

## ABSTRACT

SCHIANO, ANGELINA NICOLE. Does Vitamin Fortification Affect Light Oxidized Flavor in Fluid Milk? (Under the direction of Dr. Maryanne Drake).

Vitamin fortification of dairy products is an industry standard but can lead to the development of off-flavor compounds which are difficult to extract and detect by instrumental methods. Previous work has identified these compounds and their specific role in off-flavors but efficient extraction and quantification of these compounds remains a challenge. In the first study, three rapid methods (stir bar sorptive extraction (SBSE), solvent assisted stir bar sorptive extraction (SA-SBSE), and solid phase microextraction (SPME)) were compared for their ability to effectively recover vitamin degradation volatiles from fluid skim milk. The performance of the three methods for detecting and quantifying vitamin degradation-related volatile compounds was determined by linear regression of standard curves prepared from spiked standards of five vitamin degradation volatiles, the reproducibility on the same day and between days as measured by the average relative standard deviation (RSD) of each standard curve, and the limits of detection (LOD) and quantitation (LOQ). Measurement of vitamin degradation compounds in commercial pasteurized fortified skim milks was also conducted using each method. Based on linearity, RSD, LOD, LOQ, and ability to detect selected compounds in commercial milks, cyclohexane SA-SBSE with 10 mL sample volume is recommended for the quantitation of vitamin degradation-related volatiles in fluid skim milk.

Light oxidation is an off-flavor in milk due to photooxidation of native riboflavin and tetrapyrroles that results in an array of lipid oxidation compounds. Vitamin A has antioxidant properties which could potentially protect fortified milk from light oxidation, but whether this effect is significant in milk is unknown. The objective of the second study was to determine the role of vitamin fortification on light oxidation of high temperature short time (HTST) pasteurized

fluid skim milk. First, the aroma profiles and aroma-active volatile compounds in light-exposed vitamin premixes was determined by exposing the premixes to fluorescent (FL) or light emitting diode (LED) light and documenting the aroma profiles of each vitamin premix at each time point with a trained panel. Headspace solid phase microextraction followed by gas chromatography olfactometry and gas chromatography mass spectrometry (GCMS) was performed to characterize aroma-active compounds in light-exposed vitamin premixes. In the second experiment, commercial vitamin premixes (vitamin A, vitamin D, oil and water matrices) were used to fortify skim milk. Skim milk was pasteurized, homogenized, and packaged in high density polyethylene (HDPE) jugs. Milks were exposed to FL or LED light; light-shielded controls with and without vitamins were included. Riboflavin and vitamin A and D degradation were quantified via ultra high performance liquid chromatography. A trained panel documented sensory profiles of milks at each time point. Lipid oxidation volatile compounds were quantified via solid phase microextraction with GCMS. Vitamin-related volatile compounds were quantified via solvent-assisted sorptive stir bar extraction with GCMS. Riboflavin, vitamin A, and vitamin D degradation were consistent with previous studies. There was no impact of vitamin fortification on development of typical light-oxidation related off-flavors (cardboard and mushroom) or lipid oxidation related volatiles (hexanal and heptanal) ( $p > 0.05$ ). A perfumey/floral flavor was documented in the oil based vitamin A fortified milk suggesting that light exposure impacted the off-flavors contributed by vitamin fortification. These results indicate there is no evidence that vitamin fortification provides any protection against light oxidation off-flavors in fluid milk.

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Does Vitamin Fortification Affect Light Oxidized Flavor in Fluid Milk?

by  
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## **BIOGRAPHY**

Angelina Nicole Schiano was born on August 17<sup>th</sup>, 1993 to Jerry and Donna Schiano. She grew up in the suburbs of Philadelphia with her three sisters. Angelina attended the College of William and Mary, where she majored in Neuroscience. She became interested in sensory science after working on research involving development of taste preferences in the labs of Dr. Catherine Forestell at William and Mary and Dr. Julie Mennella at the Monell Chemical Senses center. After graduating in 2015, began a Master's degree in Food Science with a focus on sensory science under the direction of Dr. MaryAnne Drake. Away from the lab, Angelina enjoys dance, crafting, sewing, and spending time with her oversized, slightly overweight, extra-fluffy orange cat, Oliver.

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# **CHAPTER 1: LITERATURE REVIEW. SENSORY AND INSTRUMENTAL ANALYSIS OF LIGHT OXIDIZED FLAVOR IN FLUID MILK.**

## **INTRODUCTION**

Fluid milk has long been an important food around the globe, once comprising over 10% of American daily caloric intake (International Dairy Foods Association, 2008). However, fluid milk consumption in the United States has steadily declined over the past 50 years (International Dairy Foods Association, 2008). Current theories as to the declining popularity of milk include competition from other beverages as well as consumer dissatisfaction with flavor variability in milk. In order to better understand flavor variability in fluid milk, analytical and sensory techniques must be developed and applied to investigate potential sources of off-flavors. Light oxidation and vitamin fortification are two such sources of off-flavors.

## **SENSORY ANALYSIS OF FLUID MILK FLAVORS**

Sensory evaluation of milk is critical due to widespread familiarity with milk and its sensory profile. Consumers expect a bland, pleasant flavor from milk and will not accept a product which does not meet their expectations. Thus, sensory methods have been developed in order to ensure fluid milk quality and consumer satisfaction.

### **Quality Judging**

The first standardized method for the sensory evaluation of dairy products was dairy product judging and the American Dairy Science Association (ADSA) scorecard system (Clark and Costello, 2008). As branding became an established concept in the early 20th century, companies began to turn to officially recognized standards of quality in order to promote quality to consumers. In 1917, when the first National Collegiate Dairy Products Evaluation Contest was held for milk, a USDA-developed, ADSA-approved scorecard was used which, in addition to

flavor, considered bacterial content, sediment, temperature, acidity, and bottle and cap appearance (Clark and Costello, 2008). In addition to the ASDA scorecard system, several other scoring systems were used in the 1920s-1930s, and there was often debate within the industry as to how scorecards should be used. By the early 1930s, the ADSA scorecard had become the standard scorecard for judging fluid milk quality (Nelson and Trout, 1934).

Traditional quality judging techniques are defect-oriented, using one to two trained judges to document defects rather than profiling the intensities of sensory attributes. By this approach, a large number of samples can be rapidly screened for recognized sensory defects. Early research studies on milk used sensory properties quality judging techniques as better techniques were not yet available (Weaver and Kuhlman, 1935; Hening and Dahlberg, 1939; Kratzer et al., 1987). Quality defects were designed to link a designated sensory defect with a specific root cause. Quality judging techniques are useful for on-the-spot evaluations of quality in industrial settings and for judging dairy competitions, but they are of limited utility for research for numerous reasons which have been reviewed previously (Alvarez, 2009; Drake, 2004, 2007). The modern ADSA milk and cream scorecard grades milk on a 0-10 point scale, placing milks into categories of excellent (10), good (7-9), fair (4-6), poor (1-3), and unacceptable (0) (Alvarez, 2009). Points are deducted for specific defects and their perceived intensities. In cases where a milk sample exhibits multiple defects, it is typically assigned a flavor score based on the most serious defect (Alvarez, 2009).

Many of the defects found on the modern scorecard have remained unchanged from the 1934 version, although there are some changes. Due to modern dairy sanitation measures, cowy/barny/unclean flavors are rarely found and therefore are not usually printed on the scorecard, while musty/stale has been removed as a defect entirely. Astringent, a defect added

after 1934, is also rarely encountered and not usually printed (Alvarez, 2009). Cardboard and disinfectant attributes have been removed from the scorecard. The metallic defect has been clarified as metal oxidized, and light oxidized has been added as a defect. The defect cooked, once perceived as a severe defect before the widespread pasteurization of milk, is now viewed with far less criticism (Alvarez, 2009). When dairy judging contests first began, raw whole milk was evaluated. Eventually, pasteurized whole milk and subsequently, pasteurized 2% milk, replaced raw whole milk in dairy judging contests (Clark and Costello, 2009). Lacking richness, a defect from the 1934 scorecard associated with skimmed milk, has been removed from the modern scorecard, perhaps due to the change to evaluating reduced fat milks instead of whole milks. Flat, a term on the current scorecard, was added to recognize the rare adulteration of milk with water.

Descriptive sensory analysis, developed in the 1950s, has slowly replaced quality judging techniques for all published research due to its versatility, specificity, and statistical robustness. Descriptive analysis uses a group of 6-12 trained individuals to document intensities of the sensory attributes of a product. Claassen and Lawless (1992) compared the ability of a defect-oriented system panel with a descriptive analysis panel to detect differences in light-oxidized, metallic-oxidized, and rancid milk. Given the same amount of training, the descriptive analysis panel was more sensitive to differences than the traditional judging panel. Although traditional quality judging methods imply hedonics, they are not good indicators of consumer liking (Sidel et al., 1981; McBride and Hall, 1979; Bodyfelt et al., 1981; Drake, 2004). Lawless and Claassen (1993) compared the correlation of consumer liking scores to data generated by traditional dairy judging and descriptive analysis panels and reported that descriptive analysis was more likely to correlate with consumer liking (although the method itself does not attempt to predict consumer

liking). Defect quality judging remains a useful industrial quality test; its limitations and the role of modern sensory techniques have been reviewed elsewhere (Drake, 2004, 2007; Bodyfelt et al., 2008).

### **Analytical Sensory Tools**

Analytical sensory tests are objective sensory tests that document sensory profiles, sample variability, or other product features free from liking considerations. These methods can be used to objectively profile products, and have been applied extensively to fluid milk. Among the most common analytical sensory tests used are discrimination tests. Discrimination tests are simple tests that aim to identify whether a significant difference exists between two or more samples, and results can be easily ascertained by referencing published significance tables based on the binomial distribution (Lawless and Heymann, 2010). The most common discrimination tests used in the study of fluid milk have been paired-preference, duo-trio, and triangle testing, although tetrad testing has also been used (Carlisle, 2014). Bierman et al. (1956) used triangle testing methods to evaluate if irradiation treatments resulted in noticeable differences in milk and cream products. In addition, Modler et al. (1977) used triangle tests to determine if consumers could notice differences between milks with varying feed flavor intensities, followed by paired-preference tests to determine whether noticeable differences were preferred or not. Consumers could distinguish control 2% milks from milks with “pronounced” feed flavors, and preferred control milk samples. Difference testing is still widely used in quality control capacities for the milk industry, as well as in academic research (Lee et al., 2016; Yeh et al., 2017a; McCarthy et al., 2017a).

Descriptive analysis (DA) techniques are valuable methods for quantitatively and objectively profiling the sensory attributes of fluid milk. Descriptive analysis involves extensive

training (10-100h, depending on number of attributes) of panelists prior to data collection, and uses a panel of 6-12 trained panelists evaluating products together in a consistent and sensitive manner, analogous to an instrument (Chambers et al., 2004). Descriptive analysis has been used extensively for the evaluation of fluid milk samples (Claassen and Lawless, 1992; Lawless and Claassen 1993; Phillips et al., 1995a; Watson and McEwan 1995; Chapman and Boor 2001; Francis et al., 2005; Chung et al., 2008; McCarthy et al., 2017a; Lee et al., 2017). Trained panel descriptive analysis produces objective and quantitative data that can be statistically analyzed, making it an excellent technique to combine with instrumental measurements.

### **Consumer-Focused Sensory Tests: Affective Sensory Tests**

Evaluation of consumer acceptance has been integral to ensuring acceptability of various fluid milk products and treatments since the inception of hedonic scaling methods in the 1940s. Affective sensory tests measure subjective consumer responses and collect hedonic data (Lawless and Heymann, 2010). Consumer tests may be administered many ways with fluid milk, but central location tests (CLTs) and home usage tests (HUTs) are the most frequently used. True to their title, consumer evaluations are administered to untrained populations who represent the true consumer base of a product. Early studies on the hedonic qualities of fluid milk attempted to extrapolate consumer acceptance from trained panelists; however, deficiencies in the predictive ability of trained panelists for consumer populations was well documented (Bierman et al., 1956; Ellis, 1969). Practical uses of consumer tests include examining the effects of various processing methodologies (Horner et al., 1980; Gandy et al., 2008; Lee et al., 2017), flavor additions or fortifications (Campbell et al., 2003; Achanta et al., 2007), and shelf life (Hansen et al., 1980) of fluid milks to maintain adequate consumer acceptance and lead new product development.

For quantitative affective testing, the 9 point hedonic scale is most commonly used. The scale has been widely validated in multiple forms, including horizontal orientation, vertical orientation, labeled, and unlabeled (Lawless and Heymann, 2010). Typically, the scale is labeled with the following phrases in this order: like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much, and dislike extremely. While there is some criticism that consumers are less likely to use the extremes of the scale, it is accepted that the difference between each point on the scale represents an equal interval, thus numerical values can be used as response choices and parametric statistics can be used to analyze the data (Lawless and Heymann, 2010). In addition to the 9-point hedonic scale, one particularly useful consumer testing question type pertaining to product development is the just-about-right (JAR) scale. JAR scales are an excellent tool for understanding the influence of individual sensory attributes or product qualities on overall liking. JAR scales evaluate individual attributes of a product and deviation from “just-about-right” categorization to determine effects of those attributes on overall liking. There has been extensive use of JAR scales in studies investigating consumer acceptance of functional or flavored milk beverages, such as probiotic milks (Villegas et al., 2010), regionally flavored milks (Zhi et al., 2016), and coffee-flavored milks (Li et al., 2014; Li et al., 2015), as well as different pasteurization methods (Lee et al., 2017; Chapman and Boor, 2001).

Consumer studies may also focus on qualitative aspects of the consumer experience. Qualitative consumer data is often collected in the form of free-response comments, check-all-that-apply (CATA) questions, or, in some cases, from organized interview methods such as focus groups. In studies of light-induced oxidation effects on consumer liking of 2% milks, Walsh et al. (2015) employed the use of emotional CATA questions and found significantly higher

frequencies of terms such as happy, safe, warm, and whole corresponded with higher hedonic scores. Means-end-chain interviews, a specialized type of individual interviews, have been used to identify drivers of choice for fluid dairy milk over plant-based alternatives and to investigate the effect of milkfat on consumer preference for fluid milks (McCarthy et al, 2017a; McCarthy et al., 2017b). Traditional focus groups have been used to investigate drivers of liking for fluid milk, including organic label claims and the effect of aftertaste (Dimitri and Dettmann, 2010; Mobley et al., 2014; Porubcan and Vickers, 2005).

## **INSTRUMENTAL ANALYSIS OF FLUID MILK FLAVORS**

### **History of Instrumental Flavor Analysis Techniques**

Instrumental techniques have developed alongside sensory techniques. While instruments do not measure flavor, instrumental data can go hand in hand with sensory data and be applied to more clearly identify sources of flavors. As new instrumental methods for identifying and quantifying volatile compounds have developed, they have been applied as tools for understanding the mechanisms behind sensory differences. However, no instrumental method can stand on its own without sensory data as, ultimately, the human palate is more sensitive and able to grasp complex sensations far more effectively than any technology developed.

Volatile compounds are the source of aromas in fluid milk, gas chromatography is an excellent tool for identifying possible sources of milk flavors. Partition chromatography (a chromatography technique based on the differing solubilities of analytes in the stationary phase) was one of the predecessors to gas chromatography, developed in the 1940s. Gas-liquid partition chromatography involves injection of a liquid sample into a column of inert gas which moves the sample to the detector and was used in early experiments involving chromatography of milk volatiles (Jennings, 1957). Prior to partition chromatography, paper chromatography methods

were sometimes used to analyze volatiles in milk, however, these methods were extremely time consuming, required large sample volumes, and were difficult to reproduce (Wong and Patton, 1962). An early, pre-Gas chromatography olfactometry (GC-O) method for determining flavor significance of volatile compounds used a threshold test involving spraying samples into the mouths of 5 trained panelists, with the threshold determined to be the 50% positive response level (Patton and Josephson, 1957).

Modern gas chromatography was developed in 1952, and mass spectrometry became common shortly after. Methods papers published after the development of GC technology attempted to standardize sampling procedures and minimize contamination from sources outside the sample (Sundararajan et al., 1967). During the 1960s and 70s, many dairy studies utilized GC or GCMS technology to focus on feed flavors in milk, measuring the volatiles of the milk, blood, or urine of cows fed different feeds (Gordon and Morgan, 1972; Loney et al., 1963; Bassette et al., 1966). Milkfat flavor and heat-induced flavor changes, very relevant to industrial processing, were also studied (Scanlan et al., 1968). Other efforts sought to quantify levels of volatile compounds identified in milk to get a baseline for further research (Bassette and Ward, 1974). Large “laundry lists” of volatile compounds detected in dairy products and other foods were compiled; however, there was limited information on the relative importance of each compound, and new techniques were needed in order to make sense of the compounds detected (Harper, 2007).

At this time, descriptive sensory analysis was still very new, and there were no clearly established methods for combining instrumental and sensory data. As quality judging was still a common research practice, some studies attempted to relate instrumental data to the flavor scores of the milk assigned by trained dairy judges. Keller and Kylene (1972) related total peak areas of

gas chromatograms with haylage flavor (a feed flavor defect) scores, finding a correlation between total chromatogram area and intensity of off-flavor. Reddy et al. (1967) found that methyl sulfide concentrations were negatively correlated with flavor quality scores for raw milk, and that commercial pasteurization removed 95% of the methyl sulfide. These papers suggested that GC could be used as an objective method for quantifying off-flavors in fluid milk, not fully understanding the limitations of GC. As descriptive analysis became more common, it fulfilled the need for an objective measure of off-flavors and allow for more robust correlations of instrumental and sensory data.

