ABSTRACT

YEH, EILEEN BARCELON. The Role of Vitamin Fortification on Flavor of Fluid Milk. (Under the direction of Dr. MaryAnne Drake).

Fluid milk consumption in the United States has continued to decline. As a result, the level of dietary vitamin D provided by fluid milk in the U.S. diet has also declined. Undesirable flavor(s)/off-flavor(s) in fluid milk can negatively impact milk consumption and consumer product acceptability. The objectives of this study were to identify aroma active compounds in vitamin concentrates used for fluid milk, and to determine the influence of vitamin A and D fortification on flavor of milk. The aroma profiles of fourteen (14) commercial vitamin concentrates (vitamins A and D), in both oil soluble and water dispersible forms, were evaluated by sensory and instrumental volatile compound analyses. Orthonasal thresholds were determined for 8 key aroma active compounds in skim and whole milk. Six (6) representative vitamin concentrates were selected to fortify skim and 2% fat high temperature short time (HTST) pasteurized milks (vitamin A at 1500-3000 IU/qt, vitamin D at 200-1200 IU/qt, vitamin A and D at 1000/200-6000/1200 IU/qt). Pasteurized milks were evaluated by sensory and instrumental volatile compound analyses and by consumers. Fat content, vitamin content and particle size were also analyzed. The entire experiment was replicated in duplicate. Water dispersible vitamin concentrates had overall higher aroma intensities and more odor active compounds detected than oil soluble vitamin concentrates. Trained panelists and consumers were able to detect flavor differences between vitamin A, and vitamin A and D water dispersible fortified skim milks compared to unfortified skim milks. Consumers were not able to detect flavor differences in oil soluble fortified milks but trained panelists documented faint carrot flavor in oil soluble fortified skim milks at the higher vitamin A concentrations (3000-6000 IU). No differences were detected in vitamin D fortified skim milks ($P > 0.05$) and no differences were detected in all 2% milks (P

> 0.05). These results demonstrate that vitamin concentrates may contribute to off flavor(s) in fluid milk, especially in skim milk fortified with water dispersible vitamin concentrates.

The Role of Vitamin Fortification on Flavor of Fluid Milk

by
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DEDICATION

To my parents for their unconditional love and support.

BIOGRAPHY

Eileen Yeh was born in Manila, Philippines on December 24, 1991 to David Yeh and Erlinda Yeh. She grew up in Taiwan and came to North Carolina at the age of 12. After high school she attended North Carolina State University to pursue a Bachelor's Degree in Food Science and Nutrition. She joined Dr. MaryAnne Drake's lab as a graduate student in May 2014 where she worked for two and a half years before completing her Master's Degree in December 2016.

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ABSTRACT

Vitamin concentrates with vitamins A and D are used for fortification of fluid milk. Although many of the degradation components of vitamins A and D have an important role in flavor/fragrance applications, they may also be source(s) of off-flavor(s) in vitamin fortified milk due to their heat, oxygen, and the light sensitivity. It is very important for the dairy industry to understand how vitamin concentrates can impact flavor and flavor stability of fluid milk. Currently, little research on vitamin degradation products can be found with respect to flavor contributions. In this review, the role of vitamin A and D fortification on flavor and stability of fluid milk are discussed.

INTRODUCTION

Current fluid white milk consumption in the United States has steadily declined in the last 50 years (International Dairy Foods Association, 2008). As a result, the level of dietary vitamin D provided by fluid milk has also declined. Increased fortification of vitamin D in fluid milk and fortification of a larger variety of products with vitamin D have been recommended (Calvo and Whiting, 2003; Dietary Guidelines Advisory Committee, 2015). Milk is commonly displayed under fluorescent or light-emitting diode (LED) lighting in retail dairy cases. Longer storage periods increase the risk of light exposure, which increase the risk of altering milk quality before purchase (Johnson et al., 2015). Both fluorescent and LED lighting sources deliver light energy in UV and visible wavelengths regions that cause light oxidation of photosensitive molecules such as riboflavin in milk. Light oxidation is the most common source of flavor and nutritional problems in milk. Another possible source of flavor variability in fluid milk is vitamin fortification.

Origin of Vitamin Fortification in Fluid Milk

Fortification is defined as the process of adding micronutrients such as essential vitamins to food (Alvarez, 2009). The fortification of food products has been practiced for more than 80 years. Vitamin deficiencies that lead to goiter, rickets, ariboflavinosis and pellagra in the United States are reduced by the consumption of foods fortified with iodine, vitamin D, vitamin B_2 and niacin, respectively (West et al. 2002; Bishai and Nalubola, 2002). Vitamin D fortification of milk and milk products began in the 1930s. This practice was recommended by the American Medical Association's Council on Foods and Nutrition to assist in reducing rickets in children (Stevenson, 1955). Various methods such as animal feed supplementation and direct irradiation were applied to increase vitamin D content in milk, but direct addition of vitamin concentrates still proved to be most reliable and has become the accepted industry practice (Roadhouse and Henderson, 1950). The wide acceptance of milk fortification with vitamin D led to the fortification of milk with vitamin A, which was initiated in 1940s (Public Health Service, 1940). The goal of fortifying milk with vitamin A was to ensure that Americans have a constant source of vitamin A since milk provided 10% of American consumers' food energy.

