 µm cellulose filter and analyzed by high-performance liquid chromatography (Breeze 2 system, Waters Corporation, Milford, MA) with a reversed phase column (Kinelex 5u XB-C18 100 x 4.60 mm, Phenomenex). 20 µl of sample were injected with a flow rate of 1.0 mL/min implementing a gradient of two mobile phases. The aqueous phase consisted of 2%

acetonitrile and 0.15% trimethylamine (Sigma-Aldrich) in 5 mmol/L hexanesulfonic acid (EMD Millipore, Darmstadt, Germany), and 87.85% HPLC grade water adjusted to pH 2.8 with 0.1N H<sub>2</sub>SO<sub>4</sub>. The organic phase was 100% of HPLC grade methanol. The gradient was programmed to begin with 100% aqueous phase for 3 min, followed by a linear progression to 43% aqueous phase and 57% organic phase at 23 min. Following this gradient, the machine was programmed to return to 100% aqueous phase by minute 35 and allowed to equilibrate until minute 55 at which point another sample could be injected onto the column. Peaks were analyzed using a Waters 2998 photodiode array detector measuring wavelengths 254 nm, 278 nm, and 358 nm. Peak identification as well as compound concentration was determined based on individual standard curves derived from standards for each compound. Vitamin concentration was determined based on a standard curves composed of 6 concentrations of  $\geq 99.0\%$  thiamin (Sigma-Aldrich),  $\geq 98.0\%$  riboflavin (Supelco, Bellefonte, P.A.),  $\geq 99.5\%$  nicotinic acid (Sigma-Aldrich), D-pantothenic acid  $\geq 99\%$  (Sigma-Aldrich, and  $\geq 98.0\%$  cyanocobalamin (Sigma-Aldrich). Compounds were evaluated from each sample in duplicate.

#### *Volatile Compound Analysis*

Volatile compounds were determined in triplicate by GC-MS after extraction by headspace solid-phase microextraction as outlined by Campbell et al. (2011). Spray-dried WPC80 samples were rehydrated to 10% (w/v) solids, with 10% (w/v) NaCl (VWR) and 5  $\mu$ L of an internal standard composed of 81 ppm 2-methyl-3-heptanone in HPLC grade ether (Sigma Aldrich). Samples were maintained at 10°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before a 30 min exposure to a Tri Phase

(divinylbenzene/carboxen/polydimethylsiloxane) 1 cm fiber (Supelco, Bellefonte, PA) at 31 mm with 4-s pulsed agitation at 250 rpm. Fibers were injected at a depth of 50 mm for 5 min. Sampling injections and equilibrations were done by use of a CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). The column used was a Rtx-5ms column (Rtx-5mn 30 m x 0.25 mm i.d. x 0.25  $\mu$ m film thickness; Restek Bellefonte, PA). The initial GC temperature was maintained at 40°C for 3 minutes, then increased to 90°C at a rate of 10°C/min, then increased to 200°C at a rate of 5°C/min. This temperature was held for 10 min, then increased to 250°C at a rate of 20°C/min and held for 5 min. The fibers were introduced into the split/splitless injector while the column was maintained at 250°C. The MS source and transfer line were maintained at 250°C with the quadrupole at 150°C. Compounds were identified using the National Institute of Standards and Technology (2005) mass spectral database. Internal standard concentration was used to calculate the relative abundance of selected compounds (Campbell and Drake, 2013b; Jervis et al., 2012).

#### *Descriptive Sensory Analysis*

Descriptive sensory analysis of rehydrated WPC80 was conducted by a trained sensory panel (n = 8) in compliance with North Carolina State University Institutional Review Board for Human Subjects approval. Panelists were between the ages of 23 and 50 y, each with over 150 h of experience in descriptive analysis of dried dairy ingredients using the Spectrum™ descriptive analysis method and a 0 to 15 point universal intensity scale (Meilgaard et al., 2007; Drake and Civille, 2003). Powders were rehydrated to 10% solids (w:v) in deionized water and descriptive analysis utilized a previously published lexicon for dried dairy ingredients (Drake et al., 2003; 2009; Wright et al., 2009). Samples (40 mL) were



dispensed without overhead lighting into 59-mL soufflé cups (Solo Cup Co., Champaign, IL), lidded, given a randomized 3-digit code, and allowed to temper to 21°C. Panelists expectorated each sample and were provided DI water for palate cleansing. Each sample from each experimental replicate was evaluated by each panelist in duplicate. Compusense Cloud version 7.8 (Compusense, Guelph, ON, Canada) was used for data collection.

#### *Statistical Analyses*

Data were analyzed by analysis of variance with means separation using XLSTAT software (version 2015.3.01; Addinsoft, New York, NY). Fisher's least significant difference (LSD) test was used to analyze differences among sample means.

## RESULTS AND DISCUSSION

#### *Compositional analysis*

Fat concentrations of liquid whey before fat separation, following fat separation, and following ultrafiltration were  $0.19\% \pm 0.04$ ,  $0.07\% \pm 0.05$ , and  $0.381\% \pm 0.073$  respectively. Percent solids of liquid whey before separation, whey after fat separation, and whey following ultrafiltration were  $6.87\% \pm 0.62$ ,  $6.40\% \pm 0.71$ , and  $10.32\% \pm 0.65$  respectively. Fat content of powdered WPC80 was  $3.87\% \pm 0.53$ , moisture was  $4.69\% \pm 0.68$ , and the protein content was  $81.3\% \pm 0.95$ . Calcium, potassium, magnesium, sodium, and phosphorus concentrations of powdered WPC80 were not different ( $p > 0.05$ ) and were consistent with previous studies (Smith et al., 2015; Jervis et al., 2012; and Campbell et al., 2014; results not shown). The mean concentration of iron was significantly lower ( $p < 0.05$ ) in powdered WPC80 bleached with HP or LP compared to powdered WPC80 Con, AltC, and BP (7.48

$\pm 0.82$  versus  $11.49 \pm 1.16$  mg/kg respectively) which was consistent with previous research (Jervis et al., 2012).

### *Vitamin loss during processing*

Degradation of fat-soluble compounds in WPC80 (Table 2) was induced by bleaching, however over 90% of fat-soluble compound loss occurred during curd formation and cream separation (stage 2 and 3 respectively, Figure 1). Because these vitamins are primarily found in milk fat, fat-soluble vitamin loss during these stages was likely because these processes reduce the concentration of fat in whey (Bilic et al., 1988; Batra et al., 1992). Consistent with previous research, retinol,  $\beta$ -carotene, and  $\alpha$ -carotene were not impacted ( $p > 0.05$ ) by whey pasteurization (Bilic et al., 1988; Jesse, 1990). Pasteurization of whey caused 3.2% degradation to  $\alpha$ -tocopherol ( $p < 0.05$ ), which is comparable to  $\alpha$ -tocopherol degradation during milk pasteurization (MacDonald et al., 2011). Ultra-filtration and spray drying also did not impact the concentration of fat soluble compounds ( $p > 0.05$ , Labuza and Tannenbaum, 1972). WPC80 colored with AltC contained 6.2% more  $\beta$ -carotene than uncolored Con WPC80 (0.065 versus 0.061 ppm,  $p < 0.05$ ), indicating  $< 1.0\%$  of the added  $\beta$ -carotene based alternative cheese colorant was present in the final whey. As with other fat-soluble compounds, the majority of the  $\beta$ -carotene in AltC samples remained in the curd and did not partition into the whey.

Consistent with previous literature, water-soluble vitamins in powdered WPC80 had less than 20% degradation compared to pasteurized milk, except for ascorbic acid and thiamin which were reduced from 1.24 to 0.71  $\mu\text{g}/100\text{ml}$  and 43.7 to 33.06  $\mu\text{g}/100\text{ml}$  respectively (MacDonald et al., 2011; Ottaway, 2002; Sierra and Vidal-Valverde, 2001).

Concentrations of ascorbic acid in liquid whey decreased during whey pasteurization and bleaching (stages 4 and 5 respectively,  $p < 0.05$ ). Although ascorbic acid is heat labile, bleaching caused much more degradation (1.14 to 0.74 mg/100ml) than pasteurization (1.18 to 1.14 mg/ml,  $p < 0.05$ ). Concentrations of cobalamin decreased following whey pasteurization (stages 4) by 10.2% ( $p < 0.05$ ). A meta-analysis by MacDonald et al. (2011) indicated that ascorbic acid, thiamin, and cobalamin are all heat labile and susceptible to degradation during milk pasteurization. Similar heat degradation was observed in liquid whey.