Difference testing was also frequently used to provide context for instrumental data. Gordon and Morgan (1972) used retronasal and orthonasal duo trio tests to determine the threshold of principal volatile compounds detected with GC from feed flavored milk. Researchers were unable to recreate the exact feed flavor by adding in isolated volatiles they detected, demonstrating that while GC technology was excellent for detecting and quantifying volatiles in a sample, it was no substitute for sensory analyses. Forss (1969) noted in his review the difficulties of interpreting GC data, noting that in some cases the human nose was the best detector.

Gas chromatography olfactometry (GCO) is a method of combining a GC with a sniffer port, allowing a human sniffer to smell and record aroma-active compounds as they elute from a column. This technique represents the interface between sensory and instrumental analysis methodologies. The first description of a GC modified for GCO was published by Fuller et al. in 1964. To isolate the user from environmental aromas, the device was contained in a telephone booth. The first “true” GCO, which mixed humid air with the GC effluent, was developed in 1971 (Mayol, 2001). Following initial applications, semi-quantitative approaches to GCO have

also been proposed, and explored by numerous studies (van Ruth, 2001; Acree et al., 1984; Grosch, 1991). Volatile and/or non-volatile compound or rheological measurements can be combined with descriptive analysis data or consumer data and used for profiling product characteristics, differentiating between products, and optimizing product characteristics for different market segments (Lawless and Heymann, 2010).

### **Modern Gas Chromatography and Volatile Extraction Methods**

Many of the gas chromatography developments over the past 40 years have focused on improving detection of volatile compounds or the processes by which these compounds are extracted from foods. In milk and other food matrices, volatile compounds are present along with non-volatile components such as lipids, proteins, and carbohydrates which can impede extraction and analysis of these volatiles. Milk aromatics are already quite complex as the volatile components are numerous and have widely different structures, stabilities, and boiling points. The food matrix can result in decreased detection of volatiles or artifact formation during GC analysis if sample preparation and volatile extraction methods are not carefully considered. While every method will introduce some manner of bias and no one method will be ideal for all samples, selecting the best extraction method is critical in order to ensure valid results. A range of techniques volatile compound extraction exist currently.

Dynamic headspace extraction (colloquially referred to as “purge and trap”) involves removing volatiles from milk by streaming helium or other gas over the surface of the milk, trapping the volatiles on an absorbent material such as charcoal, and desorbing them using heat onto a GC column (Urbach, 1987; McGorin, 2007). Dynamic headspace extraction was first applied in order to minimize the impact of water from milk headspace on GC analysis (Urbach, 1987). Changing the gas or trapping material can alter the selectivity of the method.

Solid phase microextraction (SPME) involves exposing a thin silica fiber coated in absorbent material into the headspace of a sample before desorbing it into a GCMS. The sample is heated and stirred during exposure. Polydimethylsiloxane (PDMS) (in combination with divinyl benzene (DVB), carboxen, or both) fibers are most commonly used for dairy volatile compounds (McGorin, 2007). The polarity, volatility, and molecular weight of the target analytes all need to be considered when selecting a fiber type. Adsorbent (physically traps or bonds with analytes) and absorbent (extracts analytes by partitioning) fibers are both available. Absorbent fibers (such as PDMS) are more suitable for non-polar compounds, but have a higher capacity because analytes do not have to compete for binding sites on the fiber. Adsorbent fibers (such as PDMS/DVB or PDMS/Carboxen) trap a wider range of polarities, but also have a lower capacity due to competition for binding sites among analytes (McGorin, 2007). These adsorbent fibers include either DVB or carboxen suspended in a layer PDMS, and small pores in the material allow the fiber to trap volatile compounds. DVB or carboxen fibers without PDMS are not used or commercially available. The average pore size for a DVB-containing PDMS fiber is larger than that of a carboxen-containing fiber. In some cases a PDMS/DVB layer is applied over a PDMS/carboxen layer, expanding the size range of analytes recovered by allowing smaller volatiles to slip through the larger outer pores and be trapped by the PDMS/carboxen layer, while the PDMS/DVB layer traps larger analytes (Shirey and Mindrup, 1999). Double layering fiber phases reduces the extraction capacity of the fiber, but extends the range of analyte sizes the fiber can extract (Shirey and Mindrup, 1999).

This technique offers minimal sample preparation and is effective in recovering a wide range of compounds from a large variety of matrices. SPME-GCMS offers greater sample recoveries and resolution than dynamic headspace extraction, however the method is still prone

to recovery bias and may not be sensitive enough for all applications. SPME is also not as effective for recovering high molecular weight or semi-volatile compounds.

Solvent assisted flavor evaporation (SAFE) is a technique used for extracting volatiles from food pulps or solvent extracts. SAFE has been applied to a variety of milk products, including milk proteins and skim milk powders (Park and Drake, 2016; Smith et al., 2016). If a solvent extract from a complex matrix is concentrated under nitrogen and injected onto a GCMS without a prior cleanup step, artifact formation and co-extraction of matrix elements such as fat and protein can interfere with the analysis (Sides et al., 2000). SAFE utilizes a high vacuum distillation apparatus with a cooling trap to isolate the volatile components of foods and avoid artifact formation while preserving the typical odor of that food (Engel et al., 1999). Although SAFE can be run using an unextracted food sample which is then solvent extracted following distillation, for complex food matrices, SAFE is often performed on direct solvent extracts (DSE-SAFE). For both methods, the resulting condensate is then dried over sodium sulfate in order to remove any water contamination, separated into acidic and neutral/basic phases, condensed under liquid nitrogen, and directly injected onto a GC column (Engel et al., 1999). Following this process, sample volatiles are concentrated and water, which can damage or destroy GCMS columns and detectors, is completely removed. The SAFE extraction process is sensitive and able to extract heavy and semi-volatile compounds, however, the process is time consuming and requires large volumes of solvent which can pose health hazards and disposal issues.

Stir bar sorptive extraction (SBSE) uses small magnetic stir bars encased in glass and coated in sorbent material in order to extract volatiles. Stir bars are added to fluid samples, which are then placed onto a magnetic stir plate. For headspace solvent extraction (HSSE), stir bars are

suspended in the headspace of a solid, liquid, or gaseous sample during the absorption step. Volatiles are typically thermally desorbed and cryofocused onto a GCMS (Baltussen et al., 1999; McGorin, 2007). Cryofocusing narrows peak width, allowing for better transfer of analytes to the GC column and this increases sensitivity (Prieto et al., 2010). Liquid desorption, another option for non-volatile and/or thermally labile analytes, involves separating analytes using liquid chromatography or capillary electrophoresis, however, this technique has not been widely applied for analysis of dairy products (Prieto et al., 2010). SBSE has been used for volatile compounds analysis of a variety of dairy products, including analyzing hormones, veterinary residues, acid and alcohol volatiles, and sulfur volatiles in fluid milk (McGorin, 2007; Huang et al., 2009; Zabbia et al., 2012; Ochiai et al., 2016). Like SPME, SBSE operates off the basic principal of partitioning coefficients - the ratio of the mass of analyte in the stir bar and the mass of analyte originally in the matrix can be expressed as follows:

$$\frac{m_{\text{SBSE}}}{m_0} = \frac{\left(\frac{K_{\text{O/W}}}{\beta}\right)}{1 + \left(\frac{K_{\text{O/W}}}{\beta}\right)}$$

Figure 1.1. Equation for ratio of mass of analyte in stir bar and mass of analyte originally in sample matrix.

Where  $m_{\text{SBSE}}$  is the mass of the analyte in the stir bar,  $m_0$  is the mass of the analyte originally in the matrix,  $K_{\text{O/W}}$  is the octanal water partition coefficient (here assuming a PDMS stir bar and an aqueous sample, a scenario in which the PDMS water partition coefficient will be approximately proportional to the octanal water partition coefficient), and  $\beta$  is the phase ratio (the ratio of the volume of the matrix to the volume of the PDMS) (Baltussen et al., 1999).  $\beta$  therefore directly affects the percent recovery of the analyte. If  $K_{\text{O/W}}/\beta$  is large ( $>5$ ), then an almost perfectly quantitative extraction may occur, but if the volume of PDMS is small and the ratio is low, the

extraction recovery is also very low (50% for a  $K_{O/W}/\beta$  of 1, for example) (Baltussen et al., 1999). A typical SPME fiber contains roughly 0.5 $\mu$ L PDMS, resulting in a sub-optimal extraction for all compounds without a very high  $K_{O/W}$  value. SBSE, on the other hand allows for quantitative extraction of compounds with  $K_{O/W}$  values over 500. SBSE offers significant resolution improvement over traditional SPME methods. Currently, sorptive stir bars are available with limited coatings, and while some researchers have been successful in developing their own coatings, this practice is not efficient for many volatile studies (Prieto et al., 2010). In particular, recovery of relatively polar analytes is a challenge for SBSE (Prieto et al., 2010; Ochiai et al., 2016).

Like other extraction methods, SBSE results are heavily influenced by the sample matrix and specific extraction protocols, including pH, extraction time, salt addition, stirring speed, extraction temperature, sample volume, and stir bar coating. Extremely acidic or basic conditions (pH<2 or >9) can degrade PDMS coating, however, neutralizing a matrix can lead to analyte forms unsuitable for extraction and analysis (Prieto et al., 2010). Adding salts such as sodium chloride can improve extraction of polar analytes, but reduce extraction of non-polar analytes (Prieto et al., 2010). Multi-shot/dual mode desorption (two stir bars extracted under different conditions desorbed simultaneously) with and without sodium chloride addition has been shown to increase overall extraction efficiency in some cases (Park and Drake, 2016). Increasing the temperature during extraction can decrease the time required for extraction, but reduce both the  $K_{ow}$  partition coefficient (resulting in reduced extraction efficiency) as well as stability of the PDMS coating (resulting in contamination peaks on the resulting chromatograms) (Prieto et al., 2010). Increasing stirring speed decreases the time required for extraction, but excessive stirring speeds could damage the matrix and result in formation of artifacts. Stirring speeds of 500-750

rpm are common and little improvement in extraction has been noted with higher stirring rates although, 800 rpm stirring rates have been successful for extracting volatile compounds from dried dairy ingredients (Prieto et al., 2010; Park and Drake, 2016). Because the phase ratio ( $\beta$ , the ratio of the volume of the matrix to the volume of the PDMS) is inversely related to recovery, increasing the volume of the matrix could result in an overall worse recovery. However, if the matrix volume is too low, analytes of interest could be below the limit of detection. Thus choosing an ideal sample volume is a balancing act. For milk samples, volumes of 5-100mL are typically selected (Leon et al., 2003).

While altering the stir bar coating can improve extraction, the coatings commercially available are limited and in-house coatings are expensive, complicated to manufacture, and often unreliable (Prieto et al., 2010). Solvent-assisted sorptive stir bar extraction (SA-SBSE) is a modified SBSE technique utilizing sorptive stir bars swollen in solvent which was developed as a way to circumvent the need to manufacture in-house coatings (Ochiai et al., 2016). The PDMS stir bars are “bloated” in solvent before volatile compound extraction. The solvent bloating increases diffusion into and out of the PDMS coating, as well as partitions into the aqueous matrix to allow for increased recovery of aqueous phase (polar) analytes (Ochiai et al., 2016). SA-SBSE has been shown to improve peak resolution and extraction efficiency for both polar and non-polar analytes.

SBSE has been utilized for flavor research on a variety of dairy products including milk, yogurt, cream cheese, and dried dairy ingredients (Hoffman and Heiden, 2000; Park and Drake, 2016). The technique has been shown to offer significant improvement over SPME for detecting and quantifying sulfur and carbonyl compounds in aseptically processed fluid milk (Zabbia et al., 2012). Due to the low limits of detection obtained with SBSE, the technique has been applied for

monitoring for antibiotic, hormone, and pesticide residues in bovine milk and human breastmilk (Huang et al., 2013; Rodríguez-Gómez, 2014). SA-SBSE, a newer technique has not been widely applied to dairy research.

## **FLUID MILK AND LIGHT OXIDATION**

### **Fluid Milk Processing**

Fluid milk undergoes multiple processing steps before it reaches the consumer in order to ensure a safe product with high sensory quality and a reasonable shelf life. In the United States, the Pasteurized Milk Ordinance (PMO) outlines the regulations for producing Grade “A” fluid milk and other dairy products (FDA, 2013). Raw milk is transported via refrigerated bulk tank trucks to the dairy plant, where it is filtered, separated (skim milk and cream are separated), standardized (skim milk and cream are added back to produce lowfat or 3.25% fat whole milk), pasteurized (heated to destroy pathogens), and homogenized (mixed at high pressure resulting in small, evenly distributed fat globules) (Bylund, 1995). For fortified milks, concentrated vitamin premixes are added before pasteurization, either by batch addition or through the use of metering pumps (DPC, 2001). There are several methods of pasteurization, however in the United States the most common method is HTST (high temperature short time) pasteurization, in which milk is heated to 72°C (161°F) for a minimum of 15s (FDA, 2013). HTST pasteurized milk is characterized by a light cooked flavor (MacCurdy and Trout, 1940).

Ultrapasteurization (UP), another common method of milk pasteurization, involves heating milk to 138°C (280°F) for 2 s. Although the shelf life of UP milk is longer than that of HTST milk, many American consumers prefer HTST milk due to the increase in cooked, sulfur, eggy, caramelized, and stale flavors associated with UP milk (Lee et al., 2017; Chapman and Boor, 2001; Valero et al., 2001). Ultra high temperature (UHT) pasteurized milk uses the thermal

standards of ultrapasteurization along with aseptic packaging, resulting in a commercially sterile shelf-stable product (Burton, 1977; Boor and Murphy, 2002).

## **Fortification**

Fortification is defined as the process of adding micronutrients such as essential vitamins to food. Fortification of fluid milk began in the 1930s and 40s, when milk provided 10% of American consumer food energy (Yeh et al., 2017b). Although whole milk is not a food naturally rich in vitamin D, vitamin D fortification became common after the American Medical Association's Council on Foods and Nutrition recommended the practice in order to reduce rickets in children (Stevenson, 1955). Whole milk is a good source of vitamin A as well as of provitamin carotenoids such as  $\beta$ -carotene and lutein. The primary form of vitamin A in whole milk is all-*trans*-retinol, although 13-*cis*-retinol (75% as bioactive as all-*trans*-retinol) is also present at a low level. The fat-soluble pigment  $\beta$ -carotene can also be converted to retinol by the body (although it is only 17% as bioactive as all-*trans*-retinol). With all forms of retinol and provitamin compounds taken into account, the overall bioactivity of vitamin A in whole milk is 12 retinol equivalents/g fat (40 IU/g fat) (MacGibbon and Taylor, 2006). Because vitamin A are insoluble in water, the overall vitamin content of milk sharply decreases when whole milk is separated and standardized into skim or reduced fat milk (Rodriguez-Amaya, 2016). As reduced fat milks began to gain popularity in the 1940s, concern grew that the American population would become vitamin A deficient as they would no longer be getting their vitamin A from milk. The popularity of vitamin D fortified milk led to fortification of reduced fat and fat-free milks with vitamin A (Public Health Service, 1940). The Nutrition Labeling Act of 1990 removed the standards of identity of nonfat and lowfat milk so they instead fell under the general standard of identity for fluid milk (under this system, "reduced fat" and "nonfat" are considered nutrient

content claims) (DPC, 2001). According to 21 CFR 130.10, if a food uses both a standardized term and a label claim, nutrients must be added so that the nutrient levels are equal to those found in the standardized food. Following the NLEA, fortification of reduced fat milk with vitamin A was required in order to obtain equivalent nutrient content to that of whole milk (FDA, 2013). Fortification with vitamin D, which is not naturally found in high levels in whole milk, remained optional.

Today, vitamin A is typically added to milk as synthetic retinyl palmitate (retinol palmitate) while vitamin D is added as synthetic D<sub>3</sub>. Synthetic retinyl palmitate is synthesized as all-*trans*-retinol palmitate form, which is equally as bioactive as all-*trans*-retinol (Weiser and Somorjai, 1992). Legally, fluid milk sold in America may have anywhere from 100-150% the amount of vitamins A and D declared on the label, thus vitamin A and D-fortified reduced fat milk may contain anywhere from 2,000-3,000 IU/qt vitamin A and 400-600 IU/qt vitamin D (PMO, 2017). These vitamins are available as commercial concentrates which are typically added to fluid milk after standardization and before pasteurization (Yeh et al., 2017b).

Concentrated vitamin premixes are available in two forms: oil and water soluble (van Deutekom, 2015). Emulsifiers, antioxidants, and preservatives may be added to the vitamin premixes, often resulting in a final product including sunflower oil, corn oil, water, polysorbate 80, propylene glycol, and glycerol monooleate (Yeh et al., 2017b).

There has not been much work regarding the sensory effects of fluid milk vitamin fortification. Hanson and Metzger (2010) reported vitamin D fortification of 100 to 250 IU per serving did not affect the sensory characteristics of HTST-processed 2% unflavored milk, UHT-processed 2% chocolate milk, or low-fat strawberry yogurt. However, other studies have suggested that vitamin A fortification might contribute off-flavors, such as oily or haylike notes

(Weckel and Chicoye, 1954; Whited et al., 2002; Fellman et al., 1991). Recently, Yeh et al. (2017a) reported that when skim milk was fortified with vitamin A concentrates at levels near the upper limits of what is allowed by law (3000 IU/qt) consumers could detect differences between unfortified and fortified milks. The type of vitamin concentrate used impacted flavor – consumers were able to more easily detect differences in milks fortified with water dispersible premixes. Sensory evaluation by trained panelists confirmed that the flavor in fortified milks was described as “carrot-like” or “perfumey”. Consumers were not able to distinguish between fortified and unfortified 2% milks or milks fortified with only vitamin D. More studies utilizing sensory methodology are needed to understand the effects of vitamin fortification on fluid milk flavor and consumer acceptability.