The Need for Vitamin Fortification

Vitamin D is essential for calcium absorption and is involve in the mineralization process required for bone growth. Deficiency of vitamin D causes rickets (softening of bones) in children and osteomalacia in adults (Ceglia, 2009). Recent studies also suggest that vitamin D plays a role in prevention of prostate, breast and colorectal cancers (Grant et al., 2007; Schwartz and Skinner, 2007; Garland et al., 2006; Bouillon et al., 2006). The major vitamin D forms responsible for human health benefits are ergocalciferol (D_2) (Figure 1) and cholecalciferol (D_3) (Figure 2). These vitamin D forms are considered to be inactive until they are converted to their biologically active form, 1,25-dihydroxy vitamin D_3, in the liver or kidney (Holick, 1995).

Vitamin D is an essential vitamin that is made when the body is exposed to sunlight (Holick, 1994). However, no photosynthesized vitamin D is produced in the skin for several months of the year for those who live in northern latitudes during winter, and supplementation of vitamin D is required to prevent deficiency (Calvo et al., 2004; Weaver and Fleet, 2004). The current recommended dietary allowance (RDA) of vitamin D is 600 IU per day for people between ages 1 to 70; and 800 IU per day for elderly over 70 years old (Institute of Medicine, 2011). This range assumes no vitamin D synthesis from sun exposure. The estimated average requirement is 400 IU (Institute of Medicine, 2011), and the tolerable upper limit (UL) intake is 4,000 IU per day for all ages (National Institute of Health, 2011).

Vitamin A is needed for normal growth, vision, reproduction, and differentiation of epithelial cells. Vitamin A deficiency results in night blindness, xerophthalmia (progressive blindness caused by drying of the cornea of the eye), keratinization (accumulation of keratin in digestive, respiratory and urinary-genital tract tissues) and finally exhaustion and death (Zile and Cullum, 1983). On the other hand, excessive intake of vitamin A is toxic. If intake of vitamin A exceeds 10,000 IU per day for adults (age 19 and over), symptoms such as drowsiness, irritability, headaches and vomiting can occur (Institute of Medicine, 2011). The current RDA of vitamin A is 2,333 IU per day for females (age 14 and over) and 3,000 for males (age 14 and over) (Institute of Medicine, 2011).

Vitamin intake through the diet is often not sufficient. Vitamin D_3 is found mainly in fish products and fish liver oils (Kutsky, 1981). However foods normally consumed by human are low in vitamin D content. Fish contains approximately 120 to 500 International units (IU) of vitamin D_3 per 3-oz serving, which is 50 to 200% of the recommended daily intake level as opposed to less than 25% in unfortified grains, meats, vegetables, and breakfast cereal (Holden, 2009). Unfortified whole milk and cheese provide only 1% of the daily value of vitamin D (Holden, 2009), which is insufficient for proper nutrition. Vitamin D_3 can also be produced synthetically by purification of 7-dehydrocholesterol from animal products and converted to vitamin D_3 by irradiation (Kutsky, 1981). This synthetic vitamin D_3 is added to many foods, particularly milk products.

Vitamin A is a group of compounds that includes retinoids and carotenoids (Zile and Cullum, 1983). Vitamin A from animal sources is already in a form of retinol that can be easily absorbed by the human body, whereas vitamin A from plant sources is a carotenoid that the body can transform into a retinol (Zile and Cullum, 1983). Vitamin A palmitate (Figure 3) is a form of vitamin A found naturally in animal sources and also produced synthetically. Whole milk, butter, cheese, and eggs are also important dietary sources of vitamin A palmitate (Zile and Cullum, 1983). Vitamin A fortification in reduced and fat free milks is required because whole milk contains some vitamin A palmitate, however vitamin A levels in reduced and fat free milks are much lower since fat soluble vitamin A palmitate is removed with fat. Therefore, fortification of vitamins in dairy products has been one of the approaches to address vitamin deficiencies (Parish and Richter, 1979).

Vitamin Regulations in Fluid Milk

In 1935, vitamin fortification of milk was addressed in the U.S. Public Health Service Milk Ordinance and Code, and in 1939, Vitamin D Milk was defined as milk in which the vitamin D content was increased by a method and in an amount approved by the regulating health official (Public Health Service, 1940). The Milk Ordinance and Code did not specify any fortification levels, but did recommend monitoring vitamin D concentrations by bioassays in a laboratory approved setting as required by the health officer. In 1953, the Milk Ordinance and Code established a level of at least 400 IU per quart (qt) for vitamin D milk fortification. This document also addressed the option of fortifying milk with other vitamins and minerals under "Fortified Milk and Milk Products", although no concentrations were specified for other nutrients. The Milk Ordinance and Code continued to be revised and became the Grade "A" Pasteurized Milk Ordinance (PMO) in 1965 (Public Health Service, 1965).