#### *Bleaching treatments*

The concentration of fat soluble vitamins immediately before bleaching of each sample were compared to concentration of fat soluble vitamins immediately after bleaching of each sample (Figure 2). Liquid whey treated with HP, LP, or BP had lower concentrations of all measured fat soluble vitamins after bleaching compared to concentrations before bleaching ( $p < 0.05$ ), indicating that bleaching negatively impacted fat soluble vitamins. Because retinol,  $\alpha$ -tocopherol,  $\beta$ -carotene, and  $\alpha$ -tocopherol are prone to oxidative degradation, these results were expected (Barrefors et al., 1995; Henry et al., 1998).

The concentration of  $\beta$ -carotene before and after bleaching in liquid wheys indicated more than 12% degradation by bleaching, with BP bleaching causing the most dramatic degradation (17.4%,  $p < 0.05$ ). Similar results were observed for  $\alpha$ -tocopherol, retinol, and  $\alpha$ -carotene before and after bleaching ( $p < 0.05$ ; Kamal-Eldin and Appelqvist 1996; McCay 1985). This may be due to the increased bleaching potential of BP due to the lower bond dissociation energy of the BP O-O bond (36.9 kcal/mol at 25°C) compared to the HP O-O

bond (50.5 kcal/mol at 25°C) caused by the charge stabilization along the benzene ring during hemolysis (Bach et al., 1996; Murakami et al., 1985). This low bond dissociation energy allows for greater bleaching potential and greater oxidation in BP compared to HP or hypothiocyanate (the peroxide formed during LP bleaching). Additionally, benzoyl peroxide is more nonpolar than HP or hypothiocyanate and might bleach fat soluble compounds more readily as it solubilizes along the surface of fat globules (Jervis et al., 2012). Bleaching efficiency of BP in fat was expected, as well as destruction of natural carotenoids, as BP has been used to bleach native carotenoids in milk fat during blue cheese production (Washam et al., 1974).

No difference in  $\alpha$ -carotene degradation was observed among bleaching agents ( $p > 0.05$ ). Bleaching caused greater degradation to  $\alpha$ -tocopherol ( $p < 0.05$ ) than any other compound tested with a degradation of 25.9%, 23.8%, and 31.9% for LP, HP, and BP respectively. This was expected, as  $\alpha$ -tocopherol has been demonstrated to be an antioxidant which is more efficient at free radical scavenging during lipid peroxidation than retinol or  $\beta$ -carotene (Vile and Winterbourn, 1988). Although compound degradation occurred, the concentrations of total vitamin A and vitamin E were less than one percent of the recommended daily intake outlined by the Code of Federal Regulations (FDA, 2016). There was no difference ( $p > 0.05$ ) between concentrations of fat soluble compounds before and after the bleaching step (stage 5) for Con and AltC. This included  $\beta$ -carotene, which indicates that the alternative colorant was retained in the curd and did not leech into the whey as intended. These samples were not subjected to a bleaching agent during this step, but were held at 50°C for 1h.

Bleaching liquid whey did not impact concentrations of thiamin, riboflavin, nicotinic acid, pantothenic acid, or cobalamin ( $p > 0.05$ , Table 2). Compounds such as thiamine and riboflavin are prone to oxidation but often require a catalyst to oxidize significantly under the conditions seen in milk (Frisell et al., 1959; Lukienko et al., 2000). However, bleaching liquid whey with LP, HP, and BP caused a 28.2%, 26.4%, and 35.0% degradation to ascorbic acid respectively ( $p < 0.05$ ). As with fat soluble compound degradation, there was no difference in the degradation of ascorbic acid between LP and HP bleached liquid whey ( $p > 0.05$ ), while BP bleaching caused more degradation to liquid whey than HP or LP ( $p < 0.05$ ). This was expected as natural oxidation of ascorbic acid in dairy products before the introduction of any oxidant has been well documented (Smith and Dunkley 1961; Zulueta et al., 2007).

Concentrations of thiamin, riboflavin, nicotinic acid, and cobalamin were not different in Con or AltC wheys following 1h at 50°C ( $p > 0.05$ , Table 2). This result suggests that heating liquid whey to 50°C for 1h did not affect the concentrations of these water soluble vitamins. Concentrations of pantothenic acid were reduced by 5.6 and 4.2 percent in Con and AltC wheys respectively following 1h at 50°C ( $p > 0.05$ ). No differences were observed in pantothenic acid degradation between treatments which used bleach and treatments which did not use bleach during this stage ( $p > 0.05$ ). Consistent with previous research, this finding indicates that the 50°C heat treatment for 1h was likely responsible for the observed pantothenic acid degradation (Ottaway, 1993).

Vitamin degradation in WPC80 due to bleaching has little to no impact on nutrition labeling. Riboflavin, thiamin, niacin, cobalamin, and pantothenic acid concentrations were

not impacted by bleaching. Retinol,  $\beta$ -carotene,  $\alpha$ -carotene, and  $\alpha$ -tocopherol concentrations were impacted by bleaching, but vitamin A and E concentrations are below 1% of the recommended daily value for a typical serving size of 25g (DV, FDA, 2016). Vitamin C was found at a concentration (2.3-3.1% DV at a 252g serving) which could be labeled in the nutrition facts panel (FDA, 2016). Bleaching of WPC80 caused a 1% reduction to the percent DV for vitamin C in WPC80. Differences between bleaching agents would not impact the percent DV for vitamin C in WPC80 due to rounding rules (FDA, 2016).

#### *Bleaching efficacy*

Treatment of liquid whey with LP or BP resulted in greater norbixin degradation ( $\geq 95\%$ ) than with HP (54.6%) ( $p < 0.05$ ), consistent with previous research (Jervis and Drake, 2013; Fox et al., 2013; Campbell and Drake, 2013b, 2014; Smith et al. 2015). All measured values were consistent with results reported in previous research (Fox et al., 2013; Smith et al., 2015; Campbell and Drake 2013b). It should be noted, that although LP bleaching caused more norbixin degradation than HP bleaching, LP bleaching caused similar degradation of fat soluble vitamins to HP bleaching ( $p > 0.05$ ). The primary objective of this study was to determine the effects of current bleaching practices on vitamin concentrations. Thus, concentrations of LP, BP and HP were used which represent moderate to high concentrations of what is used by industry, consistent with previous studies. Just as bleach concentrations, temperatures and time can be optimized to minimize off flavors and to maximize norbixin destruction, it might also be possible to optimize bleach conditions to minimize vitamin destruction. Future work is needed to address the optimal dose response relationship to optimize norbixin degradation and limit vitamin degradation for each bleaching agent.

### *Descriptive sensory and instrumental volatile analysis*

Sensory profiles of rehydrated WPC80 were consistent w/ previous studies (Jervis et al., 2012; Campbell and Drake, 2013b; Smith et al., 2015) (results not shown). Bleached WPC80 had higher cardboard flavors ( $\bar{x}$  = 2.0 vs 0.9, respectively) and lower sweet aromatic flavors ( $\bar{x}$  = 1.1 vs 1.7, respectively) compared to either Con or AltC WPC80 ( $p < 0.05$ ). LP WPC80 had a low but distinct cabbage flavor ( $\bar{x}$  = 1.2). The two control WPC80 (Con and AltC) were not distinct in flavor profile ( $p > 0.05$ ).

Instrumental volatile analysis was also consistent with previous studies (Smith et al., 2015; Jervis et al., 2012; Campbell and Drake, 2013b; Croissant et al., 2009; Fox et al., 2013) (Table 3). Bleached WPC80 had higher concentrations ( $p < 0.05$ ) of many lipid oxidation volatile compounds compared with Con or AltC WPC80. Consistent with previous studies, volatile compound profiles of the three bleached WPC80 were also distinct. There were no differences in the volatile compound profiles of Con and AltC WPC80 ( $p > 0.05$ ).

## CONCLUSIONS

To ensure high quality product, it is important that WPC80 is colorless. Both chemical and enzymatic bleaching degrades vitamins and carotenoids native to fluid milk and WPC80, including  $\alpha$ -tocopherol, retinol,  $\beta$ -carotene,  $\alpha$ -carotene, and ascorbic acid. These results did not significantly influence the recommended daily intake of vitamins A, E, or C. WPC80 bleached with BP has established greater bleaching efficiency and lower effects on flavor compared to HP or LP. However, BP results in greater degradation of vitamins and carotenoids than HP or LP. Water soluble vitamins including thiamin, riboflavin, nicotinic acid, pantothenic acid, and cobalamin were not impacted by enzymatic or oxidative

bleaching agents. Enzymatic bleaching with LP can provide similar degradation of the colorant norbixin, while degrading less  $\alpha$ -tocopherol, retinol, and ascorbic acid than BP. Alternative colorants may provide a solution which allows for colorless WPC80 with minimal off flavors while also preserving compounds prone to degradation.

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## TABLES AND FIGURES

Table 1. Sampling points for whey processing.