### **Light Oxidation**

Flavor changes in milk due to light exposure have been of interest to researchers since the earliest studies on fluid milk were published. Home refrigerators began entering the market in the early 1900s, but did not become common until the mid-1940s when new design elements and mass production made them a common household appliance (Higgins, 2001). During the winter months, consumers would often leave fluid milk and other dairy products outside to keep them cold. Early studies on light oxidized flavor in milk were prompted by complaints from consumers whose milk developed off-flavors after storage outside in the sunlight. These early studies sought mainly to characterize off-flavors as well as to prove they were directly related to light exposure and not the manufacturing practices (Frazier, 1928). The use of amber glass bottles to block sunlight exposure was also investigated (Hammer and Cordes, 1920). These early studies failed to describe their sensory methodology, leaving us to assume the conclusions

on flavors detected were derived from the sensory experiences of the authors alone. This is expected since modern objective sensory methods did not exist at this time.

Early vitamin D fortification attempts in the late 1920s and early 1930s attempted to increase vitamin D content through irradiation of milk, sparking renewed interest in investigating light-induced off flavors in dairy products (Stull, 1953). Several studies published from 1930 to 1950 noted that there appeared to be a dual nature of light-induced flavor defects. The first type of flavor was a fatty, oxidized tallowy note while the second was a burnt, cabbage, mushroom note (Stull, 1953). As with earlier studies, studies published during this time were limited by their lack of sensory methodology. Trade journals made claims about temperature, bottle type, and season of milking affecting light oxidized flavor, but results were often conflicting (Doan and Myers, 1936; Tracy and Ruehe, 1931). As sensory methodology was not documented and often no uniform system was used, there was no way to objectively compare the results of the studies.

Once the off-flavors were linked to sunlight, subsequent studies sought to identify the components responsible for the light-activated flavor as well as to investigate the effect of light exposure on other properties of milk such as vitamin content (Greenbank, 1948; Stull, 1953). Weinstein et al. (1951) investigated the fact that homogenized milk was more susceptible to light oxidation than unhomogenized milk, reporting that a whey protein fraction was responsible for the light-activated flavor rather than a lipid fraction. Riboflavin (vitamin B2) was characterized in the 1930s and determined to be an essential vitamin in 1939 (Northrop-Clewes and Thurnham, 2012). Studies throughout the 1970s investigated this water-soluble vitamin and eventually determined that photooxidation (light-induced degradation of a compound in the presence of oxygen) of riboflavin was the root cause of light-induced off-flavors in fluid milk (Allen and

Parks, 1979; Bradley, 1980; Korycka-Dahl and Richardson, 1978). While searching for additional potential photosensitive components of milk, researchers wondered if added vitamin A and D could function as photosensitizers, however, while the destruction of some vitamins due to light had been previously documented, the effect of vitamin fortification on light oxidized milk flavor was not studied in-depth at this time (Wishner, 1964). From here, researchers investigated whether exposure to artificial light sources produced the same detrimental effects as exposure to sunlight, reporting that they did (Smith and MacLeod, 1955; Smith and MacLeod, 1957).

Later published studies began to incorporate modern sensory methodology. Studies often compared trained panel data against data from consumer preference or difference testing, reporting that both could detect differences between light-exposed and non-light-exposed milk, although the trained panels could detect it much sooner and often could detect it after less than 15 minutes exposure (White and Bulthaus, 1982; Bray et al., 1977; Coleman et al., 1976). DeMan (1978) measured the intensity of fluorescent light in retail dairy display cases before exposing milk in different packages to realistic light intensities of various Kelvin color temperatures of light. The Kelvin color temperature scale expresses the color of light in terms of Kelvin, and indicates which wavelengths are emitted from the light source. A trained panel was used to quantify light-induced flavors. DeMan (1978) found that warm white light and opaque packaging resulted in the least vitamin degradation and off-flavor development, but many grocery stores today use cool white light and most milk is sold in transparent plastic cartons. Olsen and Ashoor (1987) used 25 untrained panelists to evaluate light exposed milk on a 9-point hedonic scale. While a consumer testing method is appropriate for evaluating consumer liking of light-exposed fluid milk, their sample size was far smaller than is recommended today. They reported that container type, container size, and fat content had no significant effect on the flavor

and riboflavin content of milk, indicating that perhaps a more sensitive sensory method, such as descriptive analysis, would have been a more appropriate method to use. Other studies continued using the ADSA dairy judging system at the expense of sensitivity. Reif et al. (1983) judged 304 samples of fluid milk collected from retail stores in California on a five point system for flavor. The frequency of light-induced flavor criticisms for milk in cardboard containers was compared to the frequency for milk in plastic containers. While the study did find more defect point deductions for the plastic containers for light-induced flavor, the study was limited in its ability to pull apart small variations in flavor.

Advances in instrumental techniques allowed for the association of specific chemical compounds with light oxidized flavor including methionine, acetaldehyde, n-pentanal, and n-hexanal (Bradley, 1980; Van Ardt et al., 2005a; Kim and Morr, 1996). Within the past 15 years, studies have combined sensory and this baseline instrumental volatile analysis to increase understanding of the effect of light exposure on fluid milk. Vitamin and lipid degradation and off-flavors increased with light intensity (Whited et al., 2002). Acetaldehyde, methyl sulfide, dimethyl disulfide, propanal, 2-methyl-propanal, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, and 2-nonanone were associated with light-induced flavor (Webster et al., 2009). Packaging materials, fat, flavorings, and antioxidants can delay the onset of detection (Mestagh et al., 2005; Van Aardt et al., 2005a, Van Ardt et al., 2005b). LED (light emitting diode) light, although less detrimental than fluorescent or sunlight exposure, also results in light oxidized flavor and vitamin degradation in fluid milk (Brothersen et al., 2016; Martin et al., 2016). The type of plastic packaging used as well as the shade of packaging can affect light absorption and therefore light oxidized flavor (Potts et al., 2016).

These studies on light oxidation flavor integrated sensory methods to varying degrees. Mestdagh et al. (2005) described the use of a trained sensory panel screened for sensitivity to light-activated flavor in order to detect differences between lowfat UHT milk stored in different varieties of PET bottles. While the author described the use of a triangle test to determine sensory differences, the number of panelists or the training methods were not documented. Chapman et al. (2002) determined the duration of exposure needed to produce a level of light oxidized flavor in 2% milk exposed to 2,000 lx fluorescent light which could be detected by a trained panel and consumers. Training procedures for a ten person sensory panel were documented and samples were analyzed by untrained consumers using a paired difference method. They found that consumers were able to detect light oxidized flavor after an exposure time of 54 min to 2 h, while a trained panel was able to detect light oxidized flavor after as little as 15-30 minutes of exposure. Moyssiadi et al. (2004) used a trained panel of 17 people to scale off-flavors in light-exposed milk stored in several types of HDPE or PET bottles. The panel was trained to differentiate between only two attributes – burnt (light oxidized) and stale (lack of freshness), but clear differences between samples were found. Brothersen et al. (2016) utilized a Spectrum™ descriptive analysis panel to obtain a reliable picture of all the sensory characteristics of A and D fortified 1% milk milk exposed to fluorescent or LED light. Further studies on light oxidation may probe deeper into protection offered by packaging, the effects of type and color temperature of light, and the interactions between vitamin fortification and light exposure.

### **Photosensitization Mechanisms in Milk**

Photochemical reactions are initiated by the absorption of light by a light-sensitive compound. When light of particular wavelengths hits compounds sensitive to those wavelengths,

the electrons in the photosensitive compounds become excited and can result in those electrons reaching a singlet or triplet excited state (Min and Boff, 2002). The excited photosensitizers can cause photooxidation by one of two ways: by forming a free radical chain (type I mechanism), or by interacting with oxygen to form highly reactive singlet excited state oxygen (Type II reaction) (Airado-Rodríguez et al., 2011). In a type I reaction, the photosensitizer molecule is oxidized itself, and usually destroyed in the reaction, whereas in a type II reaction the photosensitizer molecule transfers energy to oxygen to form singlet oxygen and is not typically destroyed in the reaction. In milk, light oxidized flavors develop in milk after photosensitized oxidation. Low oxygen concentrations result in a larger proportion of type I reactions, while a higher oxygen concentration increases the proportion of type II reactions (Lee and Min, 2009).

Over the course of light exposure, dissolved oxygen levels in milk will decrease as the oxygen is utilized in free radical reactions. Both of these photosensitization reactions can occur simultaneously in milk, compete with each other, and lead to different volatile compounds and off-flavors (Lee and Min, 2009; Wold et al., 2015). Although photooxidation can also occur via direct interaction of lipids with UV light, this is rare in milk and other dairy products (Airado-Rodríguez et al., 2011). Riboflavin is the primary photosensitizer in milk, although naturally-occurring tetrapyrroles including protoporphyrin IX, hematoporphoryn, and a chlorophyll *a*-analogue also play a role in the development of light oxidized flavors (Airado-Rodríguez et al., 2011). Blocking wavelengths below 480 nm (primary region of riboflavin excitation wavelengths) does not prevent formation of light oxidized flavor, demonstrating that although riboflavin is a major photosensitizer in milk, it is not the only compound responsible for light oxidized flavor (Airado-Rodríguez et al., 2011; Chang and Dando, 2017).

Singlet oxygen formed through photosensitization of riboflavin (particularly at wavelengths 400, 446, 450, and 570nm) can interact with lipids and proteins in milk, forming off-flavors (Webster et al., 2009). When singlet oxygen reacts with the amino acid methionine, it breaks down to form methional and dimethyl disulfide, which contribute sulfurous off-flavors in light exposed milk (Min and Boff, 2002). Interactions between singlet oxygen and other amino acids – in particular cysteine, histidine, and tryptophan – can also lead to development of sulfurous and other off-flavors (Min and Boff, 2002). Photooxidation of methionine and cysteine result in a direct increase in mercaptans, sulfides, disulfide, and dimethyl sulfide (Min and Boff, 2002). Light-induced lipid oxidation in milk leads to the development of a cardboard flavor which has been associated with the formation of neutral aldehydes (including hexanal and heptanal), ketones, alcohols, and hydrocarbons (Johnson et al., 2015; Marsili, 1999; Karatapanis et al., 2006).

Perhaps due to the many volatile compound production pathways photosensitization of riboflavin can trigger, the concentration of certain volatile compounds cannot be used to reliably determine the length of time milk was exposed to light. Although general trends exist, there is no strong correlation between neutral aldehyde concentration, perception of off-flavors, and exposure to fluorescent light in milk products (Marsili, 1999; Karatapanis et al., 2006) Hexanal and heptanal, lipid oxidation volatile products, have been reported to be the best volatile marker of light oxidized flavor formation (Webster et al., 2009; Brothersen et al., 2016). Compounds such as 1-octen-3-ol, 1-octen-3-one, 1-heptanol, and 1-octanol, have been detected in light-exposed milks but not in unexposed controls, but concentrations are too low to detect significant differences in concentration between time points, and concentrations appear to hold steady after 12-24h of exposure (Brothersen et al., 2016). Utilization of novel volatile extraction and analysis

techniques may lead to more accurate and sensitive measurement of these volatile oxidation products.

### **Carotenoid Compounds as Antioxidants**

Not all light-absorbing compounds in fluid milk are photosensitizers. Fortified retinyl palmitate and naturally occurring carotenoids such as  $\beta$ -carotene can act as antioxidants, scavenging for free radicals and perhaps affecting light oxidation in fluid milk. Carotenoids can halt free radical reactions in several ways. The majority of the time, excitation energy from singlet oxygen can transfer to the carotenoid and dissipate as heat (Palace et al., 1999). However, if the singlet oxygen free radical is stabilized by the polyene chain of the carotenoid, the carotenoid will cleave at an unpredictable carbon along the polyene chain, resulting in the potential development of aromatic vitamin degradation compounds (Palace et al., 1999). The role of volatile vitamin degradation compounds on the flavor of light-oxidized fluid milk has not been investigated. Previous research has shown that the addition of the natural antioxidants ascorbic acid and  $\alpha$ -tocopherol delay the onset of off-flavor formation and the formation of volatile compounds associated with light oxidation in light-exposed milk, although the antioxidant potential of fortified vitamins in fluid milk has not been studied (Van Aardt et al., 2005b). Beta-carotene, however, acts as a singlet oxygen quencher in milk and other biological systems, indicating there is potential for vitamin A to display the same function (Wold et al., 2015).

### **Light-Induced Degradation of Vitamin A**

Light exposure has been shown to cause significant degradation of vitamin A and other carotenoid compounds. Whited et al. (2002) measured significant vitamin A degradation after just 2 hours when skim or reduced fat milk was exposed to fluorescent light at 2,000 or 1,000lx. Exposing the milk to a higher light intensity resulted in a greater loss of vitamin A. Endogenous

vitamin A is more stable than added vitamin A in milk, as natural vitamin A protected by fat is less exposed to oxygen than exposed, concentrated vitamin A (Bartholomew and Ogden, 1990; Thompson and Enrody, 1974). In addition to ease of fortification in a plant setting, diluting vitamin A in a premix matrix before fortification of milk also serves to protect against vitamin degradation. Other processing factors, such as increased homogenization pressure, have been shown to improve vitamin A stability (Bartholomew and Ogden, 1990). The addition of sugar, milk solids, flavorings, and colors can also prevent light-induced vitamin A degradation (Yeh et al., 2017).

### **Light Type**

Different types and colors of light are characterized by the intensities of wavelengths they emit, which in turn impacts how the light interacts with photosensitizers in milk and other dairy products. Riboflavin absorbs light wavelengths under 500 nm, while tetrapyrroles absorb across the entire visual spectrum.  $\beta$ -carotene absorbs in the 400-525nm range, indicating that it could potentially prevent photooxidation of riboflavin in milk (Airado-Rodríguez et al., 2011). Because milk photosensitizers absorb across the entire visual spectrum, any retail dairy case light is at risk for triggering light oxidized flavors in milk in translucent packaging. Typically, dairy cases in grocery stores are outfitted with fluorescent or LED lights, color temperatures around 3,500-4,000K (cool white), and intensities of 500-2,500lx depending on the location within the case. LED lights are becoming more popular as replacements for FL lights due to their energy efficiency and resulting cost savings for retail stores (Martin et al., 2016).

Fluorescent lamps are narrow glass tubes containing inert gas at very low pressure as well as a low concentration of mercury atoms. These mercury atoms fluoresce UV light when vaporized by high speed electrons; the UV light is then converted to visible light by phosphors

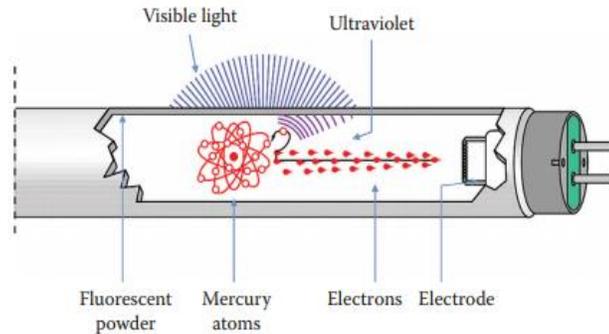


Figure 1.2. Fluorescent Lamp Tube (Kitsinelis, 2015)

(luminescent substances) coating the inside of the tube (Heidemann et al., 1993). High speed electrons are provided by a filament or electrode at either end of the lamp tube. The color temperature of the light can be modified by utilizing different blends of phosphor powders. It is necessary to maintain a steady current to the electrodes in order to ensure a steady light, thus ballasts are connected to fluorescent bulbs to moderate the electrical current (Kitsinelis, 2015).

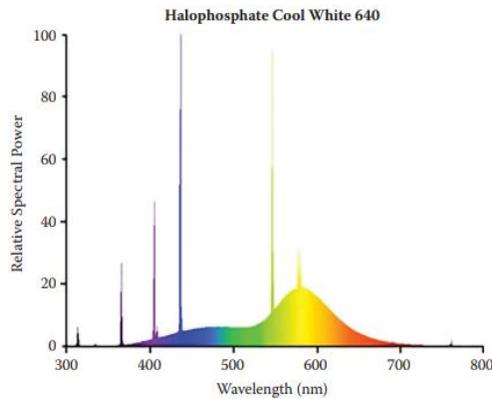


Figure 1.3. Emission Spectrum from a Fluorescent Lamp with a Phosphor (Kitsinelis, 2015)

Ballasts can vary in terms of size and efficiency; a variety of ballasts are used for dairy case lighting. The emission spectrum of a fluorescent light varies with color temperature, but

generally exhibits a “spiky” quality, with sharp high intensity emissions peaks in the UV, blue, and green/yellow regions.

LED (light emitting diode) lights have gained popularity due to their energy efficiency, low cost, and low heat output. A LED is a semiconductor, or an apparatus that conducts electricity under certain circumstances, such as at particular voltages or light intensities.