In the United States, vitamin D is generally added to fluid milk as synthetic vitamin D_3, whereas vitamin A is added as synthetic retinyl palmitate (Public Health Service, 1965). Retinyl palmitate is made from combining vitamin A acetate with methyl palmitate in the presence of sodium hydroxide (Budvari, 1996; Smith, 2016). Synthetic vitamin D_3 is made from irradiation of animal fat, usually from lanolin, the waxy secretions from sheep skin (Budvari, 1996; Holick, 1999; Smith, 2016). There are two different forms of vitamin premix: oil based and water dispersible formulations. These are available for the various dairy processing systems (van Deutekom, 2015). In general, oil based vitamin premix has to be added into the flow of milk after the separator. The water dispersible vitamin premix can be added to the flow of milk before the separator, or anywhere in the milk flow (van Deutekom, 2015). Water dispersible vitamins are not water soluble, only oil soluble. An emulsifier (polysorbate) is added into the vitamin premix to make it water dispersible. Water dispersible vitamin premix (water as the main ingredient) has a Specific Gravity >1.00 whereas oil based premix (oil as the main ingredient) has a Specific Gravity <1.00 (van

Deutekom, 2015). Most vitamin premix neither contains nor is manufactured from any genetically modified materials (van Deutekom, 2015). Oil-based vitamin premix with corn oil as the carrier may be manufactured with commodity corn oil and therefore cannot be certified as GMO free (van Deutekom, 2015). Vitamin premixes contain vitamin D_3 and/or vitamin A palmitate in a carrier generally consisting of a combination of any of the following: sunflower oil, corn oil, water, polysorbate 80, propylene glycol and glycerol monooleate. Antioxidants and/or preservatives may also be added (Murphy and Newcomer, 2001).

According to the U.S. Food and Drug Administration (FDA) regulations as specified in the PMO, the acceptable fortification concentrations were 80 to 120% of the label claims for both vitamins A and D. Over fortification of fluid milk with vitamin A and D can cause intoxication, soft tissue damage, and kidney failure (Blank et al., 1995; Jacobus et al., 1992). Based on FDA regulatory mandate, fluid milk products with levels of vitamin D over 800 IU and vitamin A over 6,000 IU in fluid milk are considered a public health threat and should be prohibited from sale and distribution. In 1978, the PMO required that each processor monitor vitamin concentrations in fortified products at least once a year in a FDA certified laboratory using standard test methods such as HPLC methods (Public Health Service, 1978).

New regulatory standards for vitamin fortification in the 1990s arose due to issues with variability in the vitamin content of retail milk. Excessive over fortification of milk with vitamin D in one processing plant resulted in human illness from consumption of these products (Jacobus et al., 1992). Several studies also found that numerous fortified milk products failed to meet label claims (Brown et al., 1992; Holick et al., 1992; Nichols, 1991). Murphy et al. (2001) found that 53-55% of fortified milk products were out of compliance with the label contents for vitamin A and for vitamin D. Milk fortification practices were not entirely consistent with the Nutritional Labeling and Education Act of 1990. As a result, the FDA revised the accepted fortification levels and mandated that vitamin concentrations for fortified fluid products must be within 100 to 150% of the label claims (Nichols, 1992), which equates to 400-600 IU per quart for vitamin D and 2000-3000 IU per quart for vitamin A (PMO 2013). If fluid milk products are found below 100% or above 150% of the required values or label claims, they should be resampled and the source of the problem determined (PMO 2013).

Fortification was not specified for low-fat or skim milks. Low-fat milk was first defined in the 1965 PMO as containing not less than 0.5% and not more than 2% fat. Skim milk was defined as milk with fat removed to a content of less than 0.5%. In the 2013 PMO, 21 CFR 130.10 states

> That nutrients must be added to the food to restore nutrient levels so that the product is not nutritionally inferior to the standardized food for products which combine a nutrient content claim, i.e., low-fat, non-fat, or reduced fat, with a standardized term, i.e., milk, sour cream, eggnog.

Therefore, vitamin A should be added to dairy products from which fat has been removed, in an amount necessary to replace the amount of these vitamins lost in the removal of fat. Unfortified whole milk is not considered to be a significant source of vitamin D since natural vitamin D concentrations in whole milk range from 0.34 IU to 0.84 IU per gram of fat (McBean and Speckmann, 1988). Thus removal of fat does not render milk nutritionally inferior for vitamin D, and therefore fortification with vitamin D is optional for all milks. If added, vitamin D must be present at 400 IU per quart (PMO 2013). Most fluid milk in the

United States is fortified with vitamin D due to the importance of vitamin D milk in human nutrition (Holick et al., 1992; McBean and Speckmann, 1988; Miller et al., 2000).