Production point	Stage of processing
Milk following pasteurization	1
Whey drained from vat	2
Liquid whey following fat separation before HTST	3
Liquid whey following pasteurization	4
Liquid whey following bleaching treatment	5
Retentate stream following ultra-filtration	6
Spray dried powder	7

Table 2. Vitamin and carotenoid concentrations before and after bleaching treatments.

Treatment	Prior to bleaching treatment <sup>1</sup>		Following bleaching treatment <sup>2</sup>				
	Traditional colorant	Alternative colorant	Con	AltC <sup>3</sup>	LP	HP	BP
α-Tocopherol (µg/ 100 mL)	1.17 <sub>A</sub>	1.13 <sub>A</sub>	1.12 <sub>A</sub>	1.14 <sub>A</sub>	0.92 <sub>B</sub>	0.86 <sub>B</sub>	0.77 <sub>C</sub>
Retinol (µg/100 mL)	1.87 <sub>A</sub>	1.92 <sub>A</sub>	1.83 <sub>A</sub>	1.87 <sub>A</sub>	1.73 <sub>B</sub>	1.75 <sub>B</sub>	1.69 <sub>C</sub>
β-Carotene (µg/100mL)	0.862 <sub>AB</sub>	0.921 <sub>A</sub>	0.945 <sub>B</sub>	0.880 <sub>AB</sub>	0.734 <sub>CD</sub>	0.756 <sub>C</sub>	0.702 <sub>D</sub>
α-Carotene (µg /100mL)	0.040 <sub>A</sub>	0.040 <sub>A</sub>	0.039 <sub>A</sub>	0.039 <sub>A</sub>	0.036 <sub>B</sub>	0.036 <sub>B</sub>	0.035 <sub>B</sub>
Ascorbic Acid (mg/100 mL)	1.14 <sub>A</sub>	1.15 <sub>A</sub>	1.13 <sub>A</sub>	1.14 <sub>A</sub>	0.82 <sub>B</sub>	0.84 <sub>B</sub>	0.74 <sub>C</sub>
Thiamine (µg/100 mL)	40.5 <sub>A</sub>	40.7 <sub>A</sub>	39.8 <sub>A</sub>	40.3 <sub>A</sub>	39.1 <sub>A</sub>	39.5 <sub>A</sub>	38.5 <sub>A</sub>
Riboflavin (µg/100 mL)	197 <sub>A</sub>	198 <sub>A</sub>	194 <sub>A</sub>	192 <sub>A</sub>	196 <sub>A</sub>	194 <sub>A</sub>	193 <sub>A</sub>
Nicotinic Acid (µg/100 mL)	86.0 <sub>A</sub>	86.1 <sub>A</sub>	85.1 <sub>A</sub>	85.9 <sub>A</sub>	83.4 <sub>A</sub>	85.3 <sub>A</sub>	84.1 <sub>A</sub>
Pantothenic Acid (µg/100 mL)	298 <sub>A</sub>	298 <sub>A</sub>	279 <sub>B</sub>	283 <sub>B</sub>	278 <sub>B</sub>	280 <sub>B</sub>	281 <sub>B</sub>
Cobalamin (µg/100 mL)	0.34 <sub>A</sub>	0.35 <sub>A</sub>	0.34 <sub>A</sub>	0.35 <sub>A</sub>	0.34 <sub>A</sub>	0.33 <sub>A</sub>	0.33 <sub>A</sub>

<sup>a-d</sup>Means in a row not sharing a common superscript are different (P < 0.05). Values are represented as the average of all 6 samples from three test runs analyzed in duplicate.

<sup>1</sup>Samples taken from each vat of whey immediately before bleaching (following pasteurization).

<sup>2</sup>Samples taken immediately after bleaching (stage 3 of processing). All treatments except AltC came from the vat of whey with traditional colorant.

<sup>3</sup>Condition labeled AltC was drawn from whey vat with alternative colorant.



Table 3. Mean relative abundance (ug/kg) of selected volatile compounds in powdered WPC80.

Treatment	Con	AltC	LP	HP	BP
Hexanal	0.18 <sub>C</sub>	0.17 <sub>C</sub>	3.9 <sub>A</sub>	1.10 <sub>B</sub>	0.50 <sub>B</sub>
Heptanal	0.09 <sub>C</sub>	0.12 <sub>C</sub>	1.5 <sub>A</sub>	0.77 <sub>B</sub>	0.25 <sub>C</sub>
Octanal	0.098 <sub>C</sub>	0.074 <sub>C</sub>	8.91 <sub>A</sub>	8.86 <sub>A</sub>	4.29 <sub>B</sub>
Nonanal	2.73 <sub>C</sub>	3.35 <sub>C</sub>	7.84 <sub>A</sub>	7.13 <sub>B</sub>	4.79 <sub>B</sub>
Diacetyl	0.644 <sub>AB</sub>	0.734 <sub>A</sub>	0.577 <sub>B</sub>	0.651 <sub>AB</sub>	0.526 <sub>B</sub>
Decanal	0.251 <sub>B</sub>	0.227 <sub>B</sub>	0.402 <sub>A</sub>	0.379 <sub>A</sub>	0.234 <sub>B</sub>
Benzaldehyde	0.214 <sub>A</sub>	0.186 <sub>A</sub>	0.326 <sub>A</sub>	0.206 <sub>A</sub>	0.255 <sub>A</sub>
DMTS <sup>1</sup>	0.039 <sub>C</sub>	0.020 <sub>C</sub>	0.087 <sub>A</sub>	0.051 <sub>B</sub>	0.054 <sub>B</sub>
Acetic acid	0.316 <sub>C</sub>	0.322 <sub>C</sub>	0.401 <sub>A</sub>	0.379 <sub>B</sub>	0.319 <sub>C</sub>
2-Pentyl furan	34.5 <sub>D</sub>	31.6 <sub>D</sub>	201 <sub>B</sub>	149 <sub>C</sub>	341 <sub>A</sub>
1-Hexen-3-one	0.467 <sub>C</sub>	0.382 <sub>C</sub>	0.903 <sub>B</sub>	1.82 <sub>A</sub>	0.69 <sub>BC</sub>
1-Pentanol	1.35 <sub>B</sub>	0.89 <sub>C</sub>	4.13 <sub>A</sub>	3.79 <sub>A</sub>	1.21 <sub>BC</sub>
1-Octen-3-one	0.03 <sub>C</sub>	0.09 <sub>C</sub>	0.62 <sub>A</sub>	0.42 <sub>B</sub>	0.11 <sub>C</sub>
DMDS <sup>2</sup>	0.002 <sub>C</sub>	0.006 <sub>B</sub>	0.012 <sub>A</sub>	0.008 <sub>AB</sub>	0.005 <sub>B</sub>
3-Methylbutanal	0.634 <sub>A</sub>	0.726 <sub>A</sub>	0.573 <sub>A</sub>	0.711 <sub>A</sub>	0.561 <sub>A</sub>
2-E-Octenal	5.42 <sub>A</sub>	2.25 <sub>B</sub>	5.08 <sub>A</sub>	6.87 <sub>A</sub>	2.96 <sub>B</sub>
2-Methylbutanal	1.14 <sub>AB</sub>	1.2 <sub>AB</sub>	1.40 <sub>B</sub>	1.37 <sub>B</sub>	0.89 <sub>A</sub>
2,3-Octanedione	1.43 <sub>A</sub>	0.634 <sub>C</sub>	1.01 <sub>B</sub>	1.12 <sub>AB</sub>	0.891 <sub>BC</sub>

<sup>a-d</sup>Means in a row not sharing a common superscript are different (P < 0.05).