Electricity flows into one end of the diode, then is converted to visible light as it passes out of the

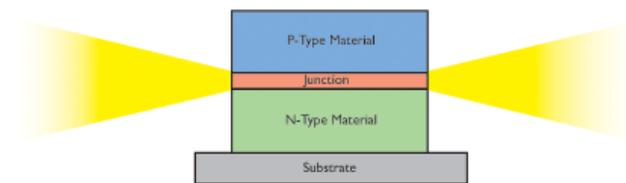


Figure 1.4. Light-emitting Diode Diagram  
(Phillips Holding B.V., 2017)

diode. Diodes only allow electric current to flow in one direction, and consist of a layer of positively charged (p-type) material stacked against a layer of negatively charged (n-type) material. As the current travels through the diode, the negatively charged atoms donate electrons to the positively charged atoms, causing release of photons and, if the diode is a light-emitting diode, producing light (Phillips Lighting Holding B.V., 2017). The material used to make a LED determines the color output of the LED.

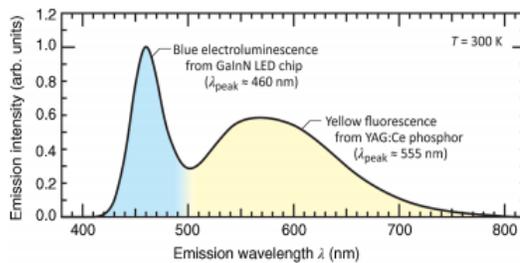


Figure 1.5. Emission Spectrum of a Phosphor-converted White LED  
(Schubert et al., 2005)

There are several ways to produce a white LED light, however, the most common method used commercially for dairy case lights is to coat a blue LED with a yellow phosphor (Schubert

et al., 2005). Phosphor-converted white LEDs produce a smooth emission spectrum, unlike fluorescent lamps which have a “spikier” emission spectrum, and emit light over the entire visible spectrum. The color temperature of phosphor-converted white LED lights can be modified by the inclusion of red, green, or blue diodes or modification of the phosphor (Schubert et al., 2005). Regardless of color temperature, LED lights generally produce a two-peak emission spectrum, with a primary peak in the blue region, a smaller secondary peak in the yellow/green region, and little if any infrared emission.

Differences in the emission spectrum of light types result in different interactions between the light and the photosensitive compounds in milk. Light oxidized flavor has been

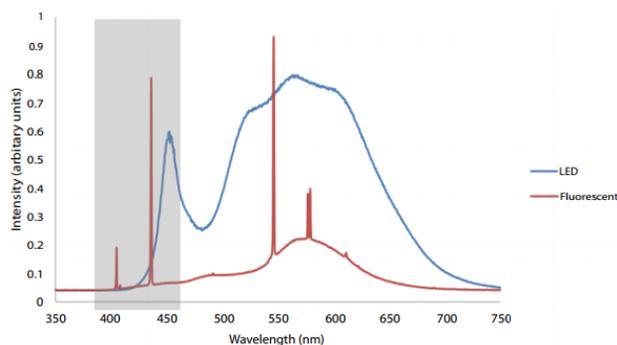


Figure 1.6. Emission Spectrum of FL and LED Light at the Same Intensity (De Jesus and Dando, 2016)

documented as less intense in milk exposed to LED light than FL light (Brothersen et al., 2016). Potts et al. (2017) found exposure to 1,460lx FL light resulted in a larger decrease in dissolved oxygen than exposure to LED light at the same intensity, and that across 4h of exposure LED light induced less oxidation in the milk. Consumer overall liking scores for 2% milk exposed to 1,460lx LED light for 4h were not significantly different from overall liking scores of light protected milk, while the milk exposed to the same intensity of FL light for the same amount of time received significantly lower consumer overall liking scores (Potts et al., 2017). While Brothersen et al. (2016) found exposure of 1% HTST milk to LED light resulted in a slower rate

of light oxidized flavor development, they found no differences in consumer liking between 2,000 lx FL and 4,000 lx LED exposed samples after 24h. Potts et al. (2017) reported that when exposing 2% milk in translucent HDPE or clear PET bottles to 1,460 lx FL or LED light for 4 h, only FL light resulted in a significant drop in consumer overall liking scores. By contrast, Martin et al. (2016) found in both skim and 2% milk, exposure to 1,200 lx of LED light resulted in significantly lower consumer liking scores after 4 h. Chang and Dando (2018) exposed HTST skim milk to 2,000 lx LED or FL light and reported that the consumer sensory threshold for a flavor difference was 12h of FL light exposure and 9h of LED light exposure. The type of light also affected the nature of the off flavors formed – LED exposure resulted in significantly higher plastic aroma while FL and LED with wavelengths under 480nm blocked light exposure resulted in significantly higher cardboard aroma and aftertaste. Consumers preferred FL exposed skim milk over LED exposed skim milk (at 2,000lx for both light types), however, while the consumers recruited purchased skim milk they did not necessarily select skim as their preferred fat content of milk, which could have influenced hedonic scores.

### **Fat Content**

Altering the milk fat content of milk changes both the nature and rate of light oxidation in milk as well as the nature of the flavors produced. Full-fat milk contains higher concentrations of fat-soluble beta-carotene and naturally occurring tetrapyrroles, which absorb light across the visible spectrum, especially in the violet region (Wold et al., 2015). Thus the action spectrum (a representation of the efficiency of light producing a photochemical reaction, similar to the absorption spectrum of the photoreactive compound but influenced by matrix effects) is influenced by these tetrapyrroles to a greater degree while the action spectrum of skim milk (which includes lower concentrations of these tetrapyrroles) is closer to that of riboflavin alone

(Wold et al., 2015). Milk fat also scatters significantly more light at lower wavelengths, and thus is more efficient at protecting compounds which would absorb in this violet region, including riboflavin, from light oxidation (Wold et al., 2015).

Increasing the fat content of milk slows light-induced vitamin A degradation. Whited et al. (2002) found that vitamin A degradation in skim milk occurred 3 times faster than reduced fat milk and 3.5 times faster than whole milk. Increasing the fat content results in increases of some light oxidation related volatiles, such as neutral aldehydes, while others such as sulfur compounds are unaffected by changes in fat content (Lee and Min, 2009; Bassette, 1976). However, due to increased sensory detection thresholds of these volatile compounds, it is not necessarily easier to detect an off-flavor resulting from these compounds. Sensory thresholds of light oxidized flavor in whole milk are usually higher than those for skim and reduced-fat milk due to the impact fat content has on thresholds of aroma-active compounds (Chapman et al., 2002). However, one study by Whited et al. (2002) reported a faster rate of off-flavor development in light oxidized whole milk and reduced-fat milk than skim.

### **Packaging Type**

A variety of packaging solutions have been developed in order to control light oxidized flavor and other off-flavors in milk by controlling light and oxygen exposure. Glass bottles, the historic method of packaging fluid milk, have largely fallen out of favor, partially due to the inability of clear glass to block any light. Modern consumers typically purchase plastic packaging, but will also accept paperboard cartons which are typically used to pack organic and ultra-pasteurized milks (Patterson, 2016). Although paperboard cartons completely block light, they can contribute cardboard off-flavors which may also be unacceptable to consumers (Leong et al., 1992). Consumers place value on food packages which adequately display the product

inside, thus resulting in a challenge for preventing light-related flavor defects in milk while still producing packages attractive to consumers (Webster et al., 2009).

Specialized light blocking packages have been developed in order to reduce the impact of light exposure. While these packages can impact light oxidation, only completely opaque packages have been successful in preventing it entirely. Polyethylene terephthalate (PET) and high-density polyethylene (HDPE) plastic jugs are most commonly used for milk packaging (Karatapanis et al., 2006). Plastics can be a single layer (monolayer) or multilayer, and pigmented plastics, such as white or yellow plastics, have also been used (Karatapanis et al., 2006). Potts et al. (2017) looked at the effect of translucent HDPE or clear PET packaging on consumer acceptability of 2% milk exposed to 1,460lx FL, reporting the HDPE packaged milks had significantly higher consumer overall liking, flavor liking, aftertaste liking, and freshness perception scores than the PET packaged milks.

UV absorbers can be added to packaging to block specific UV wavelengths without affecting package clarity (Webster et al., 2009). However, these packages do not typically block all possible excitation wavelengths of riboflavin, and do not block wavelengths in the visible spectrum which can excite naturally-occurring tetrapyrroles, and thus while these packages may have a mild effect on formation of volatiles or vitamin degradation, they ultimately do not prolong the onset of light-oxidized flavor in a significant manner (Mestdagh et al., 2004). When comparing light oxidized flavor development in milk exposed to broad spectrum light in packaging with under 4% or 20% transmission of riboflavin excitation wavelengths, Webster et al. (2009) found no significant difference in light oxidized flavor or riboflavin degradation at days 1-4 of exposure between the two packages. While a light-exposed control sample (in packaging with full transmission of the riboflavin excitation spectrum) did show higher light

oxidized flavor, there were also no significant differences in riboflavin degradation over days 1-4 of exposure between the test packages in the control, illustrating that the relationship between light oxidized flavor and riboflavin degradation is not straightforward, and that other photosensitizers play a large role in flavor development.

Oxygen scavenging packages have been investigated to attempt to reduce the dissolved oxygen concentration in milk so that less oxygen is available to form singlet oxygen after reaction with photosensitized riboflavin. The correlation between dissolved oxygen levels and development of light oxidized flavor in milk has been debated, with Potts et al. (2017) reporting a reduction in dissolved oxygen was a reliable indicator of sensory changes in 2% milk exposed to either FL or LED at 1,460lx for 4h, while While these packages do reduce the total dissolved oxygen in the milk, current oxygen-scavenging inner layers are not effective enough to significantly prevent vitamin degradation and the development of light oxidized flavor (Mestdagh et al., 2004).

## **CONCLUSION**

While vitamin fortification and light exposure are known sources of off-flavor in milk, the role of interactions between vitamin fortification and light exposure has not been evaluated. By using established sensory and instrumental methodologies to investigate the interactions between these processes, we can better understand a potential source of flavor variability in milk which could impact consumer liking. The objective of this study was to determine the role of vitamin fortification on light oxidation of fluid HTST pasteurized skim milk.

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## **CHAPTER 2: COMPARISON OF THREE RAPID METHODS FOR ANALYSIS OF VITAMIN DEGRADATION COMPOUNDS IN FLUID SKIM MILK**

### **ABSTRACT**

Vitamin fortification of dairy products, including fluid milk and fortified whey protein beverages, is an industry standard but can lead to the development of off-flavor compounds which are difficult to extract and detect by instrumental methods. Previous work has identified these compounds and their specific role in off-flavors but efficient extraction and quantification of these compounds remains a challenge. Three rapid methods (stir bar sorptive extraction (SBSE), solvent assisted stir bar sorptive extraction (SA-SBSE), and solid phase microextraction (SPME)) were compared for their ability to effectively recover vitamin degradation volatiles from fluid skim milk. The performance of the three methods for detecting and quantifying vitamin degradation-related volatile compounds was determined by linear regression of standard curves prepared from spiked standards of five vitamin degradation volatiles, the reproducibility on the same day and between days as measured by the average relative standard deviation of each standard curve, and the limits of detection (LOD) and quantitation (LOQ). Measurement of vitamin degradation compounds in commercial pasteurized fortified skim milks was also conducted using each method. Detection of selected vitamin degradation volatiles was linear in skim milk (0.005-200 ppb).  $R^2$  values differed between methods and compounds. Within day and between day percent RSD also varied with compound and method. LOD/LOQ values for all methods except SPME were lower than concentrations of selected volatile compounds typically found in commercial milk. SA-SBSE with a 10 mL sample volume provided the most consistent detection of selected compounds in commercial milks. Based on linearity, RSD, LOD, and LOQ,

cyclohexane SA-SBSE with 10 mL sample volume is recommended for the quantitation of vitamin degradation-related volatiles in fluid skim milk.

### **INTERPRETIVE SUMMARY**

Vitamin fortification of dairy products, including fluid milk and fortified whey protein beverages, is an industry standard but can lead to the development of off-flavor compounds which are challenging to detect by instrumental methods. The performance of three rapid methods (SBSE, SA-SBSE, SPME) for detecting and quantifying vitamin degradation-related volatile compounds was compared by linear regression of standard curves from spiked standards, reproducibility, LOD/LOQ, and measurement of selected volatiles in commercial fortified skim milk. SA-SBSE with 10 mL sample volume is recommended for the quantitation of these volatiles in fluid skim milk.

### **INTRODUCTION**

The flavor of high-quality fluid milk is often described as “pleasant” or “bland,” posing a particular challenge for milk manufacturers as any off-flavors present will be noticeable by consumers. Flavor variation in milk can be attributed to varied factors such as the diet of the cow, the breed of the cow, the processing methods used, and the macronutrient content of the milk. Recent studies have shown that other milk processes, such as vitamin fortification, can also create noticeable off-flavors detected by consumers (Yeh et al., 2017). According to the Nutrition Labeling Act of 1990, skim and reduced-fat milk sold in the United States are legally required to be fortified with enough vitamin A to be “nutritionally equivalent” to whole milk (DPC, 2001). Although not a legal requirement, fortification of milk of all fat levels with vitamin D<sub>3</sub> is typical industry practice.

Vitamin D in fluid milk is heat and light stable (Renken and Warthesen, 1993), but vitamin A can degrade under conditions typically experienced by commercial fluid milk (PHS/FDA, 2015; Whited et al., 2002). During light exposure, singlet oxygen free radicals are created through photosensitization of riboflavin and other naturally occurring tetrapyrroles (Airado- Rodríguez et al., 2011). These free radicals can result in lipid and protein oxidation leading to light oxidized off-flavors in milk (Lee and Min, 2009; Wold et al., 2015; Bray, 1977). Free radicals can be stabilized by the polyene chain of carotenoids such as retinyl palmitate, cleaving the vitamin at an unpredictable carbon along the chain (Palace et al., 1999; Rodriguez-Amaya, 2016). Following this cleavage, the vitamin is destroyed and downstream reactions can lead to the development of vitamin degradation compounds (Palace et al., 1999; Rodriguez-Amaya, 2016). Furthermore, the premixes themselves also contain degradation compounds. The addition of some vitamin A premixes to fluid milk imparted a floral/carroty flavor which was attributed to vitamin A degradation compounds present in the premixes themselves (Yeh et al., 2017).

Reliable detection and quantification of aroma-active compounds related to vitamin degradation in fluid milk is challenging. Commonly used methods for analyzing volatile aroma-active compounds in fluid milk include solid phase microextraction (SPME) and solvent assisted flavor evaporation (SAFE) performed on direct solvent extracts (DSE-SAFE) (McGorin, 2007). Although SPME is effective at recovering a wide range of target compounds, the technique is not sensitive enough to efficiently recover vitamin degradation-associated volatiles from fluid milk, which are present in small concentrations and are often semi-volatile (Yeh et al., 2017). The SAFE extraction process is sensitive and able to extract heavy and semi-volatile compounds,

however, the process is time consuming and requires large volumes of solvent which can pose health hazards and disposal issues (Jelén et al., 2012).

Stir bar sorptive extraction (SBSE) uses small magnetic stir bars encased in glass and coated in sorbent material to extract volatiles. Stir bars are added to fluid samples or may also be suspended in the headspace of a solid, liquid, or gaseous sample during the absorption step. Volatiles from the stir bars are typically thermally desorbed and cryofocused followed by GCMS (Baltussen et al., 1999; McGorin, 2007; Prieto et al., 2010). SBSE has been used for volatile compound analysis of a variety of products, including hormones, veterinary residues, acid and alcohol volatiles, and sulfur volatiles in fluid milk (McGorin, 2007; Huang et al., 2009; Zabbia et al., 2012; Ochiai et al., 2016). Like SPME, SBSE operates off the basic principal of partitioning coefficients - the ratio of the mass of analyte in the stir bar and the mass of analyte originally in the matrix can be expressed as follows:

$$\frac{m_{\text{SBSE}}}{m_0} = \frac{\left(\frac{K_{\text{O/W}}}{\beta}\right)}{1 + \left(\frac{K_{\text{O/W}}}{\beta}\right)}$$

Figure 2.1. Equation for ratio of mass of analyte in stir bar and mass of analyte originally in sample matrix.

Where  $m_{\text{SBSE}}$  is the mass of the analyte in the stir bar,  $m_0$  is the mass of the analyte originally in the matrix,  $K_{\text{O/W}}$  is the octanal water partition coefficient and  $\beta$  is the phase ratio (the ratio of the volume of the matrix to the volume of the PDMS) (Baltussen et al., 1999). A typical SPME fiber contains roughly 0.5 $\mu\text{L}$  PDMS, resulting in a sub-optimal extraction for all compounds without a very high  $K_{\text{O/W}}$  value. SBSE, on the other hand, allows for quantitative extraction of compounds with  $K_{\text{O/W}}$  values over 500. As such, SBSE offers potential significant resolution improvement over traditional SPME methods.

Like other extraction methods, SBSE results are heavily influenced by the sample matrix and specific extraction protocols, including pH, extraction time, salt addition, stirring speed, extraction temperature, sample volume, and stir bar coating. Adding salts such as sodium chloride can improve extraction of polar analytes, but reduce extraction of non-polar analytes (Prieto et al., 2010). Choosing an ideal sample volume is also a balancing act. For milk, volumes of 5-100mL are typically selected (Leon et al., 2003).

Solvent-assisted sorptive stir bar extraction (SA-SBSE) is a modified SBSE technique utilizing sorptive stir bars swollen in solvent which was developed as a way to circumvent the need to manufacture in-house custom coatings for stir bars (Ochiai et al., 2016). The PDMS stir bars are “bloated” in solvent before volatile compound extraction. The solvent bloating increases diffusion into and out of the PDMS coating, as well as partitions into the aqueous matrix to allow for increased recovery of aqueous phase (polar) analytes (Ochiai et al., 2016). SA-SBSE has been shown to improve peak resolution and extraction efficiency for both polar and non-polar analytes (Ochiai et al, 2016).