The fortification of milk with vitamin D almost eliminated the public health concern of rickets in the 19^{th} century. However, vitamin D deficiency has reemerged as a global health concern. Current low intakes of vitamin D and reduced time in sunlight exposure (increased time spent indoors) have resulted in inadequate vitamin D status (Dietary Guidelines Advisory Committee. 2015). There has been interest in fortifying dairy products with higher levels of vitamin D. Patterson et al. (2010) found that on average, vitamin D_3 in 2% milk was higher in 2007 compared with the vitamin D_3 levels in 2001, with a trend toward more samples of whole milk having greater than 150% of the labeled content. Hanson and Metzger (2010) reported that increasing the fortification of vitamin D from 100 to 250 IU per serving was stable over the shelf lives of HTST-processed 2% milk, UHT-processed 2% chocolate milk and low-fat yogurt, and no change in sensory characteristics was found in these products. They concluded that increasing the fortification of vitamin D in milk was a feasible strategy to increase vitamin D supplementation.

Fortifying products with higher amounts of calcium or vitamin D is permitted by FDA provided that the product and any label claims comply with FDA regulations including standards of identity (U.S. Food and Drug Administration). The addition of vitamin D to milk and yogurt is optional. Unfortified cheese and cultured dairy products are not considered good sources of vitamin D. If added, the minimum amount of vitamin D in each quart of the product must not be less than 400 IU (PMO 2013). The acceptable range for vitamin D is 400 IU to 600 IU per quart of milk (PMO 2013). For cheese, the FDA authorized the use of vitamin D_3 at levels up to 81 IU per 30 grams in cheese and cheese products that have a reference amount of 30 grams. This does not include cottage cheese, ricotta cheese, and hard grating cheese, such as Parmesan and Romano.

Vitamin A can be found in significant amounts in unfortified whole milk since it is primarily associated with the fat phase of the milk at 37.7 IU per gram of fat (McBean and Speckmann, 1988). However, milk fat removal results in vitamin A reduction in low-fat and skim milks. As demand for low-fat and skim milk products increased in the United States, there was a nutritional concern for reduction of vitamin A present in these products. This concern was addressed in the 1978 PMO (Public Health Service, 1978), which required low-fat and skim milks to be fortified with vitamin A to the nutritional equivalence of the general milk standard-containing not less than 2000 IU per quart. Vitamin A fortification is optional for whole milk, but if added, the concentration must not be less than 2000 IU per quart.

In order to ensure proper fortification of dairy products, it is important to effectively monitor product concentrations. The latest revision of the PMO (2013) mandated that each manufacturer have representative milk products assayed at least annually in a laboratory certified by the FDA with methods that are acceptable to the FDA. Currently HPLC methods are the testing procedures of choice for vitamins A and D in milk. FDA is involved in the standardization of testing procedures among laboratories and has implemented a certification program for vitamin testing laboratories. Current listings of certified laboratories can be found in the IMS Quarterly Report. Samples for vitamin testing should be sent to an approved laboratory within a few days of processing, kept cold (≤4°C) without freezing and protected from light. Once received at the laboratory, analyses should begin as soon as possible.

Fluid Milk Processing

Milk handling at the farm contributes to microbiological contamination from equipment and storage tanks used (Polyanskii et al., 2005; Hutchinson et al., 2005). If raw milk is held too long or at temperatures higher than 4°C prior to pasteurization, milk will develop off-flavors from the growth and metabolism of various microbial contaminants (Walker, 1988; Buchrieser and Kasper, 1993; Tetra Pak, 2003)

Before milk pasteurization was introduced in the mid-19th century, milk was a source of infection, as it is a perfect growth medium for micro-organisms. Thus, milk pasteurization is widely practiced in order to destroy the most heat-resistant non-spore-forming human pathogens likely to be present in raw milk. Pasteurization is a process invented by the French scientist Louis Pasteur, who applied heat to destroy pathogens in food. For dairy products, pasteurization is the process of heating milk to a specific temperature at the corresponding specific time without affecting the physical and chemical properties of the milk. The original method of pasteurization was vat pasteurization, which heats milk to 63°C in a large tank for at least 30 minutes. It is currently used primarily for preparing milk for making starter cultures in the processing of cheese, yogurt and for pasteurizing ice cream mixes (Tetra Pak, 2003).

The most common method of pasteurization in the United States today is High Temperature Short Time (HTST) pasteurization, which uses metal plates and hot water to heat milk to at least 72°C for not less than 15 seconds, followed by rapid cooling. Higher Heat Shorter Time (HHST) is a process similar to HTST pasteurization, but it uses slightly different equipment and higher temperatures for a shorter time (83°C for 15 seconds). For Ultra Pasteurization (UP), product is heated to not less than 138°C for two seconds. UP products result in longer shelf life but still requires refrigeration. Ultra High Temperature (UHT) aseptic processing involves heating the milk with commercially sterile equipment and filling it under aseptic conditions into hermetically sealed packaging. The resulting products are shelf stable and do not require refrigeration until opened (PMO, 2013).

Processing of milk products requires gentle handling and correct designed process line to ensure good milk quality. Figure 4 shows a typical process flow chart in a fluid milk processing plant.

Chilled raw milk enters the pasteurizer via a balance tank and is pumped to a plate heat exchanger where it is pre-heated before it continues to a separator, which produces skim milk and cream. Fat separation works by the influence of centrifugal force, which the fat globules (cream) are less dense than the skim milk, move inwards through the separation channels toward the axis of rotation. The skim milk then move outwards and leaves through a separate outlet. Standardization then takes place in an in-line system where fat content of the cream is set to the required level and is kept constant by the control system. The control system consists of a density transmitter, flow transmitter, and regulating valves (Tetra Pak, 2003).