<sup>1</sup>Dimethyltrisulfide

<sup>2</sup>Dimethyldisulfide

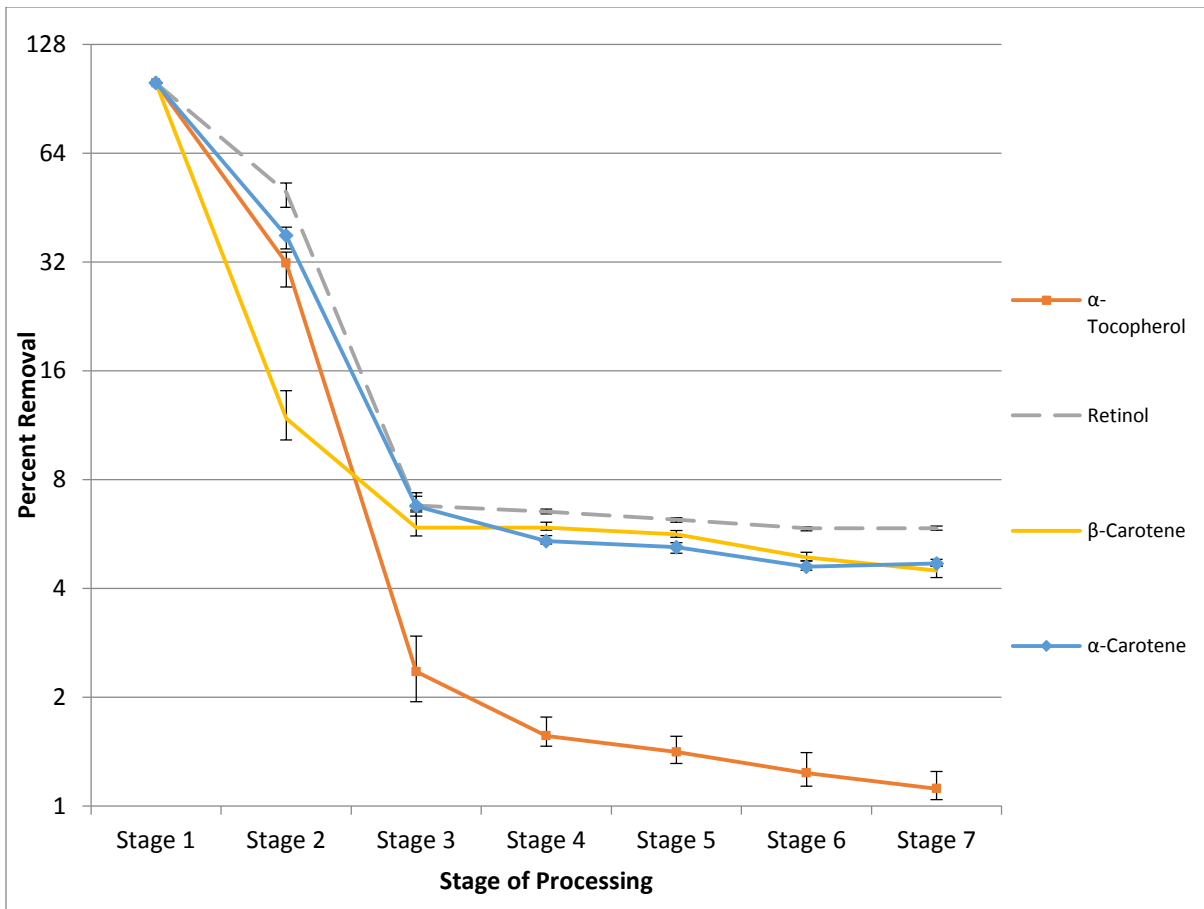


Figure 1. Mean percentage of fat soluble vitamin loss throughout processing of CON whey. Percent degradation indicates the percentage of starting compound (measured in Stage 1) measured during each stage. Values are represented as the mean of all three reps measured in duplicate (n=6).

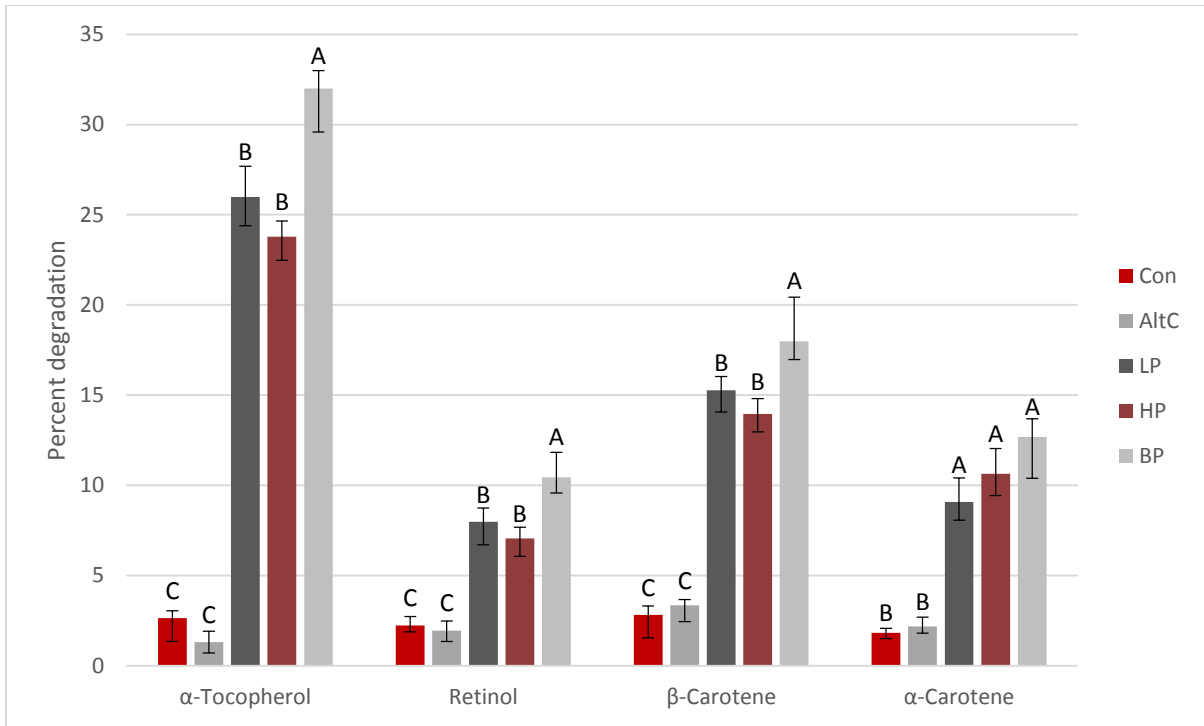


Figure 2: Mean percent degradation of fat-soluble compounds in liquid whey after bleaching treatment relative to concentrations before bleaching. <sup>a-d</sup>Means within a compound not sharing a common superscript are different ( $P < 0.05$ ). Mean percent degradation was determined by the ratio of the mean ( $n=6$ ) concentration at stage 5 of processing compared to the mean ( $n=6$ ) concentration at stage 4 of processing for each compound (table 1).

**CHAPTER 3: SIMULTANEOUS CAROTENOID AND VITAMIN  
ANALYSIS OF MILK FROM TRADITIONAL MIXED RATION FED  
COWS OPTIMIZED FOR XANTHOPHYLL DETECTION**

**Simultaneous carotenoid and vitamin analysis of milk from traditional mixed  
ration fed cows optimized for xanthophyll detection**

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## INTERPRATIVE SUMMARY

Carotenoid concentrations are lower in bovine milk from traditional mixed ration (TMR) fed cows than pasture fed cows. Lower concentrations of carotenoids can prevent measurement of lutein and zeaxanthin above the limit of quantification using HPLC techniques. This paper addresses a method which optimized chromatographic lutein and zeaxanthin measurement without influencing concentrations of other carotenoids or fat soluble vitamins. This paper demonstrates a simple and efficient method to measure lutein and zeaxanthin concentrations in TMR fed bovine milk.

## ABSTRACT

Concentrations of retinol,  $\alpha$ -tocopherol, and major carotenoids in dairy products are often determined simultaneously by liquid chromatography. These compounds have different polarity and solubility, thus extracting them simultaneously can be difficult and inefficient. Methods often use hexane as part of the final extraction solution, which solubilizes  $\beta$ -carotene and retinol well, but is less efficient at solubilizing lutein. Carotenoid concentration in bovine milk varies between pasture and traditional mixed ration (TMR) fed cows. In milks with low carotenoid concentrations, the xanthophylls lutein and zeaxanthin may not be completely resolved using common extraction techniques. A simple UPLC method was developed to optimize chromatographic sensitivity of lutein and zeaxanthin in bovine milk without decreasing sensitivity to other vitamins or carotenoids. The developed method evaluates lutein, zeaxanthin,  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol simultaneously through reversed phase UPLC-PDA. Solvents were evaluated for optimal xanthophyll extraction (diethyl ether, dichloromethane, hexane, and tetrahydrofuran). Common saponification

temperatures (40-60°C) and concentrations of KOH in caustic solutions (10%-50% KOH w/v) were evaluated. The limit of detection (LOD) and limit of quantification (LoQ) was defined as 3:1 and 10:1 signal to noise ratio respectively. All experiments were performed in triplicate. Milks solubilized in solutions containing diethyl ether had greater concentrations of lutein than hexane or tetrahydrofuran (THF) based solutions ( $p < 0.05$ ), with peak areas above LoQ values ( $p < 0.05$ ). The solution containing diethyl ether solubilized similar concentrations of retinol,  $\alpha$ -tocopherol, and  $\beta$ -carotene when compared to other solutions ( $p > 0.05$ ). The optimal saponification procedure was a concentration of 25% KOH at either 40°C or 50°C. The proposed method allows for the simultaneous determination of carotenoids from milk with increased lutein and zeaxanthin sensitivity without sacrificing recovery of retinol,  $\alpha$ -tocopherol, and  $\beta$ -carotene.