SBSE has been utilized for flavor research on dairy products including milk, yogurt, cream cheese, and dried dairy ingredients (Hoffman and Heiden, 2000; Park and Drake, 2016). The technique has demonstrated significant improvement over SPME for detecting and quantifying sulfur and carbonyl compounds in aseptically processed fluid milk (Zabbia et al., 2012). SA-SBSE, a newer technique has not been widely applied to dairy research. The objective of this study was to compare three methods (SA-SBSE, SBSE, and SPME) to rapidly and effectively recover vitamin degradation volatiles from fluid milk.

The performance of the three methods (SA-SBSE, SBSE, and SPME) for detecting and quantifying vitamin degradation-related volatile compounds was evaluated by linear regression

of standard curves prepared from spiked standards, the reproducibility on the same day and between days as measured by the average relative standard deviation of each standard curve, and the limits of detection (LOD) and quantitation (LOQ).

## **MATERIALS AND METHODS**

Reagent-grade solvents (cyclohexane, methanol, and dichloromethane) and pure chemical standards (2-methyl-3-heptanone, alpha-phellandrene, beta-cyclocitral, beta-damascone, beta-ionone, and alpha-irone) were purchased from Sigma-Aldrich (St. Louis, MO). These compounds were selected because they were identified as key aroma-active vitamin degradation compounds contributing to vitamin-related off-flavors in fluid milk by Yeh et al. (2017). HPLC-grade water was purchased from Fluka (Seelze, Germany). Unfortified raw skim milk (<0.5% fat) was obtained from the North Carolina State University Dairy Enterprise System (Raleigh, NC). Skim milk was high temperature short time (HTST) pasteurized at 72°C for 15 s using a Microthermics EHVH pasteurization unit (Microthermics, Raleigh, NC) with a two-stage homogenizer (GEA Niro Soavi, Parma, Italy). Commercial HTST and ultrapasteurized (UP) skim milks (n=4) were purchased locally (Raleigh, NC) and were within shelf life. All milks were prepared with overhead lights off to prevent light oxidation.

### **Solid-Phase Microextraction**

The headspace SPME-GCMS system was an Agilent 7820 GC with a 5975 MSD (Agilent Technologies Inc., Santa Clara, CA) with a ZB5-ms (5% phenyl-arylene stationary phase, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Zebron, Phenomenex). The sample preparation and SPME GC-MS method were modified from the method used by White et al., 2013. Samples were injected using a CTC Analytics CombiPal Autosampler (Zwingen, Switzerland). Five milliliters of milk with varying concentrations of vitamin degradation

compound standards were added to 20 mL amber glass SPME vials (Microliter Analytical, Suwanee, GA) in triplicate. Vials were equilibrated for 25 min at 40°C with 4s pulses of 250rpm agitation. A 1 cm SPME fiber (divenylbenzene/carboxen/polydimethylsiloxane; Supelco, Bellefonte, PA) was used for all analyses. The fiber was retracted and injected at 5.0 cm in the GC inlet for 5 min. Initial GC oven conditions were 40°C for 3 min with ramp rates of 10°C/min to 90°C, 5°C/min to 200°C held for 10 min, and 20°C/min to 250°C held for 5 min. A combination of scanning from 40 to 200 *m/z* and selective ion mode for ions 93 (alpha-phellandrene), 121 (alpha-irone), 137 (beta-cyclocitral), and 177 (beta-damascone, beta-ionone) was performed to identify the compounds of interest. Samples were evaluated in triplicate. The entire analysis was repeated on a second day.

#### **Sorptive Stir Bar Extraction and Solvent-Assisted Sorptive Stir Bar Extraction**

SA-SBSE and SBSE analyses were performed using an Agilent 7890B series GC/Agilent inert 5977A MSD equipped with a ZB5-ms column (5% phenyl-arylene stationary phase, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Zebron, Phenomenex). The SBSE GC-MS program was modified from the method used by Park and Drake, 2016. Stir bars were injected using an autosampler (MPS Autosampler, Gerstel, Inc.) and desorbed on a TDU (Gerstel, Inc.) at 250°C for 10min. Volatile compounds were cryogenically focused at -120°C and analyzed using GCMS. Initial GC oven conditions were 40°C for 3 min with ramp rates of 10°C/min to 90°C, 5°C/min to 200°C held for 10 min, and 20°C/min to 250°C held for 5 min. Purge time was set to 1.2 min using helium as the carrier gas at a constant flow rate of 1 ml/min. A 3 min solvent delay was included in the MS acquisition parameters. A combination of scanning from 40 to 200 *m/z* and selective ion mode for ions 93 (alpha-phellandrene), 121 (alpha-irone), 137 (beta-cyclocitral), and 177 (beta-damascone, beta-ionone) was performed to identify compounds of

interest. 10x0.5mm PDMS-coated magnetic stir bars and TDU tubes (Gerstel) were conditioned prior to analysis for 1h at 300°C. Stir bars were cleaned following desorption by soaking the bars 3 times in a 50:50 vol/vol methanol:dichloromethane solution for 1 h, after which the stir bars were allowed to dry overnight before thermal conditioning as previously described.

For SBSE, stir bars were added to 5 or 10 mL milk spiked with varying concentrations of vitamin degradation compounds in a 20 mL amber glass vial (Microliter Analytical) and rotated on a magnetic stir plate at 900 rpm for 60 min at 25°C. Two sample volumes (10 mL and 5 mL) were used to determine the effect of sample volume on efficiency of vitamin compound extraction. After sample extraction on the stir plate, stir bars were rinsed with HPLC grade water and dried briefly before thermal desorption as described previously.

For SA-SBSE, batches of 10 stir bars were submerged in 20 mL of cyclohexane (99.9% purity) for 30 min before being briefly dried and added to 5mL milk spiked with varying concentrations of vitamin degradation compounds as previously described. Bloat time was determined by bloating stir bars in cyclohexane and weighing them periodically; the ideal bloat time was the time after which the stir bars no longer significantly increased in weight. Previous literature (Ochiai et al., 2016) suggested that cyclohexane would be an appropriate solvent for this application; this was verified by preliminary work testing the efficacy of cyclohexane, hexane, diethyl ether, and dichloromethane. Preliminary experiments were conducted with stir bars bloated in each solvent followed by recovery of vitamin degradation compounds in spiked samples and detection of compounds in commercial HTST and UP milks. Following preliminary experiments, cyclohexane was selected.

To determine that the relationship of vitamin degradation compound concentration and signal from the instruments was linear for each extraction method, five vitamin degradation

compounds were spiked into unfortified HTST pasteurized skim milk over a wide range of concentrations (200-0.005 ppb) and extracted and quantified by each of these methods. The concentrations were prepared by making a stock solution of known concentration of the vitamin degradation compounds and then making dilutions over the previously stated range. Each concentration was evaluated in triplicate by each extraction method. The entire experiment was repeated on a second day. Measurement of vitamin degradation compounds in commercial HTST and UP pasteurized fortified bovine milks was conducted using all three methods. Concentrations of vitamin degradation compounds extracted using these methods was determined using relative response factors previously determined with calibration standards during linear regression of standard curve experiments.

### **STATISTICAL ANALYSIS**

All data was analyzed using XLSTAT software (version 2018.1, Addinsoft, New York, NY). Solvent uptake by stir bars to determine maximum bloom time was analyzed by ANOVA with means separation between each time point. Differences among weights at each bloom time were determined using Tukey's honestly significant difference test at 95% confidence. Data was analyzed by ANOVA with means separation between the methods for each vitamin degradation compound in commercial skim milks. Differences among sample means were determined using Tukey's honestly significant difference test at 95% confidence.

Linearity of the standard curves for each method was determined using linear regression. The same day reproducibility was determined by the relative standard deviation (RSD) between peak areas of compounds from spiked unfortified milks analyzed on the same day. The between day reproducibility was determined by RSD between peak areas of compounds from spiked unfortified milk samples analyzed on different days. The LOD and LOQ were defined as a

signal-to-noise ratios of 3:1 and 10:1, respectively, for each vitamin degradation compound in skim milk. The LOD and LOQ determinations were generated from spiked unfortified skim milk to account for background interference to be calculated into these limits. Three compounds (beta-damascone, beta-ionone, and alpha-ionone) selected produced a signal in unfortified skim milk, likely due to residual breakdown products of endogenous retinol found in whole milk before separation (MacGibbon and Taylor, 2006). LOD and LOQ were determined in these signal-producing compounds using the AOAC alternative definition of the LOD and LOQ (AOAC, 2016). LOD was interpreted as the blank (unfortified) milk value plus three times the standard deviation of the blank, while LOQ was interpreted as the blank milk value plus ten times the standard deviation of the blank.

## RESULTS

Detection of selected vitamin degradation volatiles was linear in skim milk (0.005-200 ppb).  $R^2$  values differed between methods and compounds (**Table 1**). Alpha-phellandrene curves were associated with the lowest  $R^2$  values, regardless of method, while beta-damascone was associated with the highest  $R^2$  values, regardless of method. Of all the methods, SA-SBSE with a 10 mL extraction volume and SPME had the most consistently high  $R^2$  values. The SA-SBSE with 10 mL extraction volume was the only method with  $R^2$  values  $\geq 0.90$  for all compounds tested, indicating that all compounds can be reliably extracted across this range.

$R^2$  values for the 5 mL extraction volume SBSE methods were distinctly lower than  $R^2$  values of the other methods, regardless of stir bar bloating. Increasing the volume of matrix in a SBSE extraction can result in poorer recovery in some cases as the phase ratio (ratio of the volume of the matrix to the volume of the PDMS) is inversely related to recovery (Prieto et al., 2010). However, a sample volume which is too low can also result in very poor recovery of

analytes at concentrations near their limit of detection (Prieto et al., 2010). For milk, sample volumes of 5-100 mL are typically selected (Leon et al., 2003). The headspace in the amber glass vials used for sample extraction could also have contributed to the increased linearity resulting from a 10 mL extraction volume. Holding the vial size consistent while decreasing the sample volume results in greater headspace within the vial. Volatile compounds are partitioned between the headspace and the sample matrix, leaving less in the sample matrix to partition into the absorbent stir bar.

Slopes of the standard curves varied by compound regardless of the method used, in some cases varying by orders of magnitude (**Table 1**). The variability of slopes indicates that using a single internal standard to quantify vitamin degradation compounds in fluid milk, especially at the levels typically found in commercial milks, will provide less accurate results than standard curves prepared with the compound of interest. Of the methods tested, SPME had the least variability in slopes of the standard curve, indicating that quantification with a single internal standard would be more likely to produce accurate results with SPME than a stir bar method, provided that concentrations are within limits of detection.

Within day, percent RSD varied with compound and method (7-52%) (**Table 2**). The bloated stir bar method with 10 mL sample volume was the only method to produce < 20 % RSD for all selected compounds. Between day % RSD values tended to be higher than within day values (**Table 2**).

LOD/LOQ values for all methods except SPME were lower than concentrations of selected volatile compounds typically found in commercial milk (**Table 1**). All three methods tested were able to detect selected volatile compounds at concentrations lower than the consumer detection threshold for the compounds in fluid skim milk (Yeh et al., 2017). The LOD and LOQ

values obtained with SPME were higher than the other methods tested, the LOD and LOQ values obtained for the two stir bar methods were largely similar within the range of compounds tested. Alpha-phellandrene, the most polar compound tested, showed considerable improvements in LOD and LOQ when SA-SBSE was used over SBSE, consistent with previous work (Ochiai et al., 2016).

Concentrations of selected compounds in commercial milks averaged across the 5 milks tested is presented in **Table 3**. Concentrations of selected compounds varied with the commercial milk tested, as expected (data not shown). Average concentrations across the samples tested are presented in order to demonstrate the effect of method on compound detection rather than the effect of sample. Alpha-phellandrene was not detected in commercial skim milks by any of the methods. While beta-damascone and beta-ionone were consistently detected by some of the methods (SA-SBSE and 10 ml/5 mL SBSE, respectively), other methods did not detect the compounds consistently enough to result in an average across all 5 milk samples above the previously determined LOQ.

## CONCLUSIONS

SA-SBSE with a 10 mL sample volume provided the most consistent detection of selected compounds in commercial milks, although the levels of compounds detected were not consistently higher than other methods ( $p > 0.05$ ). The SA-SBSE method provided more consistent detection of the selected compounds than SBSE. None of the selected compounds were detected in any of the 5 commercial milks tested using SPME, consistent with previous findings (Yeh et al., 2017).

Rapid methods are able to reliably extract and quantify vitamin degradation-related volatile compounds in fluid milk at low concentrations, however, the method used can greatly

affect results. Based on linearity, RSD, LOD, and LOQ, cyclohexane SA-SBSE with 10 mL sample volume is recommended for the quantitation of vitamin degradation-related volatiles in fluid skim milk. Although SPME produced linear standard curves and low RSD values, the LOD and LOQ offered by the method is not low enough to accurately track concentrations of vitamin degradation compounds in fluid milk, making the method unsuitable for this application. The reduced sensitivity of SPME is likely due to the lower volume of PDMS coating on a fiber vs. a stir bar, resulting in sub-optimal extraction of compounds without a high partition coefficient between the headspace and the fiber (Baltussen et al., 1999). However, if the expected concentrations of these volatiles are higher, SBSE or SA-SBSE offers no significant improvement over SPME for analysis.

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## **CHAPTER 3: DOES VITAMIN FORTIFICATION IMPACT LIGHT OXIDIZED FLAVOR IN SKIM MILK?**

### **ABSTRACT**

Light oxidation is an off-flavor in milk due to photooxidation of native riboflavin and tetrapyrroles that results in an array of lipid oxidation compounds. Recent work has established that water dispersible vitamin A fortification can result in off-flavors in fluid skim milk due to vitamin A degradation products in the vitamin premix. The objective of this study was to determine the role of vitamin fortification on light oxidation of high temperature short time (HTST) pasteurized fluid skim milk. First, the aroma profiles and aroma-active volatile compounds in light-exposed vitamin premixes was determined by exposing the premixes to fluorescent (FL) or light emitting diode (LED) light at 2,000 lx at 4°C for 0, 2, 4, 8, or 24 h. A trained panel (n=8) documented aroma profiles of each vitamin premix at each time point. Headspace solid phase microextraction followed by gas chromatography olfactometry and gas chromatography mass spectrometry (GCMS) was performed to characterize aroma-active compounds in light-exposed vitamin premixes. In the second experiment, commercial vitamin premixes (vitamin A, vitamin D, oil and water matrices) were used to fortify skim milk (3,000 IU/946 mL vitamin A, 600 IU/946 mL vitamin D). Skim milk was pasteurized, homogenized, and packaged in 1892 mL high density polyethylene (HDPE) jugs. Milks were exposed to FL or LED light at 2,000 lx at 4°C for 4, 12, 24, or 48 h. Controls with and without vitamins and light shielding were included. Riboflavin and vitamin A and D degradation were quantified via ultra high performance liquid chromatography. A trained panel (n=8) documented sensory profiles of milks at each time point. Lipid oxidation volatile compounds were quantified via solid phase micro-extraction with GCMS. Vitamin-related volatile compounds were quantified via solvent-

assisted sorptive stir bar extraction with GCMS. Riboflavin, vitamin A, and vitamin D degradation were consistent with previous studies. There was no impact of vitamin fortification on development of typical light-oxidation related off-flavors (cardboard and mushroom) or lipid oxidation related volatiles (hexanal and heptanal) ( $p>0.05$ ). A perfumey/floral flavor was documented in the oil based vitamin A fortified milk suggesting that light exposure impacted the off-flavors contributed by vitamin fortification. These results indicate there is no evidence that vitamin fortification provides any protection against light oxidation off-flavors in fluid milk.

### **INTERPRETIVE SUMMARY**

Light oxidation and vitamin fortification are two sources of off-flavors in fluid milk, however it is unknown if the antioxidant potential of vitamin A affects the development of light-oxidized flavor. HTST pasteurized skim milk was fortified with water dispersible or oil based vitamin A or D premix and exposed to FL or LED light. Unfortified and unexposed controls were included. There was no impact of vitamin fortification on development of typical light-oxidation related off-flavors or lipid oxidation related volatiles.

## INTRODUCTION

Fluid milk has long been an important food around the globe, once comprising over 10% of American daily caloric intake (International Dairy Foods Association, 2016). However, fluid milk consumption in the United States has steadily declined over the past 50 years (International Dairy Foods Association, 2016). Current theories as to the declining popularity of milk include competition from other beverages as well as consumer dissatisfaction with flavor variability in milk (McCarthy et al., 2017a). Light oxidation and vitamin fortification are two such sources of flavor variability in milk.

Whole milk is a good source of vitamin A as well as of provitamin carotenoids such as  $\beta$ -carotene and lutein, however, as these compounds are insoluble in water, the overall vitamin content of milk decreases when whole milk is separated and standardized into skim or reduced fat milk (Rodriguez-Amaya, 2016). Skim and reduced fat milks are legally required to be fortified with vitamin A to be “nutritionally equivalent” to whole milk (DPC, 2001). Fortification with vitamin D, although not legally mandated in the United States, is common due to the historical precedent of preventing rickets in children by supplementing milk with vitamin D (Public Health Service, 1940). Today, vitamin A is typically added to milk as synthetic retinyl palmitate while vitamin D is added as synthetic D<sub>3</sub>. Legally, fluid milk sold in America may have anywhere from 100-150% of the amount of vitamins A and D declared on the label (PHS/FDA, 2015). These vitamins are available as commercial concentrates which are typically added to fluid milk after standardization and before pasteurization (Yeh et al., 2017b).