After standardization, the cream is divided into two streams. One stream is routed to the homogenizer while the surplus cream is passed to the cream treatment plant. The original cream with 40% fat content is diluted with skim milk prior to homogenization, thus the final fat content of the cream to be homogenized should be a maximum of 18%. Following homogenization, the 18% cream is mixed in-line with the surplus volume of skim milk to achieve 3% before pasteurization. The standardized milk is then pumped to the heating section of the heat exchanger where it is pasteurized, and temperature is recorded continuously. If the pasteurization temperature drops, a temperature transmitter activates the flow diversion valve which brings the milk back to the balance tank to be reheated (Tetra Pak, 2003). After pasteurization, milk continues to a cooling section in the heat exchanger. The cold milk is then pumped to the filling machines for packaging, storing and product distribution (Tetra Pak, 2003).

Accurate pasteurization procedure and correct cooling are essential processes in the treatment of milk for longer shelf life. Temperature and pasteurization time must be specified precisely in relation to PMO requirements. Practically all milk marketed in the United States is both pasteurized and homogenized. The 2013 PMO defines "Homogenized" as follows:

> The term "homogenized" means that milk or the milk product has been treated to insure breakup of the fat globules to such an extent that, after 48 hours of quiescent storage at 7°C (45°F), no visible cream separation occurs on the milk; and the fat percentage of the top 100 milliliters of milk in a quart, or of proportionate volumes in containers of other sizes, does not differ by more than 10% from the fat percentage of the remaining milk as determined after thorough mixing.

The fat globule size before homogenization is 1 to 10 microns and after homogenization is 0.2 to 2 microns. Typically 90% of fat globules need to be less than 1.5 microns to achieve proper stability during storage (Walstra and Jenness, 1984). The homogenization process involves forcing milk through a small cavity at a high pressure, usually at about 60°C when the fat is liquid and lipase inactivated. Homogenization is less effective at lower temperatures when the fat is partially solid. Usually the fat globules occur in clumps after just a single stage of homogenization, due to fragments of whey and casein in the walls of fat globules, some are surrounded by a layer of protein. A two-stage homogenization system disperses the clumps and reduces the viscosity (Tetra Pak, 2003). Homogenization temperatures normally applied for fluid milk are 55°C to 70°C, and homogenization pressures is 500 psi for second stage and up to 2000 psi total for the first stage (Tetra Pak, 2003).

Vitamin Processing in Fluid Milk

Vitamins can be added into the pasteurizing vat, the HTST constant-level tank, or on a continuous basis into the pipeline after standardization. The addition of vitamins usually occur after separation and standardization, and before pasteurization. Homogenization will then take place after pasteurization to allow the vitamins to be distributed evenly throughout the milk. Two vitamin addition procedures can be used: the batch addition and addition with metering pumps. The batch procedure requires accurate measurement of the fortified milk volume, accurate measurement of the vitamin concentrate, and proper mixing. The metering pump procedure requires the pumps in the HTST unit to be activated only when the unit is in forward-flow (PMO 2013).

Under or over fortification can occur when vitamins are added before separation and standardization, resulting in low fat product being under fortified and high fat product being over fortified. This occurs because vitamin A and D are fat-soluble, they will gradually become more concentrated in the milk fat portion of the milk. Therefore, it is recommended to add the vitamins after separation and standardization (PMO 2013).

Many HTST systems now have in-line fat standardization, which allows continuous switching from milk being fortified with vitamin D to milk being fortified with both vitamin A and D (Figure 5). Figure 5 shows a two speed vitamin fortification installation using two pumps and two vitamin concentrate sources. This allows changing from different vitamin concentrates and different speed pumps via the adjustment of three-way valves. Separate pumps, tubing and check-valves are used when multiple types of vitamin concentrates are injected, and prevent milk from contaminating the vitamin concentrate.

Methods of Vitamins A and D Analysis

A list of the official AOAC, CEN, and ISO methods available for determining fat soluble vitamins has been reported (Blake, 2007). These procedures involve mostly liquid chromatography (LC), but also include spectrophotometric and gas chromatographic (GC) techniques. In the various official procedures for fat soluble vitamins, extractions are usually made either by saponification or by direct solvent extraction. Saponification removes the fat portion of milk and facilitates extraction by releasing carotenoids, retinoids, tocopherols and vitamin D compounds from the matrix. Saponification is generally performed with the addition of antioxidants such as ascorbic acid, pyrogallol, butylated hydroxyl toluene (BHT), or hydroquinone to reduce oxidation losses, along with nitrogen flushing (Blake, 2007). After saponification, extraction takes place with organic solvents such as hexane, petroleum ether, ethyl ether or mixtures of these substances (Table 1). Vitamin D can also be directly extracted with organic solvents without saponification process. These organic solvents are methyl dichloride alone, or mixed with methanol and hexane alone, or mixed with ethyl ether, or chloroform (Kazmi et al., 2007). However, vitamin D recoveries from direct extraction were lower than those obtained by saponification (Delgado et al., 1992; Hagar et al., 1994). Vitamin D_3 is insoluble in water, but soluble in 95% ethanol, acetone, fats and oils, and readily soluble in chloroform and ether. Retinyl palmitate is also insoluble in water, but soluble in chloroform, ether, and vegetable oil (corn oil), and slightly soluble in alcohol.