## INTRODUCTION

Carotenoids are a class of plant derived pigments which have a wide range of polarity, solubility, and stability (Kohlmeier and Hastings, 1995). Carotenoids are classified as either xanthophylls or carotenes, with xanthophylls containing oxygen atoms and carotenes being pure hydrocarbons without oxygen atom components (Gill and Indyk, 2008). The carotene  $\beta$ -carotene and xanthophylls lutein and zeaxanthin are among the most common carotenoids found in milk (Gill and Indyk, 2016). These compounds are primarily synthesized in plants and many are found in bovine milk due to the carotenoids in dairy cow diets (Nozière et al., 2006). Understanding carotenoid concentration in milk is important, as accurate detection of  $\beta$ -carotene, lutein, and zeaxanthin in bovine milk can serve as a marker for diet quality of dairy cows (Martin et al., 2005; Prach et al., 2002). Additionally, lutein and

zeaxanthin consumption is also vital for infants, as these are the primary carotenoids composing the macular pigment in the rod outer segment of the human eye, serving as a filter and an antioxidant (Rapp et al., 2000; Jewel et al., 2004). Although bovine milk does contain enough lutein to contribute to this nutritional need, understanding lutein content of milk may aid in determining total lutein in dairy based infant formulas.

Retinol,  $\alpha$ -tocopherol and carotenoids in bovine milk are often determined simultaneously by solvent extraction followed by liquid chromatography (Oliver et al., 2000; Indyk, 1987; Chauveau-Duriot et al., 2010; Giuliano et al., 1992; Jinno and Lin 1995; Ollilainen et al., 1989; Blake et al., 2007). However, unlike  $\beta$ -carotene, the xanthophylls lutein and zeaxanthin have been reported as difficult to quantify in milks with low carotenoid content using this approach. Nozière et al. (2006) reported that lutein was detected in milks from silage fed cows but below the limit of quantification (LoQ). Chauveau-Duriot et al. (2010) reported that lutein in whole milks was below the LoQ and occasionally below the limit of detection (LoD) using a UPLC-PDA method. Percent recovery for lutein was below 70% for this method (Chauveau-Duriot et al., 2010). Gill and Indyk (2008) designed a method to determine lutein in milk with a percent recovery of lutein > 95%, however this method was not optimized for fat soluble vitamins or carotenes. Gill and Indyk (2008) reported consistent lutein values above the LoQ suggesting that increasing the recovery rate might improve the likelihood of detecting lutein above the LoQ. Several factors that may limit lutein recovery rates are saponification parameters and lutein solvent affinity. Although efficient chromatographic methods exist to determine lutein content in bovine milk alone, no paper to our knowledge addresses the difficulties of optimizing chromatographic xanthophyll



detection in bovine milk with trace levels of carotenoids without decreasing detection of other carotenoids in a simultaneous extraction (Gill and Indyk, 2006).

Lutein concentration in bovine milk is dependent on the type of cow feed and total fat content (Havemose et al., 2004). Traditional mixed ration (TMR) fed milk contains approximately 20-60% less lutein than pasture fed cows (Nozière et al., 2006; Havemose et al., 2004). When accounting for carotenoid concentrations in bovine milk, the feeding source of the cow must be taken in to account. Lower concentration of total lutein in TMR milks may prevent accurate lutein determination above the LoQ. For this method to be viable, lutein should be consistently detected in TMR fed milks.

Saponification is often necessary to remove carotenoids from lipids in samples containing fat, however this process may encourage lutein degradation (Gill et al., 2016; Khachik et al., 1997; Guiliano et al., 1992). Although Khachik et al. (1997) stated that lutein and zeaxanthin were extremely susceptible to caustic solutions above room temperature, Indyk (1987) demonstrated that saponification is needed for optimal recovery of  $\beta$ -carotene and retinol. Thus, determining the temperature and KOH concentration during saponification may contribute to increased lutein and zeaxanthin recovery rates, while preserving  $\beta$ -carotene and retinol recovery rates. Saponification has been performed at temperatures as high as 70°C in milk for carotenoid detection, however Gill and Indyk (2008) demonstrated that these saponification parameters reduced the percent recovery of lutein. Granelli and Helmersson (2008) demonstrated 30°C was sufficient for complete recovery of  $\beta$ -carotene, despite the claim by Indyk (1987) that high temperature saponification was needed for complete extraction of carotenoids and retinol from milk fat.

Although xanthophylls and carotenes are chemically similar, the electron density on the oxygen atoms of xanthophylls can influence polarity and solubility (Yan et al., 2007; Mortensen and Skibsted, 1996). Simultaneous extraction of carotenoids and fat soluble vitamins in bovine milk may be influenced by these solubility differences (Craft and Soares, 1992; Indyk, 1987; Gill et al., 2016). Previous methods have used extraction solutions containing hexane, dichloromethane, tetrahydrofuran (THF), acetonitrile, and ethanol (Granelli and Helmersson, 1996; Giuliano et al., 1992; Chauveau-Duriot et al., 2010; Granelli and Halmerson, 1996). However, Craft and Soares (1992) demonstrated that lutein solubilized poorly in hexane as well as having reduced solubility in dichloromethane compared to  $\beta$ -carotene. Although rarely used in carotenoid extraction of milk, previous research has shown that diethyl ether could efficiently solubilize both lutein and  $\beta$ -carotene (Craft and Soares, 1992; Oliver and Palou, 2000). Diethyl ether has been used to extract retinol and  $\alpha$ -tocopherol in similar applications, indicating potential use as an extractant (Ollilainen et al., 1989; Salo-Väänänen et al., 2000). Hexane, dichloromethane, and THF have all been used in the literature to extract carotenes, xanthophylls, and vitamins from milk. However, no study to our knowledge has determined if a particular solvent optimizes lutein and zeaxanthin extraction without affecting  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol extraction.

Simultaneous carotenoid and fat soluble vitamin determination may not consistently detect xanthophylls above the LoQ. This may be due to aggressive saponification parameters and inadequate solubility. The objective of this study was to determine a chromatographic method which optimized lutein detection without reducing the detection of other carotenoids

and vitamins. To meet this objective, extraction solvents and saponification parameters were optimized in whole TMR fed bovine milk. The optimization parameters, extraction solvents and saponification parameters were compared against extraction solvents and saponification parameters commonly used. Both TMR and pasture based milks were then used to evaluate the optimized method.

## MATERIALS AND METHODS

### *Experimental overview*

The sample preparation and UPLC conditions which were determined to be ideal for measurement of the carotenoids and fat soluble vitamins were identified. Following the description of this procedure, the performance of the UPLC method was verified. This was done through determination of percent recovery, reproducibility, LoD, and LoQ. The methods used to optimize the extraction and saponification parameters for lutein and zeaxanthin extraction are then described. Finally, the verification of the method through measurement of commercial bovine milks is described.

### *Reagents*

Reagent grade ethanol, ethyl acetate, and hexane were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade tetrahydrofuran (THF) and acetonitrile with a purity of  $\geq 99.0\%$  were also purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade water, diethyl ether, hexane, ethanol, and methanol were purchased from Fluka (Seelze, Germany). Reagent grade KOH was purchased from VWR (Randor, PA). Standards used were  $\geq 99.0\%$

crystalline retinol,  $\geq 99.5\%$   $\beta$ -Carotene,  $\geq 99.5\%$  zeaxanthin,  $\geq 99.5\%$  lutein, and  $\geq 99.0\%$   $\alpha$ -tocopherol purchased from Sigma-Aldrich (St. Louis, MO).

#### *Sample preparation and extraction*

The optimized method for lutein, zeaxanthin,  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol detection was performed by UPLC-PDA (photodiode array detection). All milks were extracted and prepared with overhead lights off to prevent light oxidation. Three mL of each milk was incorporated into a container with ethyl alcohol and a saponification solution (containing 25% KOH diluted with water) at a rate of 3:5:3 mL (v:v:v). Samples were flushed with nitrogen, capped with a Teflon-lined cap, and sonicated for 5 min. Samples were allowed to sit in a 40°C water bath for 30 min as a saponification step. After saponification, 6 mL of hexane: ethyl acetate (70:30) was poured into each vial, vortexed, sonicated for 5 min, and centrifuged at 800 x g for 10 min. The organic layer was then extracted and the aqueous phase was treated with an additional 3 mL of hexane: ethyl acetate (70:30). This complex was then vortexed, sonicated for 5 min, and centrifuged again. The resulting organic phase was extracted and added to the original organic component. A third extraction was performed by adding 3 mL of hexane: ethyl acetate (70:30) to the aqueous layer of the sample and then vortexed, sonicated, and centrifuged as previously outlined. The third organic layer was extracted and added to the organic component and evaporated to dryness under nitrogen. The dried sample was then dissolved in 700  $\mu$ L of a solution containing HPLC grade ethanol, acetonitrile, diethyl ether, and water were added at a concentration of 58:18:20:4. Samples were then filtered with a 0.20  $\mu$ m nylon filter (VWR,

Randor, PA) and placed in HPLC vials (Sigma-Aldrich, St. Louis, MO). Finally, 3.0  $\mu$ L were injected into the UPLC for analysis.