Concentrated vitamin premixes are available in two forms: oil and water soluble (Yeh et al, 2017b). Yeh et al. (2017a) reported that when skim milk was fortified with water-dispersible, but not oil-based, vitamin A concentrates at levels near the upper limits of what is allowed by law

(3000 IU/qt), consumers could detect flavor differences between unfortified and fortified milks. Sensory evaluation by trained panelists confirmed that the vitamin A-related flavor in fortified milks was described as “carrot-like.” Consumers were not able to distinguish between fortified and unfortified 2% milks or milks fortified with only vitamin D. The atypical carrot off-flavor was attributed to vitamin A degradation compounds. Thus, vitamin fortification, particularly water dispersible vitamin A fortification, may be a source of atypical flavors encountered in skim milk.

Light exposure, in contrast, is a well- and long-established source of off-flavor formation in fluid milk. When milk is exposed to light, photochemical reactions are initiated by the absorption of light by light-sensitive compounds within the milk. Riboflavin is the primary photosensitizer in milk, although naturally-occurring tetrapyrroles including protoporphyrin IX, hematoporphyrin, and a chlorophyll *a*-analogue also play a role in the development of light oxidized flavors (Airado-Rodríguez et al., 2011). When light of particular wavelengths hits compounds sensitive to those wavelengths, the electrons in the photosensitive compounds become excited and can result in photooxidation reactions occurring through formation of a free radical chain or through interactions with oxygen forming highly reactive singlet excited state oxygen (Airado-Rodríguez et al., 2011; Min and Boff, 2002). Both of these photosensitization reactions can occur simultaneously in milk, compete with each other, and lead to off-flavors which are unpleasant to consumers (Lee and Min, 2009; Wold et al., 2015; White and Bulthaus, 1982; Bray, 1977; Chang and Dando, 2018; Potts et al.; 2017).

Not all light-absorbing compounds in fluid milk are photosensitizers. Fortified retinyl palmitate and naturally occurring carotenoids such as  $\beta$ -carotene can act as antioxidants, scavenging for free radicals and perhaps affecting light oxidation in fluid milk (Palace et al.,

1999). Carotenoids can halt free radical reactions in several ways. The majority of the time, excitation energy from singlet oxygen can transfer to the carotenoid and dissipate as heat (Palace et al., 1999). However, if the singlet oxygen free radical is stabilized by the polyene chain of the carotenoid, the carotenoid will cleave at an unpredictable carbon along the polyene chain, destroying the vitamin and resulting in the potential development of aromatic vitamin degradation compounds (Palace et al., 1999). Light exposure causes significant destruction of vitamin A and other carotenoid compounds in fluid milk. Whited et al. (2002) measured significant vitamin A degradation after just 2 h when skim or reduced fat milk was exposed to fluorescent light at 2,000 or 1,000 lx.

The role of volatile vitamin degradation compounds on the flavor of light-oxidized fluid milk has not been fully investigated. Previous research has shown that the addition of natural antioxidants ascorbic acid and  $\alpha$ -tocopherol delayed the onset of light-induced off-flavor formation and the formation of volatile compounds associated with light oxidation in light-exposed milk, but the antioxidant potential of fortified vitamins in fluid milk has not been studied (Van Aardt et al., 2005). Vitamin fortification and light exposure are known sources of off-flavors in milk, but possible interactions between vitamin fortification and light exposure have not been evaluated. The objective of this study was to determine the role of vitamin fortification on light oxidation of fluid high temperature short time (HTST) pasteurized skim milk.

## **MATERIALS AND METHODS**

### **Experimental Overview**

Two experiments (experiments 1 and 2) were included in this study. The purpose of experiment 1 was to determine the sensory profiles and aroma-active volatile compounds in

light-exposed vitamin premixes. For this objective, we performed descriptive analysis, headspace extraction, and identification of volatile compounds by GC-MS and gas chromatography olfactometry (GC-O) on 4 different vitamin premixes (water dispersible A palmitate, A palmitate in an oil matrix, water dispersible D<sub>3</sub>, and D<sub>3</sub> in an oil matrix) exposed to FL or LED light. The purpose of experiment 2 was to determine the possible impact of vitamin A and D fortification on light oxidized flavor development in skim milk. For this objective, skim milk was fortified with one of the 4 vitamin premixes, HTST pasteurized, and exposed to FL or LED light. We evaluated skim milk because previous work on vitamin fortification demonstrated that skim milk was at highest risk for vitamin-related off-flavors and because fat plays a protective role in both vitamin degradation and light oxidized flavor development (Yeh et al., 2017a; Gaylord et al., 1986). If vitamin fortification impacts the off-flavor(s) produced by light exposure, it will be detected in skim milk. Descriptive analysis, vitamin analysis, and quantification of selected volatile compounds by GCMS were conducted on light-exposed milks with and without added vitamins.

### **Commercial Samples and Chemical Standards**

Duplicate lots of four commercial vitamin premixes (vitamins A palmitate and D<sub>3</sub>) in both oil based and water dispersible matrices were utilized. All premixes were within 45 d of manufacture, with >9 mo of remaining shelf life and had been previously characterized (Yeh et al., 2017a). Upon arrival, samples were stored at room temperature in the dark until analysis. All chemical standards were obtained from Sigma Aldrich (Milwaukee, WI). The vitamin A water dispersible premix contained: water, polysorbate 80, vitamin A palmitate, citric acid, and sodium benzoate. The vitamin A in oil premix contained sunflower oil and vitamin A palmitate. The

vitamin D water dispersible premix contained water, propylene glycol, polysorbate 80, and vitamin D<sub>3</sub>. The vitamin D in oil premix contained sunflower oil and vitamin D<sub>3</sub>.

### **Construction of Light Box**

A light box (88 cm long, 57 cm wide, and 31 cm deep) was constructed and covered with laser cloth (ThorLabs, Inc., Newton, NJ) so that all ambient light was blocked from the box as described by Brothersen et al. (2016). One box was fitted with fluorescent (FL) lights at 2,000 lx (3500 K, General Electric, Boston, MA) and the other was fitted with light emitting diode (LED) refrigerator case strip lights at 2,000 lx (3500 K, International Light Technologies, Peabody, MA). Light intensities were verified from previous literature (Whited et al., 2002; DeMann, 1978) and by taking light intensity values of commercial dairy cases at ten stores in the Raleigh-Durham, NC area. A light intensity of approximately 2,000 lx is also listed as the reference light intensity for vitamin A degradation in the Pasteurized Milk Ordinance (PHS/FDA, 2015). The two boxes were placed in a walk-in refrigerator and held at 4°C. The fluorescent light box was outfitted with a 17W 2300 RPM reversible cooling fan (North American Cable Equipment, Inc, West Chester, PA) in order to maintain a constant temperature ( $\pm 1^\circ\text{C}$ ) inside the box. Temperature and lux values were continuously recorded inside the boxes with a datalogging probe (MSR Electronics GmbH, Seuzach, Switzerland).

## **Experiment 1**

### **Light Exposure of Vitamin Premixes**

The four vitamin premixes were exposed to FL or LED light in the previously described light boxes for 0, 2, 4, 8, or 24 h at 4°C. Vitamin premix (19 mL) was added to clear 20 mL glass vials (Microliter Analytical, Suwanee, GA), which were flushed for 10 s under nitrogen gas to minimize oxygen exposure before capping. Vials were shielded with high-density polyethylene

(HDPE) plastic milk jugs (Upstate Niagara Cooperative, Inc., Buffalo, NY) to ensure that the wavelengths of light the premixes were exposed to were representative of the wavelengths milk in commercial dairy cases would be exposed to through plastic packaging. This process was replicated twice.

### **Descriptive Analysis of Light-Exposed Vitamin Premixes**

All sensory testing was deemed exempt by the North Carolina State University Institutional Review Board for human subjects. Ten mL samples of light-exposed vitamin premixes were evaluated orthonasally in 60 mL lidded soufflé cups (Solo Cup Company, Champaign, IL) labeled with 3-digit blinding codes. Samples were evaluated by 6 panelists (4 females, 2 males, ages 22-49 y) each had more than 150 h of previous experience with the sensory analysis of food aromas and flavors using the Spectrum™ descriptive analysis method (Meilgaard et al., 2007). The lexicon for vitamin premixes developed by Yeh et al. (2017a) was applied. Each premix was evaluated by each panelist in duplicate. Compusense Cloud (Compusense, Inc., Guleph, CA) was used for data collection.

### **Headspace Extraction of Volatile Compounds from Light Exposed Vitamin Premixes**

Volatile compounds were extracted from vitamin premixes by headspace solid-phase microextraction (SPME) followed by gas chromatography mass spectrometry (GCMS) using the methods of Yeh et al. (2017a) with modifications. All injections were made on an Agilent 7820 GC with a 5975 MSD (Agilent Technologies Inc., Santa Clara, CA) with a ZB-Semivolatiles column (5% phenyl-arylene stationary phase, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Zebron; Phenomenex, Torrence, CA). Volatile compounds were extracted using a CTC Analytics CombiPal Autosampler (Zwingen, Switzerland). Five milliliters of vitamin premix was added to 20 mL amber glass SPME vials (Microliter Analytical, Suwanee, GA) in

triplicate. Vials were equilibrated for 25 min at 40°C with 4 s pulses of 250 rpm agitation. A 1 cm SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane; Supelco, Bellefonte, PA) was used. The SPME fiber was exposed to the samples for 40 min at 3.1 cm depth. The fiber was retracted and injected at 5.0 cm in the GC inlet for 5 min. Scanning from 30 to 200  $m/z$  was performed to identify selected aroma-active compounds of interest as described by Yeh et al. (2017a). Each vitamin premix was evaluated in triplicate.

### **GC-O**

Headspace SPME, followed by gas chromatography olfactometry (**GC-O**), was performed to characterize aroma-active compounds in light-exposed vitamin premixes as described by Yeh et al. (2017a) with the modification of the use of a ZB-Semivolatiles column (30 m length x 0.25 mm i.d. x 0.25  $\mu\text{m}$  film thickness; Zebron; Phenomenex Inc.). Ten milliliters of vitamin concentrate was added to 40 mL amber vials (28 x 98 mm; Supelco Inc.) with a stir bar. The vials were heated at 40°C for 3 min with constant stirring. A SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane; Supelco Inc.) was exposed in each sample at 2 cm for 30 min. The fiber was then injected on an Agilent 6850 GCO flame-ionization detector equipped with an olfactometer port (Agilent Technologies Inc.). The GC method used an initial temperature of 40°C for 3 min. The temperature was then increased at a rate of 10°C/min to 150°C, followed by 30°C/min to 200°C, and held for 5 min. The flame ionization detector sniffing port was held at a temperature of 300°C, with a helium carrier gas flow of 2 mL/min, and the port was supplied with humidified air at 30 mL/min. Each sample was evaluated in triplicate by three highly experienced sniffers (each with >50 h previous experience with GCO) who recorded retention time, aroma character, and perceived intensity of each aroma event.

## **Compound Identification for Light Exposed Vitamin Premixes**

Retention indices, mass spectra, and odor properties of unknown compounds were compared with those of authentic standard compounds (Sigma Aldrich) analyzed under identical conditions on GC-MS and GC-O. Tentative identifications were based from comparisons of the mass spectra of unknown compounds with those in the National Institute of Standards and Technology mass spectral database (NIST MS Search 2.0, NIST/EPA/NIH Mass Spectral Library, <http://webbook.nist.gov>) or from matching retention index values of unknowns against those of authentic standards. To calculate retention indices, an alkane series (C<sub>8</sub> – C<sub>20</sub> Sigma Aldrich) was used (van den Dool and Kratz, 1963).

## **Experiment 2**

### **Pilot Plant Manufacture of Vitamin-Fortified Milk**

The four vitamin premixes were used to fortify skim milk. Unfortified skim milk was also processed. Raw skim milk was obtained from the North Carolina State University Dairy Enterprise System (Raleigh, NC). Raw milk proximate analyses included somatic cell count (SCC) (Somatoscope, Delta Instruments, Drachten, the Netherlands), coliforms, aerobic plate count, fat content, and total solids content. Coliform and aerobic plate counts were conducted using standard methods (Wehr and Frank, 2004; method numbers 7.070 and 6.040 respectively) with Petrifilm plates (Coliform Count Plates and Aerobic Count Plates; 3M, St. Paul, MN). Plates were incubated at 32°C for 24 h before being counted. Fat and total solids content were measured using a Fourier-transform mid-infrared milk analyzer (LactoScope FTIR; Delta Instruments BV).

Vitamin premixes were added to the raw skim milk. Raw milks for each run were batched as follows: control with no vitamin addition, A<sub>w</sub> at 3,000 IU/qt (946 mL), A<sub>o</sub> at 3,000 IU/qt (946

mL),  $D_w$  at 600 IU/qt (946 mL),  $D_o$  at 600 IU/qt (946 mL). These fortification levels represent the maximum level of fortification for each vitamin allowable by the United States Food and Drug Administration, 150% of label claims for skim and reduced-fat milk, thus representing a “worst case” scenario for flavor differences resulting from vitamin fortification (PHS/FDA, 2015). A Microthermics EHVH pasteurization unit (Microthermics, Raleigh, NC) with a two-stage homogenizer (GEA Niro Soavi, Parma, Italy) was used to process the milks. Each batch was preheated to 60°C, homogenized (first stage at 17.3 MPa and second stage at 3.4 MPa), and pasteurized (73°C for 15 s) before cooling to 10°C. Final products were collected in 946 mL HDPE milk jugs (ULINE, Pleasant Prairie, WI) and then placed at 4°C. Milks were tested for complete pasteurization by the alkaline phosphatase test (Wehr and Frank, 2004; method 14.060). Microbial quality of the pasteurized milk was evaluated using the methods described previously.

### **Exposure of Milks in Light Box**

After a 24 h equilibration period at 4°C, pasteurized fortified and unfortified milks were placed in the light boxes. The temperature in each light box and the light intensity at the surface of the milk jugs was continuously recorded with a datalogging probe (MSR Electronics GmbH, Seuzach, Switzerland) to ensure that temperature and light intensity remained constant throughout the exposure period. Samples were removed after 4, 12, 24, and 48 h. Once removed, jugs were covered with lasercloth to prevent additional light exposure and stored at 4°C for the remainder of the 48 h exposure period. Control samples for the four fortified treatments and unfortified treatment were kept covered in lasercloth at 4°C during the entire exposure period to prevent any light exposure. After the 48 h exposure period, all milks were immediately prepared for descriptive sensory analysis. The remaining milk was transferred to 50mL vials (Fisher

Scientific, Hampton, NH) and immediately frozen at -80°C. Vitamin and volatile compound analyses were completed within 40 days. The entire experiment was replicated twice.

### **Vitamin A and D Analysis**

Vitamin analysis was conducted in a laboratory with UV-filtered orange lighting to prevent light oxidation and destruction of vitamins during extraction and analysis. Vitamin analysis was conducted in accordance with the procedure published by Chen et al. (2015). A 15 mL test portion of fortified milk was added to a 250 mL amber glass jar (Sigma Aldrich) along with 10 mL of 50% (wt/wt) KOH (Sigma Aldrich) and 20 mL of ethanol (Sigma Aldrich) containing 2% (w/vol) pyrogallol (Sigma Aldrich). Samples were saponified in a water bath held at 60°C for 2 h and mixed by inversion every 15 min. Samples were extracted twice successively with 20% diethyl ether in hexane (vol/vol). Thirty mL solvent was added to the saponified milk, which was allowed to settle, washed with a 50 mL aliquot of deionized water, mixed by inverting eight times, and allowed to settle again. A 15 mL aliquot of the solvent phase was transferred to a light-shielded beaker for each extraction. The collected solvent phase was transferred to a 125 mL glass separatory funnel and washed with 50 mL deionized water. A 10 mL aliquot of the washed solvent was transferred to a 50 mL plastic centrifuge tube (Fisher Scientific, Hampton, NH), dried at room temperature under a nitrogen gas stream, and reconstituted in 15% deionized water in methanol (vol/vol) (Sigma Aldrich) by sonicating for 5 min followed by vortexing for 30 s. The reconstituted samples were filtered through a 0.2 µm nylon filter (Sigma Aldrich). A 200 µL aliquot of the top layer of the samples was transferred to a glass insert inside a 2 mL amber glass vial (Verex; Phenomenex, Inc.) for UPLC analysis. The concentration of retinyl palmitate (vitamin A) and vitamin D<sub>3</sub> was measured by reverse-phase ultra-performance liquid chromatography (UPLC; Acquity H-Class; Waters Corporation,

Miliford, MA) with photodiode array (**PDA**) detection (325 nm for retinyl palmitate and 265 nm for vitamin D<sub>3</sub>) and an Acquity BEH C18 1.7 µm 2.1 x 50 mm column. A methanol/acetonitrile gradient (Sigma Aldrich) was used as the mobile phase. The concentration of retinyl palmitate (µg/mL) was calculated using a relative response factor determined with calibration standards (0.05-5µg/mL) (Sigma Aldrich). The concentration of vitamin D<sub>3</sub> (µg/mL) was calculated using a relative response factor determined with calibration standards (0.001-0.05µg/mL) (Sigma Aldrich).