For over 20 years, high performance liquid chromatography (HPLC) has been the method of choice for the determination of total fat-soluble vitamins such as vitamins A and D. However, HPLC methods raise environmental and economic concerns due to the large amount of organic solvents used. Thus the continued search to improve HPLC further have led to ultra-high performance liquid chromatography (UHPLC). In comparison with HPLC, UHPLC uses narrow bore, shorter columns, less run time, lower flow rate, lower injection volume and solvent volume/sample, smaller particle size, and much higher back pressure (Bohoyo-Gil et al., 2012; Rivera and Canela-Garayoa, 2012). Thus the UHPLC method has several advantages over conventional chromatography, which include faster analyses due to shorter retention times, narrower peaks giving increased signal-to-noise ratio, higher resolution and sensitivity (Rivera and Canela-Garayoa, 2012). UHPLC also saves at least 80% of mobile phase compared to HPLC (Chen and Kord, 2009). Similar to the HPLC methods, UHPLC methods also have to undergo special attention to method standardization, validation, sampling, and sample preparation to guarantee data reliability, since the smaller the analytical sample, the more difficult it is to guarantee a representative sample. In milk, the UHPLC method provided similar concentrations of vitamin A to that obtained by an HPLC method (Chauveau-Duriot et al. 2010).

Numerous methods have been reported for analysis of vitamins A and D individually in foods (Staffas and Nyman, 2003; Hite, 2003; Chase et al., 2004; Thompson et al., 2010; Hooir et al., 2004; Perales et al., 2005). A number of studies have investigated the feasibility of simultaneous analysis of multiple fat-soluble vitamins in one LC-based assay (Gomis et al., 2000; Heudi et al., 2004; Chavez-Servin et al., 2006). Currently, an HPLC method for simultaneous determination of vitamins A and D_3 in fluid milk is developed and validated

(Chen and Reddy, 2015). Table 1 provides a summary of vitamin analysis methods in different food matrices. All the methods are quite similar. In general, samples are saponified to hydrolyze the lipids, vitamins are extracted and separated using analytical reversed-phase chromatography with photo-diode array detection. Variations arise from the different extraction solvents used such as either hexane or ether/petroleum ether.

In all of these procedures, it is important to avoid vitamin losses due to light oxidation. Significant errors may result from poor use of glassware in vitamins analysis. Therefore, the use of fluorescent lighting fitted with an appropriate UV filter in the laboratory is recommended. Low-actinic glassware (amber colored glassware which is used to protect contents from light) can also be used to reduce vitamin losses (Castanheira et al., 2006).

Photooxidation of Fluid Milk

Photooxidation of milk occurs under the presence of both artificial light and sunlight (Webster et al., 2009). This process can occur very quickly in milk and can influence consumer perception of milk flavor (Fellman et al., 1991; Heer et al., 1995; Chapman, 2002; Chapman et al., 2002; Duncan and Webster, 2010; Walsh et al., 2015). Fellman et al. (1991) reported that when both 2% and skim milk samples were exposed to light, the samples received lowered preference scores compared to non-exposed control samples. Additionally, when both fortified and non-fortified 2% and skim milk samples were tasted by 10-15 trained panelists after exposure to light, the flavor was not significantly different, but were in the unacceptable range (Fellman et al., 1991). Chapmen et al. (2002) reported that untrained consumers detected light-oxidized flavor defects between 54 minutes and 2 hours of fluorescent light exposure of milks. Walsh et al. (2015) reported that light-exposed milk was rated lower in acceptability than milk that did not receive light-exposure. Additionally, the experience of drinking high-quality milk without light-oxidized flavor is characterized by positive emotional terms such as content, good, calm, satisfied, pleased, and happy. In contrast, experience of drinking light-exposed milk is characterized by negative emotional terms such as disgusted and worried (Walsh et al., 2015). These studies illustrate that light exposure is of particular concern in milk and it can be detrimental to consumer product acceptability.

Light-oxidized flavor is defined as off-flavor compounds produced in milk as a result of light exposure. These off-flavor compounds include methional, mercaptan, dimethyl sulfide, disulfides, methanethiol, methionine sulfoxide, and sulfur compounds (Forss, 1979). There are two types of photooxidation reactions that cause light-oxidized flavor defects (Hansen et al., 1975). One type involves proteins and amino acids that occurs rapidly within minutes, and result in flavors that are characterized as burnt protein and burnt cabbage. The other type occurs more slowly and involves oxidation of unsaturated fatty acids. Resulting flavors are perceived as similar to old vegetable oil, cardboard flavor (Heer et al., 1995).