#### *Chromatographic conditions*

Lutein,  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol were analyzed by high performance liquid chromatography by UPLC (Acquity UPLC H-Class, Waters Corporation, Milford, MA) with a C18 reversed phase column (Acquity UPLC® BEH C18 1.7  $\mu$ m, 2.1 mm X 50 mm, 130Å, Waters). The column temperature was maintained at 32°C  $\pm$  0.3°C, while flow rate was maintained at 0.30 mL/min (Acquity UPLC H-Class, Quaternary Solvent Manager, Waters Corporation, Milford, MA). Aliquots of each sample were then eluted with a mobile phase composed of ethanol, acetonitrile, diethyl ether, and water at a concentration of 58:18:20:4 respectively. Peaks were analyzed by photodiode array detection (Acquity UPLC H-Class, Photodiode Array (PDA) Detector, Waters Corporation, Milford, MA) with wavelengths monitored at 448nm for  $\beta$ -carotene, 325 nm for retinol, 264 nm for  $\alpha$ -tocopherol, and 452 nm for lutein and zeaxanthin (Craft and Soares, 1992; Guiliano et al., 1992).

Six calibration standards were prepared for each compound tested. Standards included  $\geq$  99.0% crystalline retinol,  $\geq$  99.5%  $\beta$ -Carotene,  $\geq$  99.5% zeaxanthin,  $\geq$  99.5% lutein, and  $\geq$  99.0%  $\alpha$ -tocopherol. Standards were diluted with a 58:18:20:4 solution containing HPLC grade ethanol, acetonitrile, diethyl ether, and water. Compound concentrations in each sample were interpolated from these curves.

#### *Method performance*

The performance of this method was evaluated by the percent recovery, the linear regression of the standard curve, the reproducibility between days, the reproducibility on the same day, and the LoD and LoQ. The percent recovery of all compounds was determined for this method by spiking water with each standard and subjecting them to extraction conditions (n=6). The linear regression and r<sup>2</sup> of the standard curves for each compound were determined from the calibration standards described in the chromatographic conditions. Reproducibility between days was determined by the residual standard deviation (RSD) between peak areas of compounds from whole commercial bovine milk extracted and analyzed on different days (n=6). Similarly, the reproducibility on the same day was determined by the RSD between peak areas of compounds extracted and analyzed from whole commercial bovine milk on the same day (n=6). The LoD and LoQ were defined as a signal-to-noise ratio of 3:1 and 10:1 respectively in whole commercial bovine milk (n=6, Chauveau-Duriot et al., 2010; Gill and Indyk, 2008).

#### *Optimization of method parameters*

Carotenoid and fat soluble vitamin extraction procedures require solubilizing in a diluent prior to chromatographic analysis. This is typically performed using a solution containing several solvents, as this step must adequately solubilize all compounds for proper injection (Gill and Indyk, 2008; Oliver and Palou, 2000; Zammarreño et al., 1992). These solutions typically contain ethanol, acetonitrile, and/or water as well as a strong nonpolar solvent such as hexane, THF, or dichloromethane. The addition of these strong nonpolar solvents could be responsible for the solubility of xanthophylls in these solutions, as ethanol, acetonitrile, and water do not efficiently solubilize these compounds (Craft and Soares,

1992). Thus, to verify that the final diluent was optimized for lutein and zeaxanthin, common nonpolar solvents were compared at a concentration of 20% in a standardized diluent solution containing ethanol, acetonitrile, and water (58:18:4). Methods vary slightly in specific concentrations of ethanol or acetonitrile, however the focus of this optimization was the ability of the nonpolar solution to solubilize compounds specifically, therefore a standardized solution was developed containing ethanol, acetonitrile, water, and the solvent being tested at a concentration of 58:18:4:20 respectively (Oliver and Palou, 2000; Guiliano et al., 1992; Gill and Indyk, 2008). Additionally, THF is a strong non-polar solvent which was observed to cause excessive peak broadening when used in high concentrations (Jinno and Lin, 1995). This may explain why procedures working with THF typically use it with another solvent such as hexane or methanol (Guiliano et al., 1992; Jinno and Lin, 1995). Thus, a solution of THF: Hexane (50:50) was prepared similar to conditions seen in literature, and added at the same rate (20% added to the standard solution) as the other solvents (Jinno and Lin, 1995). Samples from one lot of TMR milk were prepared and dried using the proposed protocol and solubilized in each of the solvents tested, before being evaluated using the proposed UPLC procedure for total concentration of lutein, zeaxanthin, retinol,  $\alpha$ -tocopherol, and  $\beta$ -carotene. This experiment required the measurement of compounds in various solutions, however the standard curve used in the proposed method is specific to the proposed extraction solution. Thus, for this experiment, individual standard curves for each compound were developed in each solution, to ensure accuracy between measurements.

The high fat content in whole bovine milk, carotenoids and fat soluble vitamins must be released from the lipid matrix before compounds can be quantified. This is often done through alkaline hydrolysis, which has been shown to degrade both lutein and zeaxanthin. Several commonly used KOH concentrations and sample temperatures were compared against the proposed saponification parameters for milk. Three temperatures and three concentrations of KOH were determined which encompassed most variability among published carotenoid methods (Granelli et al., 1996; Ollilainen et al., 1989). The temperatures selected were 25°C, 40°C, 50°C, and 60°C. Typically, samples for this procedure are made caustic by adding a concentrated KOH solution to the container with the sample and ethanol. The concentration of the solution used vary, but are typically 25-50% KOH (w:v, Giuliano et al., 1992; Gill and Indyk, 2006; Granelli and Helmersson, 1996; Chauveau-Duriot et al., 2010). To test the impact of KOH concentration on lutein recovery, KOH solutions were prepared with 10%, 25%, and 50% KOH (w/v) and added during the saponification procedure with one of the three temperatures.

#### *Method validation*

Once the proposed method had been optimized for lutein and zeaxanthin determination, the method was validated by evaluating concentrations of the lutein, retinol,  $\beta$ -carotene, and  $\alpha$ -tocopherol in 10 commercial pasteurized whole bovine milks. Five of the milks were TMR fed milks and five were pasture fed milks. Fat and protein content was evaluated by FTIR prior to extraction to ensure consistent fat and protein concentration.

#### *Statistical analysis*



All statistical values were determined by analysis of variance with means separation using XLSTAT software (version 2015.3.01; Addinsoft, New York, NY). Differences among sample means were determined through Fisher's least significant difference (LSD) tests.

## RESULTS

### *Method performance*

The performance of the developed method is summarized in Tables 1- 3. Mean percent recovery of lutein, zeaxanthin, retinol,  $\beta$ -Carotene, and  $\alpha$ -tocopherol was 101.2%, 94.5%, 98.0%, 105.4%, and 99.7% respectively. Repeatability of samples between days was determined through the analysis of six samples on different days. Mean RSD for reproducibility of peak area between days for lutein was 3.25% and 3.79% for zeaxanthin (Table 2). Repeatability between samples on the same day was determined through the analysis of six replicates (Table 2). Mean residual standard deviation (RSD) of repeatability on the same day of peak area for lutein and zeaxanthin were 1.45% and 1.86% respectively. The RSD for reproducibility of peak area analyzed on the same day was below 3.0% for all compounds tested. These RSD values are similar to those reported by Gill and Indyk (2008), Chauveau-Duriot et al. (2010), and Indyk (1987).

The LoD and LoQ of Lutein under the imposed conditions were 0.34  $\mu\text{g}/100\text{ mL}$  and 0.77  $\mu\text{g}/100\text{ mL}$  respectively (Table 3). The values are similar to the LoQ for lutein reported by Gill and Indyk (2008) of 1.4  $\mu\text{g}/100\text{g}$ , but lower than other reported LoQ values for lutein (Chauveau-Duriot et al., 2010). The LoD and LoQ of zeaxanthin for the current method were 0.26  $\mu\text{g}/100\text{ mL}$  and 0.74  $\mu\text{g}/100\text{mL}$  respectively, which is also lower than previously published LoQ values (Chauveau-Duriot et al., 2010; Indyk, 1987). The LoQ for  $\beta$ -carotene,

retinol, and  $\alpha$ -tocopherol were within ranges observed in other published methods (Chauveau-Duriot et al., 2010; Indyk, 1987).