### **Riboflavin Analysis**

Riboflavin analysis was conducted in accordance with the procedure published by Silva et al. (2005). 0.75 mL 33% trichloroacetic acid (Sigma Aldrich) was added to 0.5 mL milk in a 2 mL Eppendorf tube (Sigma Aldrich). Eppendorf tubes were vortexed for 30 s and centrifuged for 10 min at 10,000 x g force in a benchtop centrifuge. The supernatant was filtered through a 0.2 µm nylon filter (Sigma Aldrich) into a 2 mL amber HPLC vial for UPLC analysis. Reverse phase chromatography with a Waters HSS C18 1.8 µm 2.1 x 100 mm column and a mobile phase gradient consisting of 100% methanol (Sigma Aldrich) and 150 mM dibasic potassium phosphate solution (adjusted to pH 7 with phosphoric acid and filtered through a 0.2 µm nylon filter) (Sigma Aldrich) was used to analyze samples. The concentration of riboflavin in milks was measured by ultra-performance liquid chromatography (**UPLC**; Acquity H-Class; Waters Corporation) with fluorescence (**FLR**) detection using excitation at 420 nm and emission at 530 nm. The concentration of riboflavin (µg/mL) was calculated using a relative response factor determined with calibration standards (0.10-3 µg/mL) (Sigma Aldrich).

### **Descriptive Analysis of Light-Exposed Milks**

All sensory testing of milks was deemed exempt by the North Carolina State University Institutional Review Board for human subjects. Descriptive sensory analysis of milk flavor was conducted on milks using a trained descriptive sensory panel and an established milk flavor language (Croissant et al., 2007; McCarthy et al., 2017b). Descriptive analysis was conducted 3 days post-processing, following the 24 h equilibration period shielded from light and the 48 h period during which some of the samples were exposed to FL or LED light for 2, 4, 12, 24, or 48 h. Panelists (n=8, 6 females, 2 males, ages 22-49 y) each had more than 150 h of previous experience with the sensory analysis of food aromas and flavors using the Spectrum<sup>TM</sup> descriptive analysis method (Meilgaard et al., 2007). A total of forty milks were evaluated in duplicate for each experimental replication: ten light exposure treatments for each fortification treatment as well as unfortified milks (FL or LED for each vitamin premix for 0, 4, 12, 24, and 48 h). Milks were dispensed (60 mL) directly into three-digit-coded 120 mL lidded soufflé cups (Solo Cup Company, Champaign, IL) for evaluation. Preparations were conducted with overhead lights off to avoid exposure to light. Samples were evaluated by each panelist in duplicate in a randomized order. No more than six samples were evaluated in a session followed by a 30 min rest between sessions for each panelist. Compusense Cloud (Compusense, Inc., Guelph, CA) was used for data collection.

### **Volatile Compound Analysis of Light-exposed Milks**

Volatile compounds were extracted from light-exposed milks by headspace solid-phase microextraction (SPME) followed by GCMS analysis as described by Yeh et al. (2017a). All injections were made on an Agilent 7820 GC with a 5975 MSD (Agilent Technologies Inc., Santa Clara, CA) with a ZB5-ms (5% phenyl-arylene stationary phase, 30 m length x 0.25 mm

i.d. x 0.25  $\mu\text{m}$  film thickness; Zebron; Phenomenex). Samples were injected using a CTC Analytics CombiPal Autosampler (Zwingen, Switzerland). Five milliliters of milk and 20  $\mu\text{l}$  internal standard solution (2-methyl-3-heptanone in ether at 81 mg/kg; Sigma Aldrich) was added to 20 mL amber glass SPME vials (Microliter Analytical, Suwanee, GA) in triplicate. Vials were equilibrated for 25 min at 40°C with 4 s pulses of 250 rpm agitation. A 1 cm SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane; Supelco, Bellefonte, PA) was used for all analyses. The SPME fiber was exposed to the samples for 40 min at 3.1 cm depth. The fiber was retracted and injected at 5.0 cm in the GC inlet for 5 min. A combination of scanning from 40 to 200  $m/z$  and selective ion mode for ions 56 (hexanal), 70 (heptanal), and 85 (2-methyl-3-heptanone internal standard) was performed to identify compounds of interest. Each milk sample was evaluated in triplicate.

Compounds previously identified in literature to be related to light oxidation (hexanal and heptanal) were identified and quantified by relative abundance (Webster et al, 2009; Kim and Morr, 1996; van Aardt et al., 2005). Aldehydes such as hexanal and heptanal have the lowest sensory threshold of light-induced lipid oxidation volatiles, and as such are generally considered key markers for lipid oxidation in fluid milk (Marsili, 1999; Lee and Min, 2009). Hexanal and heptanal concentrations have been associated with light oxidized flavor in fluid milk by various studies (Marsili, 1999; Bassette, 1976; Mestdagh et al., 2004; Lee and Min, 2009). MassHunter Software (Agilent Technologies Inc., Santa Clara, CA) was utilized. Compounds were verified using retention times and mass spectra of authentic standards. To calculate retention indices, an alkane series ( $\text{C}_8 - \text{C}_{20}$  Sigma Aldrich) was used (van den Dool and Kratz, 1963).

## **Solvent-Assisted Sorptive Stir Bar Extraction (SA-SBSE) Analysis of Vitamin A-Fortified Milks**

Previous results (Yeh et al., 2017a) as well as sensory and GCO results from the current study suggested that flavors specific to vitamin A fortification and degradation were present in some of the vitamin A fortified milks with and without light exposure. SA-SBSE analysis was used to detect and quantify aroma-active compounds related to vitamin degradation (alpha-phellandrene, beta-cyclocitral, beta-damascone, beta-ionone, and alpha-irone) (Yeh et al., 2017a) in both water-dispersible and oil-based vitamin A-fortified milks (Ochiai et al., 2016). Each milk was evaluated in triplicate. Prior to analysis, 10x0.5 mm PDMS-coated magnetic stir bars and thermal desorption unit (TDU) tubes (Gerstel) were conditioned for 1 h at 300°C. Next, batches of 10 stir bars were submerged in 20 mL of cyclohexane (99.9% purity, Fair Lawn, NJ) for 30 min. Bloat time was determined by bloating stir bars in cyclohexane and weighing them periodically; the ideal bloat time was the time after which the stir bars no longer significantly increased in weight. One (1) bloated stir bar was added to each 10 mL milk in a 10 mL amber glass vial (Microliter Analytical) and rotated on a magnetic stir plate at 900 rpm for 60 min at 25°C. After sample extraction, stir bars were rinsed with HPLC grade water (Fisher Chemical) and dried briefly.

Stir bars were injected using an autosampler (MPS Autosampler, Gerstel, Inc.) and desorbed on a TDU (Gerstel, Inc.) at 250°C for 10 min. Volatile compounds were cryogenically trapped at -120°C and analyzed using an Agilent 7890B series GC/Agilent inert 5977A MSD equipped with a ZB5-MS column (5% phenyl stationary phase, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Zebron; Phenomenex, Torrance, CA). Initial GC oven conditions were 40°C for 3 min with ramp rates of 10°C/min to 90°C, 5°C/min to 200°C held for 10 min, and

20°C/min to 250°C held for 5 min. Purge time was set to 1.2 min using helium as the carrier gas at a constant flow rate of 1 ml/min. A combination of scanning from 40 to 200  $m/z$  and selective ion mode for ions 93 (alpha-phellandrene), 121 (alpha-ionone), 137 (beta-cyclocitral), and 177 (beta-damascone, beta-ionone) was performed to identify compounds of interest. Compounds were identified by comparison with the 2014 NIST mass spectral library (NIST, 2014) as well as by comparison with retention index and time of authentic standards injected under identical conditions. Concentration of selected compounds was calculated using a relative response factor determined with calibration standards (0.05 ng/mL-200 ng/mL) (Sigma Aldrich).

### **STATISTICAL ANALYSIS**

Data were analyzed as a split plot experiment with fortification type as the whole plot factor and duration of lighting as the sub plot factor. A separate analysis was conducted for each type of lighting. Fixed effects in the model were fortification type, duration of lighting and their interaction. Random effects included were batch replication, fortification type by batch replication interaction and duration of lighting by fortification type by batch replication interaction. For descriptive analysis data, fixed effects were again fortification type, duration of lighting and their interaction. Random effects were batch replication, panelist by batch replication interaction, fortification type by panelist by batch replication interaction, and duration of lighting by fortification type by panelist by batch replication interaction. All analyses were conducted with SAS (version 9.4, Cary, NC).

## RESULTS

### Experiment 1

#### Descriptive Analysis of Light Exposed Vitamin Premixes

Sensory data was consistent with the findings of Yeh et al. (2017a). Higher overall aroma intensities were noted in water-dispersible premixes compared to oil soluble premixes ( $p < 0.05$ ) (results not shown). Vitamin A premixes had higher overall aroma intensities than vitamin D premixes regardless of matrix ( $p < 0.05$ ). Floral and carrot aromas were detected in both types of vitamin A premixes ( $\bar{x} = 4.5, 4.0$  for water dispersible and  $3.0, 3.5$  for oil-based for floral and carrot aromas, respectively). Exposure to FL or LED light resulted in significant muting of the overall aroma of both types of vitamin A premixes after 4 h ( $\bar{x} = 3.0$  vs.  $2.5$  (vitamin A oil based),  $4.5$  vs.  $2.5$  (vitamin A water dispersible). ( $p < 0.05$ ). Exposure of vitamin A oil premixes to FL light resulted in development of a vinegar aroma ( $\bar{x} = 3.0$ ) after 4h which was not detected after exposure to LED light. Exposure to FL or LED light resulted in muting of the overall aroma in both types of vitamin D premixes after 8 h ( $\bar{x} = 2.4$  vs  $2.0$  (vitamin D oil based),  $2.5$  vs.  $2.0$  (vitamin D water dispersible)) ( $p < 0.05$ ). The vinegar aroma detected in the oil-based vitamin A premixes following 4 h FL light exposure was the only discernible sensory effect of light exposure other than overall decreases in aroma impact.

#### Instrumental Volatile Analysis

Instrumental results were also similar to those noted by Yeh et al. (2017a). More aroma events were recorded for water-dispersible premixes than oil-based premixes, and for vitamin A premixes than for vitamin D premixes. Twenty-eight aroma-active compounds were detected in the vitamin premixes – 15 terpenes (alpha-phellandrene, limonene, beta-damascone, beta-ionone, beta-ionone epoxide, dihydroactinidiolide, alpha-irone, beta-cyclohomocitral, alpha-copaene,

alpha-pinene, 2-carene, isogeraniol, citronellal, beta-cyclocitral, and isopulegol), 8 aldehydes (hexanal, heptanal, (E)-2-heptanal, octanal, (E)-2-octanal, (E)-2-nonanal, nonanal, and (E)-2-decanal), one ketone (6-methyl-5-heptenone), one fatty alcohol (1-octanol), and three hydrocarbons (3,7-dimethyl-1-octene, 1-dodecene, and dodecene) (results not shown). The aroma-active compounds documented here were similar to those published in Yeh et al. (2017a), and included the key aroma compounds attributed to the floral and carrot aromas previously published by Yeh et al. (2017a).

No new compounds were detected in light exposed premixes which had not been detected in unexposed premixes. Volatile compounds in experiment 1 were not measured quantitatively, as the purpose was to identify potential additional compounds formed during light exposure not found in light shielded premixes in order to aid in signal searching during subsequent experiments. Sensory and instrumental data from experiment 1 suggested that changes in the aroma of vitamin premixes with light exposure were likely due to changes in the concentration of volatile compounds found in unexposed premixes rather than the formation of new compounds over the course of light exposure.

## **Experiment 2**

### **Proximate Analysis of Pilot Plant Manufactured Skim Milk**

Raw milk fat content was <0.5%, total solids content was  $9.3 \pm 0.00\%$  w/w. Total plate counts and coliform plate counts in raw milks averaged  $9 \times 10^2$  CFU/mL and 23 CFU/mL, respectively. Somatic cell counts in raw milks were < 200,000. Mean fat and total solids content of pasteurized fortified milks were <0.05% and  $9.2 \pm 0.1\%$  w/w, respectively. Alkaline phosphatase results indicated complete HTST pasteurization of all milks. No coliforms were

detected in pasteurized milks. Total plate counts in pasteurized milks were less than  $10^2$  CFU/mL.

### **Vitamin Degradation**

Vitamin A and D degradation were consistent with previous work (Whited et al., 2002; Renken and Warthesen, 1993) (results not shown). No significant vitamin D degradation was observed across the exposure period with either light type ( $p > 0.05$ ). After 48 h of light exposure, vitamin A loss in water-dispersible fortified milk was 54% and 61% for LED and FL exposed milk, respectively. Vitamin A loss in oil-fortified milks was 37% and 45% for LED and FL exposed milks, respectively. These results are consistent with the findings of Brothersen et al. (2016), Whited et al. (2002), and Fellman et al. (1991). Native riboflavin in milks was also degraded following light exposure. After 48 h of light exposure, the percent loss of riboflavin was 52% for FL exposed milks and 30% for LED exposed milks, similar to previous studies which also noted increased riboflavin degradation for FL vs LED light (Gaylord et al., 1986; Lee et al, 1998; Palanuk et al., 1988).

### **Descriptive Analysis of Light Oxidized Milk**

Typical HTST pasteurized milk flavors (cooked/milky, sweet aromatic, sweet taste, salty taste, astringency) did not change with storage time, fortification, or light exposure ( $p > 0.05$ ) (results not shown). Typical light oxidation flavors (cardboard, mushroom/serumy) were only detected in light exposed milks. For cardboard and mushroom/serumy flavors, time was a significant effect but not fortification or fortification\*time, suggesting that fortification had no impact on the development of these typical light oxidation flavors. These flavors were not detected in milks unexposed to light. Cardboard flavors increased to an intensity of  $\bar{x} = 2.4 \pm 0.3$  and  $1.7 \pm 0.2$  while mushroom/serumy flavors increased to an intensity of  $\bar{x} = 1.5 \pm 0.4$  and  $0.5 \pm$

0.2 after 48 h exposure to FL or LED light, respectively. Brothersen et al. (2016) also reported larger light oxidation sensory effects in FL exposed milks compared to LED exposed milks.

Significant effects were documented for fortification, time, and fortification\*time for carrot and perfume/floral flavors. Carrot flavor was only detected in milks fortified with water-dispersible vitamin A premix (regardless of light exposure), consistent with the findings of Yeh et al. (2017a). Initial carrot flavor ( $\bar{x} = 1.1 \pm 0.2$ ) decreased with FL light exposure to 0.5 after 48 h (results not shown). Carrot flavor in water dispersible vitamin A fortified skim milk ( $\bar{x} = 1.1 \pm 0.2$ ) did not change following exposure to LED light ( $p > 0.05$ ) (results not shown).

Perfume/floral flavor was detected only following light exposure in milks fortified with oil-based vitamin A premix. Initially, perfume/floral flavor was not detected in any of the skim milks. Intensities increased to  $\bar{x} = 1.5 \pm 0.3$  and  $0.5 \pm 0.2$  following exposure to FL or LED light, respectively (Figs. 1-2).

### **SPME-GCMS**

Time was the only significant factor for hexanal and heptanal; no significant interactions were observed. Fortification had no effect on concentrations of hexanal or heptanal ( $p > 0.05$ ), but exposure to FL or LED increased concentrations of these lipid oxidation compounds ( $p < 0.05$ ) (Figs. 3-4). Hexanal concentrations in milks stored at 4°C for 48 h in the dark were  $26.3 \pm 3.2$  ppb, compared to  $136.1 \pm 11.0$  ppb for milks exposed to FL light for 48 h and  $92.6 \pm 13.2$  ppb for milks exposed to LED light for 48 h. Heptanal concentrations in milks stored at 4°C for 48 h in the dark were  $4.3 \pm 2.5$  ppb, compared to  $23.0 \pm 5.8$  ppb for milks exposed to FL light for 48 h and  $12.8 \pm 4.1$  ppb for milks exposed to LED light for 48 h.

## SA-SBSE

Selected vitamin degradation compounds were detected in milks fortified with either oil-based or water-dispersible vitamin A, but not in unfortified milks (Fig. 5). Concentrations of vitamin degradation volatiles were not consistently different in oil-based vitamin A fortified or water-dispersible vitamin A fortified milks ( $p>0.05$ ). Alpha-phellandrene concentrations were not affected by fortification or exposure to either FL or LED light ( $p>0.05$ ). Concentrations of alpha-irone and beta-damascone were not impacted by fortification or LED light exposure ( $p>0.05$ ), but were impacted by FL light exposure ( $p<0.05$ ). Fortification type did not impact concentrations of alpha-irone ( $p>0.05$ ), which decreased across exposure to FL light ( $p<0.05$ ). Concentrations of beta-damascone in vitamin A fortified milks exposed to LED light decreased across light exposure for water-dispersible vitamin A fortified milks ( $p<0.05$ ).

A significant time\*fortification interaction was observed for beta-cyclocitral and beta-ionone concentrations in milks exposed to FL light. Beta-cyclocitral and beta-ionone levels increased in oil-based vitamin A fortified milks with light exposure ( $p<0.05$ ), but did not change in water-dispersible vitamin A fortified milks ( $p>0.05$ ). This result follows the trend observed for vitamin degradation off flavors in vitamin A fortified milks: perfumey/floral off-flavor increased in oil-based vitamin A fortified milks with FL light exposure, but was not detected in water-dispersible vitamin A fortified milks. Although FL and LED exposed milks were not directly compared in this study, in milks exposed to LED light, concentrations of beta-cyclocitral and beta-ionone were lower at all timepoints for both vitamin A fortification treatments than milks exposed to FL light.