Dimethyl disulfide was mainly responsible for the light-induced off-flavor of milk. This compound was formed by the singlet oxygen oxidation of methionine in milk (Jung et al., 1998). Light acts as a pro-oxidant by producing free radicals which can react with unsaturated fatty acids. Oxygen then readily reacts with the lipid radicals to form hydroperoxides. Low molecular weight aldehydes and ketones are then formed upon cleavage of fatty acids (Nawar, 1985; Saxby, 1993).

Vitamin Destruction by Photooxidation

The light visible spectrum is the portion of the range of all possible frequencies of electromagnetic radiation that is visible to the human eye. The human eye can typically respond to wavelengths from 390 nm to 700 nm (Starr, 2005). Light exposure at wavelengths below 500 nm causes the destruction of light-sensitive vitamins such as riboflavin, vitamin A and vitamin C, and induces chemical reactions that affect milk proteins and lipids, and results in the development of unpleasant flavors in foods (Fanelli et al., 1985; Sattar et al., 1977; Schroder et al., 1985). Dairy products are very sensitive to light oxidation because of the presence of riboflavin (vitamin B_2). Milk contains large amounts of riboflavin, a water-soluble vitamin with an average concentration between 1.36 and 1.75 mg/L (Dimick 1982; Zygoura 2004). Riboflavin is a vitamin in which only slight changes can be made in structure without loss of vitamin activity. Light oxidation dramatically alters the structure of riboflavin and reduces its level in milk (Bekbolet 1990). Riboflavin is the most studied photosensitizer in milk (Sattar et al., 1977; Webster et al., 2009), and this strong photosensitizer is able to absorb visible and UV light and transfer this energy into highly reactive forms of oxygen known as singlet oxygen (Figure 6) (Boff and Min 2002). Figure 6 shows light energy reacting with riboflavin creating sensitized riboflavin (*Riboflavin), which reacts with triplet oxygen ($^3O_2$), creating singlet oxygen ($^1O_2$). Singlet oxygen is highly reactive and can react with a material, resulting in its degeneration, and is capable of oxidizing phenols, sulfides, and amines.

Excitation of riboflavin occurs when exposed to light at 250, 270, 370, 400, 446, and 570 nm wavelengths (Kyte, 1995). The cascade of oxidation reactions lead to significant losses of valuable vitamins (vitamin A, B_2, C, D and E) and amino acids, and also leads to lipid oxidation and formation of strong off-flavors (Borle et al., 2001). As a result, off-flavors may be linked to the decrease in nutritional value of milk. Therefore, light protection of photosensitive molecules in milk is needed to protect milk flavor and nutrient quality.

Protection from Light Exposure

It is essential to protect milk with light-protective packaging since both added vitamins and naturally presented vitamins (such as riboflavin) in milk are destroyed rapidly when the milk is exposed to light (Cadwallader and Howard, 1998). In the United States, milk is commonly packaged in waxed paperboard, high-density polyethylene (HDPE) or polyethylene terephthalate (PET). The HDPE transmits 57% to 60% of light wavelengths between 300nm and 700 nm (van Aardt et al., 2005). The packaging material frequently used for single-serve milk products, PET, transmits up to 75% to 85% of visible light. Packaging material can have a protective effect on milk quality through blocking or reducing the transmission of certain light wavelengths (Webster et al., 2009). Therefore, it is important to develop packaging materials that are consumer friendly but also block the most damaging wavelengths to milk quality.

Webster et al. (2009) reported a loss of total riboflavin between 52.6% and 67.5% in 2% milk in light-exposed and specialized film packaging treatments within 3 days of refrigerated storage; and 95% to 98% of the riboflavin was degraded at the end of the study (21 days). Johnson et al. (2015) studied the effectiveness of titanium dioxide (TiO_2)-loaded high-density polyethylene (HDPE) to reduce light-induced oxidation of extended-shelf life milk. They found that riboflavin concentration decreased 10.5% in the light-protected control and 28.5% in the high TiO_2 packaged milk past 29 days of light exposure, but losses were greater than 40% for all other packages. The rate of vitamin degradation caused by photooxidation can also be greatly reduced by using amber or brown glass bottles, pigmented plastic containers formulated with specific light barriers and colored paper cartons. Allen and Parks (1979) found that riboflavin in whole milk was reduced by 75% when exposed to 32 hours of fluorescent light (2690 lux), whereas Webster et al. (2009) showed a riboflavin loss between 52.6% and 67.5% after 72 hours of exposure in bottles wrapped with protective films. Hoskin (1988) also suggested that shielding a major portion of the main body of the bottle with a material that blocks the damaging wavelengths of light can reduce light oxidation. In another study, Cladman et al. (1998) covered approximately 55% of the surface of the bottle with translucent labels, and the labels reduced visible light transmission by 75%. Even after 6 days of storage, the level of lipid oxidation in the milk in the labelled bottles was 40-50% lower than in the unlabeled bottles and the vitamin A content was 20-30% higher in the labelled bottles compared to unlabeled bottles.