#### *Optimization of method parameters*

Concentrations of carotenoids and fat soluble vitamins were measured in TMR milks following extraction with one of each extraction solution (Table 4). Standardized extraction solutions containing ethanol, acetonitrile, and water were evaluated with 20% diethyl ether, dichloromethane, hexane alone, or THF: hexane (50:50 v:v) Milks extracted with the solution containing diethyl ether had more lutein than samples extracted with hexane or THF/hexane ( $p < 0.05$ ), although no difference in lutein content was seen between samples extracted in solutions containing diethyl ether and those extracted with solutions containing dichloromethane ( $p > 0.05$ ). Milks extracted with solutions containing diethyl ether contained more zeaxanthin than those extracted with any other solution ( $p < 0.05$ ). Milks extracted with solutions containing hexane or THF/hexane consistently measured zeaxanthin concentrations below the LoQ. Concentrations of  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol concentrations were consistent between extraction solutions ( $p > 0.05$ ). Diethyl ether was the most efficient solvent for xanthophyll extraction due to increased zeaxanthin extraction; however dichloromethane may be as effective as diethyl ether in lutein extraction when zeaxanthin is not needed.

To assess saponification parameters, concentrations of KOH and temperatures common in literature for lutein extraction from milk were evaluated (Table 5). Percent recovery of lutein was optimal under several KOH concentrations and temperatures including 10% KOH at 50°C, 25% KOH at 40°C, 25% KOH at 50°C, and 50% KOH at 40°C ( $p < 0.05$ ).

Zeaxanthin recovery was also optimal under 25% KOH at 40°C, as well as at 10% KOH at 40°C ( $p < 0.05$ ). Concentrations of  $\beta$ -carotene and retinol were generally lower in samples subject to 10% KOH solutions, or lower temperatures ( $p < 0.05$ ). This supports previous claims that saponification is necessary to fully separate fat soluble compounds from milk fat (Indyk 1987; Gill and Indyk 2008; Chauveau-Duriot et al., 2010). Ideal saponification parameters were determined to be either a 25% KOH at 40°C, 50°C, or 60°C as well as 50% KOH at 40°C, 50°C, or 60°C ( $p < 0.05$ ). Although Gill and Indyk (2008) demonstrated that increased saponification time can reduce total recovery of lutein, Granelli and Helmersson (1996) reported that saponification of 30 min was adequate for  $\beta$ -carotene extraction from whole milk. Thus, the variables of temperature and KOH concentration were evaluated for 30 min to determine an optimal range. Thirty minutes was long enough to observe lutein and zeaxanthin concentrations drop in high temperature treatments, likely due to degradation. Retinol and  $\alpha$ -tocopherol concentrations were both optimal under saponification parameters involving 25-50% KOH and temperature at 40-60°C. Thus the ideal saponification parameters were determined to be 25% KOH at 40°C or 50°C for xanthophyll extraction without compromising the extraction of other compounds. A temperature of 40°C was selected for the presented method, although a temperature of 50°C should provide similar extraction efficiency.

#### *Method validation*

The concentrations of lutein, zeaxanthin,  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol were determined in ten commercial pasteurized milks using the developed method (Table 6). Five of these samples were from pasture fed cows and five were from TMR fed cows. As

anticipated, concentrations of carotenoids were higher in pasture fed cows than in TMR fed cows ( $p < 0.05$ , Table 6). Using this method, lutein was consistently found above the LoQ with concentrations ranging from 1.97-2.71  $\mu\text{g}/100\text{mL}$  in pasture fed milks to 1.52-1.83  $\mu\text{g}/100\text{mL}$  in TMR fed milks. These results were similar to those reported by Nozière et al. (2006) who observed a 20% increase in carotenoids in pasture fed milk when compared to TMR fed milk. Lutein concentration for TMR fed milks was similar to values observed by Gill and Indyk (2006). Zeaxanthin was detected in 66% of the milks tested, but only 56% were measured above the LoQ ranging from 0.75-1.08  $\mu\text{g}/100\text{mL}$ . Only three commercial milks from TMR fed cows had zeaxanthin above the LoD by this method and only one contained zeaxanthin above the LoQ. Several papers have reported similar findings, with zeaxanthin consistently not being detected or being below the LoQ (Gill and Indyk, 2008; Chauveau-Duriot et al., 2010; Nozière et al., 2006). This problem may be solved by increasing the initial sample volume, increasing zeaxanthin into an observable range. However, increased volumes might encourage coelution with lutein and other peaks using the proposed method.

Consistent with other published research, approximately 70% of the measured carotenoid weight was from  $\beta$ -carotene (Calderón et al., 2006). Mean concentration of  $\beta$ -carotene in pasture fed milk was 63% higher than it was in TMR fed milk ( $p < 0.05$ ). This was similar to the 65% reduction observed by Bergamo et al. (2003). Concentrations of  $\alpha$ -tocopherol ranged from 24.9-63.5  $\mu\text{g}/100\text{mL}$  in samples tested. Concentrations of  $\alpha$ -tocopherol in pasture fed milk were 39% higher than in TMR fed milk, consistent with previous research (Bergamo et al., 2003). Although  $\alpha$ -tocopherol was the only form of

vitamin E reported in this study,  $\gamma$ -tocopherol was identified in several pasture fed milks, consistent with findings made by Chauveau-Duriot et al. (2010). Concentrations of retinol between pasture and TMR fed milks were not different ( $p>0.05$ ), with concentrations ranging from 17.1-34.2  $\mu\text{g}/100\text{mL}$ . These results were comparable to ranges observed by others (Zammarreño et al., 1992; Bergamo et al., 2003).

## CONCLUSIONS

An efficient method was developed to consistently detect lutein simultaneously with other carotenoids and vitamins in milk. Zeaxanthin was detected in 66% of milk tested, but only 56% of the samples were above the LoQ. Extraction solutions containing diethyl ether had the highest concentration of zeaxanthin ( $p<0.05$ ), and extraction solutions containing diethyl ether or dichloromethane had the highest concentration of lutein ( $p<0.05$ ).

Concentrations of  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol were unaffected by extraction solution ( $p>0.05$ ). Optimal saponification parameters for xanthophyll detection were 40-50°C with a caustic solution containing KOH concentrations of 25% KOH. These parameters were also optimal for  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol extraction from the fat matrix of milk as well. This method was developed and verified to consistently measure lutein above the LoQ in TMR milks without affecting recovery of  $\beta$ -carotene, retinol, and  $\alpha$ -tocopherol.

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## TABLES AND FIGURES

Table 1. Percent recovery and linear regression of standard curves in water spiked with each standard and extracted using the proposed method.

Compounds	Percent recovery	Linear regression of the standard curve	r <sup>2</sup>
Lutein	101.2 + 3.2	y = 3129.7x - 3608.6	0.9987
Zeaxanthin	94.5 + 1.8	y = 3247.2x - 1666.8	0.9971
β-Carotene	105.4 + 3.1	y = 2649.0x - 2639.2	0.9989
Retinol	98.0 + 2.4	y = 1854.4x - 2257.3	0.9977
α-Tocopherol	99.7 + 2.9	y = 3470.5x - 4601.0	0.9980

Table 2. Retention time, wavelength for measurement, and reproducibility of method in whole pasteurized bovine milk (n=6).

Compounds	Mean Rt (min)	wavelength for measurement (nm)	Reproducibility between days	Reproducibility on same day
			(RSD%)	(RSD%)
			Peak area	Peak area
Lutein	1.35	452	3.25	1.45
Zeaxanthin	1.43	452	3.79	1.86
$\beta$ -Carotene	3.23	448	2.02	2.02
Retinol	1.56	325	3.13	2.08
$\alpha$ -Tocopherol	1.82	264	4.17	1.31

Rt retention time

RSD residual standard deviation

Table 3. Limit of detection (LoD) and quantification (LoQ) of the proposed method in whole pasteurized bovine milk (n=6).

Compounds	LoD ( $\mu\text{g}/100\text{mL}$ )	LoQ ( $\mu\text{g}/100\text{mL}$ )
Lutein	0.34	0.77
Zeaxanthin	0.26	0.74
$\beta$ -Carotene	0.30	0.63
Retinol	0.33	0.83
$\alpha$ -Tocopherol	0.57	1.19

Table 4. Concentrations of carotenoids and vitamins extracted from TMR milk with standard extraction solution (ethanol: acetonitrile: water 58:18:4) as well as 20% of the solvent or solution specified (n=12).