## **DISCUSSION**

### **Experiment 1**

Descriptive analysis of light exposed vitamin premixes demonstrated that across light exposure, new aroma characters were not detected in vitamin premixes, with the exception of a vinegar aroma in water dispersible vitamin A premix. Instead, a muting of all aromas was noted in the vitamin premixes with exposure to either FL or LED light. Decreases in aroma intensity might be caused by degradation of aroma-active compounds or by the formation of additional oxidation related volatiles that impact sensory thresholds.

The aroma-active compounds documented by GCO in the light exposed vitamin premixes were consistent with those published by Yeh et al. (2017a) and included the same key aroma compounds with floral and carrot aromas. While volatile compounds in this experiment were not measured quantitatively, the lack of additional volatile compounds formed in the premixes after light exposure suggested that no new aroma-active compounds were formed in vitamin premixes which are not already present in the unexposed premixes.

### **Experiment 2**

There was no impact of vitamin fortification on development of typical light-oxidation related off-flavors (cardboard and mushroom/serumy), indicating no evidence that vitamin fortification provides any effects regarding light oxidation flavors. Vitamin D in oil or water dispersible form did not contribute detectable off-flavors to skim milk regardless of light exposure, consistent with previous studies (Yeh et al., 2017a). However, the development of perfumey/floral flavor in the oil based vitamin A fortified milk and the decrease in intensity of carrot flavor in the water dispersible vitamin A fortified milk suggests that light exposure impacted the specific off-flavors contributed by vitamin fortification.

Although our statistical model did not directly compare FL and LED fortified milks, differences were noted in the levels of carrot and perfumey/floral flavors between the vitamin A fortified milks exposed to the two light sources. Perfumey/floral flavor in oil-based vitamin A fortified milks took longer to develop with exposure to LED than FL light (48 h vs 12 h, respectively). Carrot flavor intensity did not change during LED light exposure, but decreased with FL exposure. The decrease may be due to further degradation of the aroma-active vitamin A degradation compounds presumed to cause this flavor, or simply due to other oxidation-related flavors increasing and thus decreasing sensory perception of the carrot flavor. Cardboard and mushroom/serumy off-flavors developed faster after exposure to FL vs. LED light for all fortification types (4 h vs 12 h, respectively). Previous studies have also demonstrated that FL exposure was more destructive to milk vitamin content and flavor (Brothersen et al, 2106).

Hexanal and heptanal are two volatile compounds indicative of lipid oxidation in fluid skim milk. Lipid oxidation is a part of light oxidation milk chemistry and as expected, these compounds were higher in light-exposed milks compared to unexposed milks. Like sensory perception of light oxidized flavors, hexanal and heptanal concentrations were higher at every time point for FL exposed milks than LED exposed milks, consistent with previous studies (Brothersen et al., 2016). Fortification had no effect on hexanal and heptanal concentrations, indicating no evidence that vitamin fortification provides any protective effects regarding formation of light oxidation-related volatiles.

Aroma-active volatile vitamin A degradation compounds with carrot and floral aromas were detected in vitamin A fortified skim milks but not unfortified controls (Fig. 5). Each of the selected compounds behaved differently when exposed to light in skim milk, indicating that the mechanisms surrounding formation of aroma-active vitamin A degradation volatiles are complex

and heavily influenced by light type and matrix. Furthermore, many degradation products of vitamin A are also light sensitive (Ly et al., 2008; Uriarte et al., 2018), and thus aroma-active compounds formed with light exposure could subsequently break down with additional light exposure into low levels of additional compounds, at or below limits of detection, further confounding analysis of these compounds. More changes in concentrations of these volatiles were noted for samples exposed to FL light than LED light, which could perhaps be another demonstration of the more damaging effects of FL light on milk and its components compared to LED light. Vitamin A and other carotenoids have demonstrated antioxidant capacities in other systems, but there is no evidence that vitamin fortification provides any protection against light oxidation off-flavors in fluid milk.

## **CONCLUSIONS**

Off-flavors were documented in skim milk fortified with water dispersible Vitamin A, consistent with previous studies. There was no impact of vitamin fortification on development of typical light-oxidation related off-flavors, riboflavin degradation, or development of lipid oxidation related volatiles. A newly-documented perfumey/floral flavor was documented in the oil based vitamin A fortified milk following light exposure that may suggest light exposure impacts the specific off-flavors contributed by vitamin fortification. These results demonstrate that the dairy industry should exercise care when selecting vitamin A premixes for milk fortification.

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## APPENDICES

**Table 2.1.** Slopes of linear regression equations, R<sup>2</sup> values, limits of detection, and limits of quantitation for selected compounds

Method	SA-SBSE		SBSE		SPME	
	10 mL	5 mL	10 mL	5 mL	5 mL	
Alpha-phellandrene	Slope	0.000181	0.000089	0.000322	0.000439	0.000003
	R <sup>2</sup>	0.885	0.439	0.679	0.482	0.831
	LOD	1.00	5.00	5.00	5.00	2.50
	LOQ	5.00	5.00	25.00	25.00	5.00
Beta-cyclocitral	Slope	0.000033	0.000030	0.000153	0.000216	0.000006
	R <sup>2</sup>	0.930	0.676	0.927	0.870	0.987
	LOD	0.01	0.01	0.01	0.01	1.00
	LOQ	0.05	0.05	0.05	0.05	2.50
Beta-damascone	Slope	0.0000203	0.0000198	0.0000396	0.0000541	0.0000068
	R <sup>2</sup>	0.986	0.634	0.975	0.960	0.989
	LOD	0.34	0.46	0.73	0.83	2.50
	LOQ	0.59	1.00	1.57	1.60	2.50
Beta-ionone	Slope	0.000013	0.000015	0.000019	0.000024	0.000007
	R <sup>2</sup>	0.986	0.727	0.989	0.894	0.934
	LOD	0.10	0.12	0.13	0.20	2.50
	LOQ	0.11	0.18	0.23	0.35	2.50
Alpha-irone	Slope	0.000038	0.000047	0.000049	0.000065	0.000017
	R <sup>2</sup>	0.980	0.798	0.987	0.743	0.938
	LOD	0.21	0.31	0.38	0.71	2.50
	LOQ	0.26	0.49	0.66	1.55	5.00

LOD/LOQ values expressed in parts per billion.

Results are averaged across samples run in triplicate on two different days.

**Table 2.2.** Within and between day % RSD for selected compounds by method (n=6 replicates across 2 days)

	Method	SA-SBSE		SBSE		SPME	
		Sample Volume	10 mL	5 mL	10 mL	5 mL	5 mL
Alpha-phellandrene	Within Day RSD		18.3%	52.1%	24.9%	24.0%	12.6%
	Between Day RSD		35.0%	90.2%	79.5%	50.2%	16.7%
Beta-cyclocitral	Within Day RSD		19.7%	13.7%	16.7%	13.8%	7.4%
	Between Day RSD		33.8%	43.9%	22.7%	20.0%	6.9%
Beta-damascone	Within Day RSD		13.3%	30.7%	15.7%	21.3%	12.3%
	Between Day RSD		26.8%	70.6%	30.1%	33.4%	25.5%
Beta-ionone	Within Day RSD		7.9%	16.1%	12.7%	11.1%	17.8%
	Between Day RSD		23.1%	39.7%	28.7%	12.2%	30.5%
Alpha-irone	Within Day RSD		8.9%	17.8%	20.9%	14.6%	14.0%
	Between Day RSD		21.2%	37.5%	47.3%	16.9%	13.0%

**Table 2.3.** Quantification of selected compounds in commercial skim milks

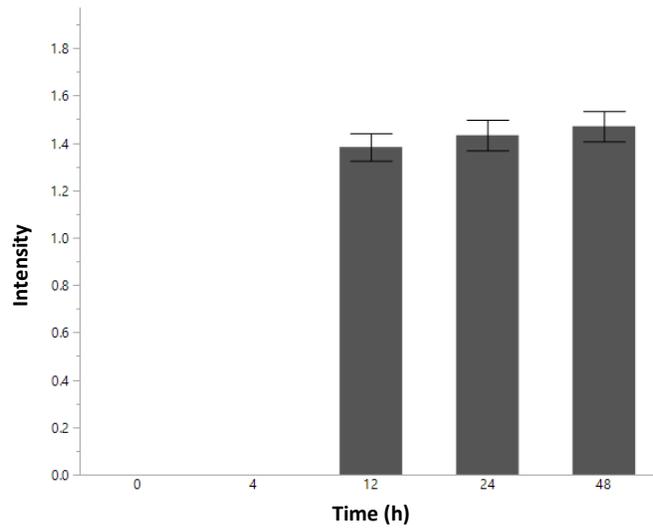
Method	SA-SBSE		SBSE		SPME
	10 mL	5 mL	10 mL	5 mL	5 mL
Alpha-phellandrene	ND <sup>1</sup>	ND	ND	ND	ND
Beta-cyclocitral	0.08b	0.05b	0.23a	0.05b	ND
Beta-damascone	0.68a	NQ <sup>2</sup>	NQ	NQ	ND
Beta-ionone	0.12a	0.19a	NQ	NQ	ND
Alpha-irone	3.98ab	6.72a	5.99ab	2.17b	ND

Values presented are an average of the 5 commercial milks tested (ppb).

a-b Means in a column not sharing a common letter are statistically different ( $p < 0.05$ ).

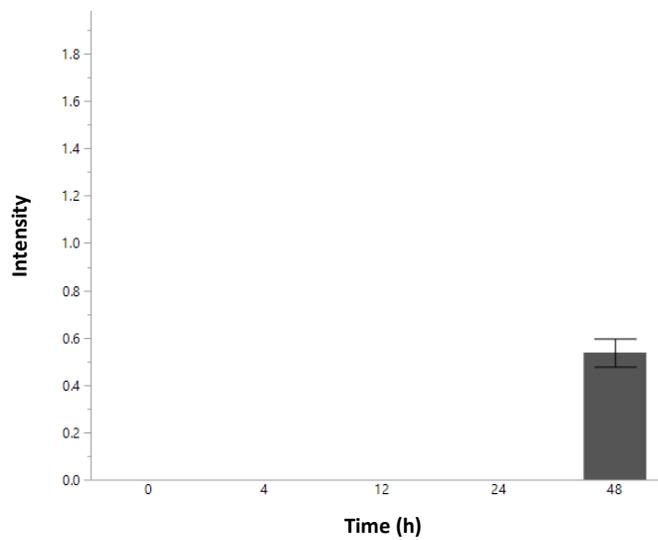
<sup>1</sup>ND = not detected.

<sup>2</sup>NQ = not quantified. Compounds were detected, but were measured below LOQ.



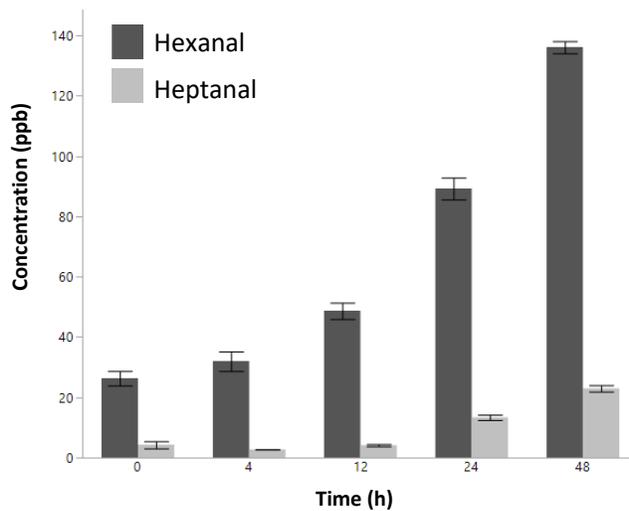
**Figure 3.1. Perfumey/Floral flavor in oil-based vitamin A fortified skim milks exposed to FL Light**

*Perfumey/floral flavor was scored on a 0 to 15 point intensity scale. Most fluid milk flavors fall between 0 and 4 on this scale (McCarthy et al., 2017b; Croissant et al., 2007). Standard error bars are included.*

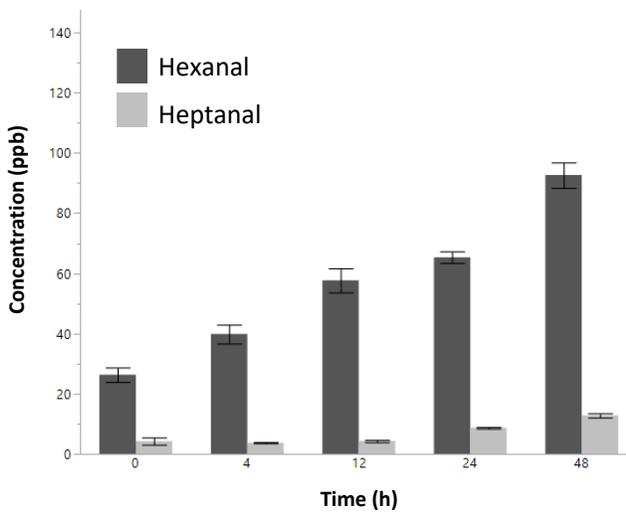


**Figure 3.2. Perfumey/floral flavor in oil-based vitamin A fortified skim milks exposed to LED Light**

*Perfumey/floral flavor was scored on a 0 to 15 point intensity scale. Most fluid milk flavors fall between 0 and 4 on this scale (McCarthy et al., 2017b; Croissant et al., 2007). Standard error bars are included.*

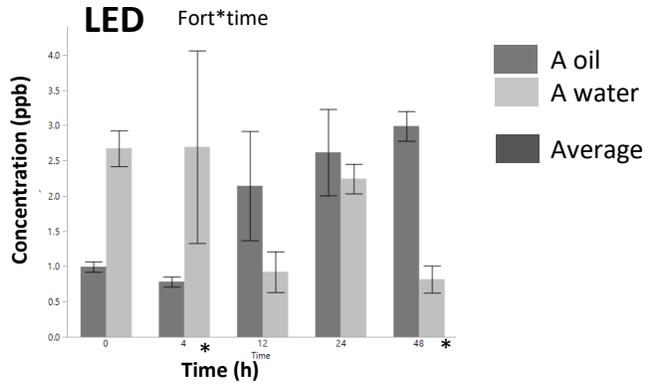
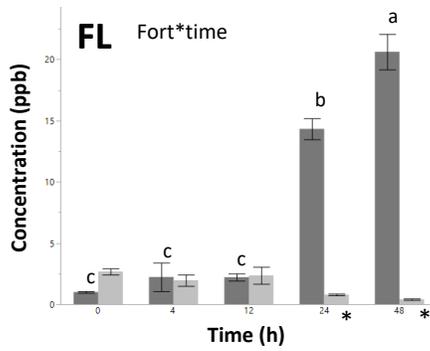


**Figure 3.3. Average hexanal and heptanal concentrations in skim milks exposed to FL light**  
*Standard error bars are included.*

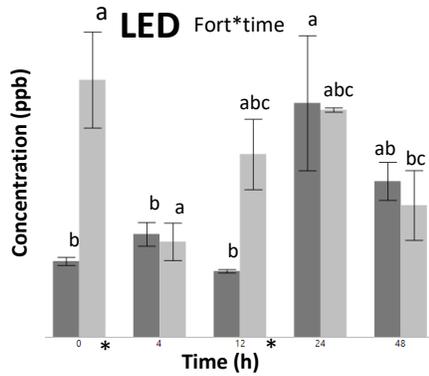
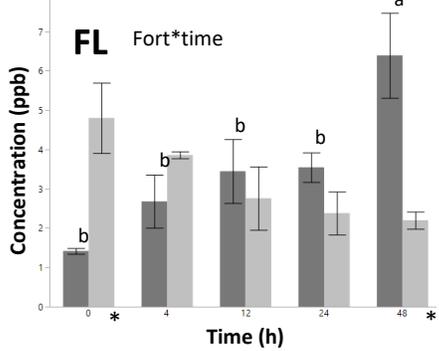


**Figure 3.4. Average hexanal and heptanal concentrations in skim milks exposed to LED light**  
*Standard error bars are included.*

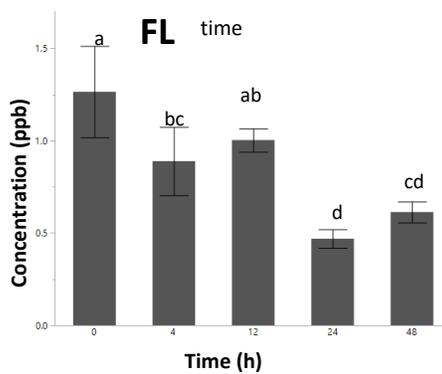
### Beta-Cyclocitral



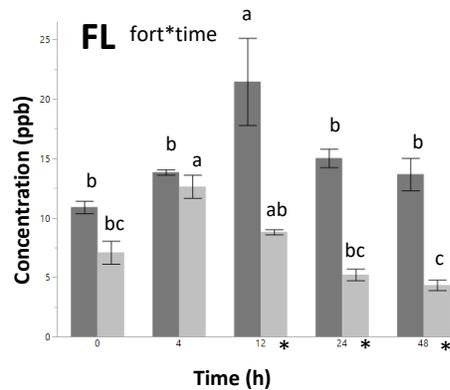
### Beta-Ionone



### Alpha-Ironone



### Beta-Damascone



\* indicates a significant difference between fortification types within each time point ( $p < 0.05$ ).  
 Times without a common letter within a fortification type are significantly different ( $p < 0.05$ ).  
 Times without letters indicate a fortification type not significantly impacted by time.  
 Standard error bars are included.

**Figure 3.5. Selected aroma-active vitamin degradation compounds in vitamin A fortified light-exposed skim milks**