Solution	Lutein Mean (µg/100mL)	Zeaxanthin Mean (µg/100mL)	β-Carotene Mean (µg/100mL)	Retinol Mean (µg/100mL)	α-Tocopherol Mean (µg/100mL)
Diethyl Ether <sup>1</sup>	1.94A	0.79A	8.96A	22.36A	42.18A
Hexane	1.62C	NQ	8.52A	21.08A	39.40A
Hexane/THF	1.71BC	NQ	8.84A	18.98A	39.70A
Dichloromethane	1.81AB	0.76B	9.04A	22.91A	41.05A
pr> F	0.017	< 0.0001	0.337	0.315	0.798
r <sup>2</sup>	0.502	0.998	0.324	0.342	0.113

<sup>1</sup>Diethyl ether is the solvent used for the proposed method

NQ not quantified. Samples were detected, but were measured below LoQ

<sup>a-c</sup>Means in a column not sharing a common letter are different (p<0.05)

Table 5. Impact of saponification parameters for 30 min. on carotenoid and fat soluble vitamin concentrations in TMR milk (n=36).

Saponification parameters <sup>1</sup>	Lutein Mean (µg/100mL)	Zeaxanthin Mean (µg/100mL)	β-Carotene Mean (µg/100mL)	Retinol Mean (µg/100mL)	α-Tocopherol Mean (µg/100mL)
10%-25°C	1.64BC	NQ	7.35C	18.75CD	38.41BC
10%-40°C	1.48C	0.78AB	6.81C	18.21CD	38.37BC
10%-50°C	1.88A	NQ	6.57C	18.16CD	39.84BC
10%-60°C	1.57C	NQ	7.06C	20.32ABCD	40.89AB
25%-25°C	1.22D	NQ	8.07BC	17.43D	40.18ABC
25%-40°C <sup>2</sup>	2.00A	0.82A	9.74A	23.24A	43.83A
25%-50°C	1.84AB	0.75B	10.34A	22.03AB	42.30AB
25%-60°C	1.58C	NQ	9.18AB	20.55ABC	39.64BC
50%-25°C	1.61C	0.74B	7.29C	18.41CD	36.64C
50%-40°C	1.87A	ND	10.11A	22.44AB	40.18ABC
50%-50°C	1.15D	ND	10.70A	21.01ABC	40.57ABC
50%-60°C	1.12D	ND	9.92A	19.85BCD	41.47AB
pr > F	< 0.0001	< 0.0001	< 0.0001	0.005	0.05
r <sup>2</sup>	0.878	0.979	0.802	0.613	0.471

<sup>1</sup>Represented as percent KOH used in each treatment followed by the temperature at which the treatment was done for 30 min.

<sup>2</sup>Saponification of 25% KOH at 40°C for 30 min is the process used in the proposed method.

ND not detected

<sup>a-c</sup>Means in a column not sharing a common letter are different (p<0.05)

NQ not quantified. Samples were detected, but were measured below LoQ

Table 6. Concentrations of vitamins and carotenoids of ten whole commercial bovine milks (n=30).

Sample number	TMR or Pasture <sup>1</sup>	Lutein Mean (µg/100mL)	Zeaxanthin Mean (µg/100mL)	β-Carotene Mean (µg/100mL)	Retinol Mean (µg/100mL)	α-Tocopherol Mean (µg/100mL)
1	TMR	1.59CD	ND	8.06C	17.30D	35.48D
2	TMR	1.73CD	NQ	8.84C	18.11CD	32.80DE
3	TMR	1.81C	0.76D	8.41C	18.60CD	39.82D
4	TMR	1.57D	ND	9.04C	18.81CD	39.90D
5	TMR	1.77CD	ND	5.89D	33.14A	26.30E
6	Pasture	2.29B	0.87BC	11.52B	20.39CD	49.56BC
7	Pasture	2.68A	1.07A	12.48B	26.05B	52.70B
8	Pasture	2.24B	0.81CD	13.08AB	20.91CD	56.85AB
9	Pasture	2.20B	0.80CD	12.28B	19.19CD	62.42A
10	Pasture	2.55A	0.92B	14.62A	21.50C	40.98CD
	pr > F	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	r <sup>2</sup>	0.927	0.988	0.915	0.872	0.873

ND not detected

NQ not quantified. Samples were detected, but were measured below LoQ

<sup>a-c</sup>Means in a column not sharing a common letter are different (p<0.05)

<sup>1</sup>Total mixed ration

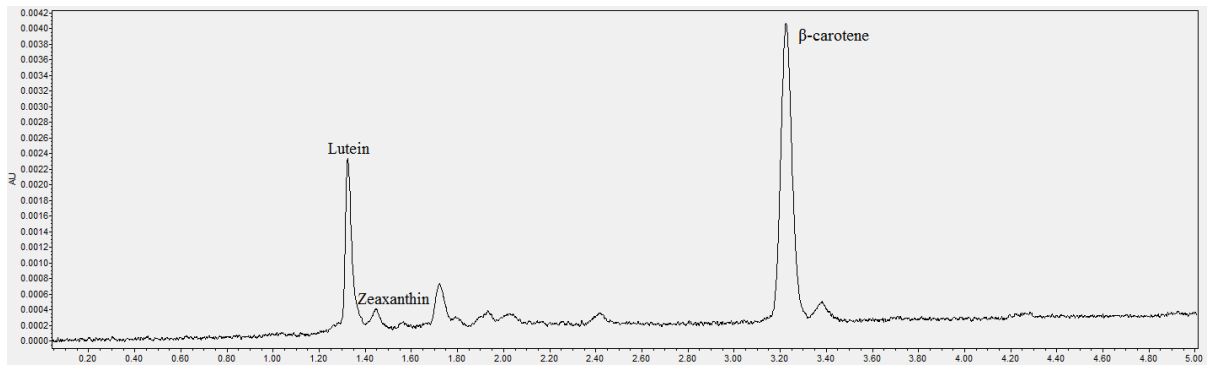


Figure 1. Chromatogram of lutein, zeaxanthin, and  $\beta$ -carotene from TMR milk sample.

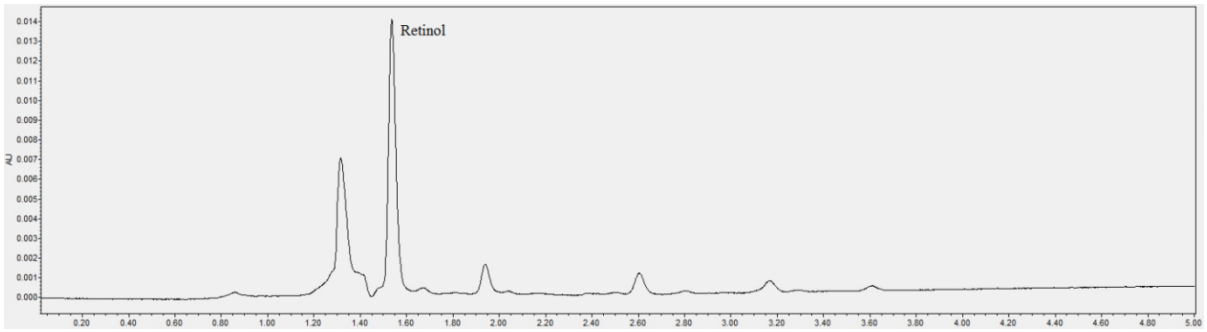


Figure 2. Chromatogram of retinol in TMR milk sample.



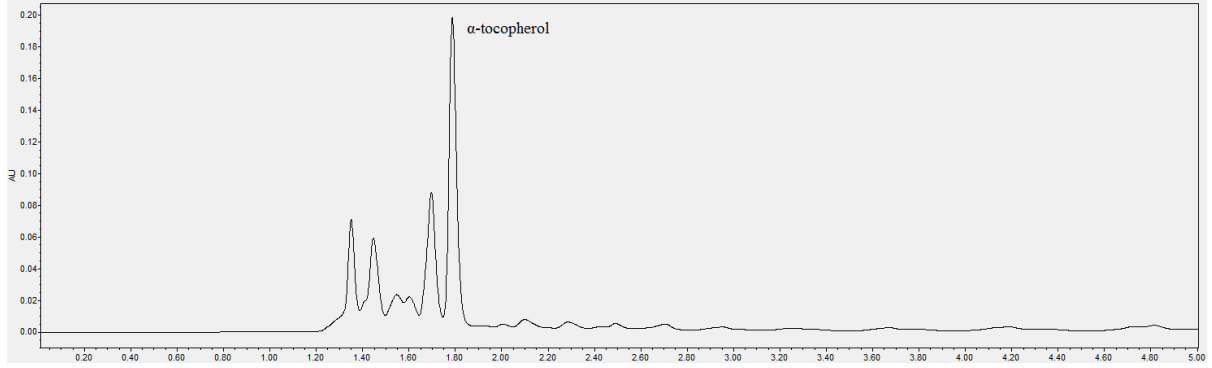


Figure 3. Chromatogram of  $\alpha$ -Tocopherol in TMR milk sample.