Dunkley et al. (1962) measured relative transmission of fluorescent light through various packaging materials. Clear glass transmitted 13 times more light between 400 and 600 nm than an uncolored fiberboard carton. They also found that milk cartons designed with large areas printed with inks of yellow, red, orange and brown do not transmit light at the critical wavelengths. In another study, a comparison of uncolored and 8 solid colored paperboard cartons demonstrated that the darker colors offered significantly more protection from light exposure when compared to lighter colors such as uncolored and yellow (Coleman, 1975). Thompson and Erdody (1974) also demonstrated that vitamin A in fortified low fat milk was rapidly destroyed after light exposure. Losses up to 60% vitamin occurred in milk in glass following 200-ft c fluorescent light exposure for three hours. The use of light-protective packaging is a major approach to protect sensory quality and nutritional values of fluid milk beverages.

Another approach in controlling or preventing light oxidation in milk involves the use of antioxidants. Antioxidants such as tocopherols, ascorbyl palmitate, and ascorbic acid help control oxidation in dairy products. Raw milk naturally contains alpha-tocopherol and ascorbic acid at approximately 13-30 mg/kg and less than 20 mg/kg respectively (Rosenthal et al., 1993; van Aardt et al., 2005). van Aardt et al. (2005) found that the addition of alpha-tocopherols and ascorbic acid both at levels of 0.025% resulted in limited light-induced flavor in milk after 10 hours of light exposure compared to controls with no added antioxidants. Metal chelators in milk, such as the milk protein lactoferrin, also serve to provide protection against oxidation (Let et al., 2006).

Studies have shown that whole milk products have slower rates of vitamin A losses compared to low-fat or skim milk products (Senyk and Shipe, 1981; deMan, 1981; Gaylord et al., 1986; Whited et al., 2002). Reduced vitamin A degradation in higher fat samples is likely due to greater light scattering by higher number of fat globules. Senyk and Shipe (1981) indicated that light at wavelengths of 400-500 nm penetrated 40-50% deeper into skim milk than into whole milk. Measureable vitamin A losses occurred at 2, 4, and 16 hours at 2000 1x fluorescent light for nonfat, reduced fat and whole milk, respectively (Whited et al., 2002). Although the presence of milk fat appears to slow down vitamin A degradation in fluid products, it dramatically reduced the flavor quality of milk after exposure to light. Moderate light-oxidized flavors were detected by 17 trained panelists after 4 hours of light exposure at 2000 1x in whole and reduced fat milk and after 8 hours in nonfat milk. By 16 hours at 2000 1x, relative light-oxidized flavor development was lower in nonfat milk than in whole or reduced fat milk (Whited et al., 2002).

Natural vitamin A in whole milk was more stable to light than added vitamin A due to natural vitamin A is found in milk fat globules whereas added retinyl palmitate is dispersed in the water phase of milk, which could be more prone to oxidation due to greater contact with oxygen (Thompson and Erdody, 1974). Vitamin D loss occurred at a rate much slower than other vitamins in milk. Using the same system of exposing milk samples in test tubes at the same light intensity of 300 ft-c, Gaylord et al. (1986) reported the rate constant for riboflavin loss was 0.0616 per hour and retinyl palmitate was 0.0298 per hour, whereas Renken and Warthesen (1993) reported the rate constant for vitamin D loss was 0.0009 per hour. Chocolate milk products also have reduced vitamin A degradation, either due to protection by carrageenan alone or in combination with chocolate color and/or flavor. Vitamin A protection in chocolate milk is due to increased light scattering by the additional particles. Chocolate flavor components of chocolate milk can also reduce development of light-oxidized off-flavors (Chapman et al., 1998). These findings demonstrate that exposure of fluid milk to light can adversely affect both flavor quality and nutritional value of fluid milk products.

Effect of Vitamin Addition on Fluid Milk Flavor

Proteins, lipids and carbohydrates are the precursors of aroma compounds that contribute to milk flavor. There are many factors that can influence milk flavor, including milk handling, processing and storing (Strobel et al., 1953; Nursten, 1997). Other factors that may contribute to off-flavors in milk are bacterial growth, chemical composition of milk, chemical changes, and addition of foreign material (Bodyfelt et al., 1988). Pasteurized milk typically results in cooked flavor, especially immediately after processing, but the intensity of the cooked flavor diminishes during storage (Badings et al., 1981; Boelrijk et al., 2003). A few studies have suggested that added vitamin A concentrate imparted a detectable off-flavor, particularly in skim and low-fat milk, and occasionally in whole milk products (Weckel and Chicoye, 1954; Whited et al., 2002). Consumers have reported that vitamin A fortified milk has an oily, haylike flavor (Fellman et al., 1991).

Effect of Vitamin Addition on Flavor of other Dairy Products

A few studies have reported no significant effect of added vitamins on flavor perception of other dairy products. Banville et al. (2000) and Upreti et al. (2002) reported that the flavor perception of vitamin D fortified Cheddar cheese was similar to that of unfortified cheese flavor at two months of aging. Ganesan et al. (2011) found no alteration in cheese flavor with vitamin D_3 addition even after aging for nine months. No effect on the sensory characteristics of HTST-processed 2% milk, UHT-processed 2% chocolate milk and low-fat strawberry yogurt was found by vitamin D fortification from 100 to 250 IU per serving (Hanson and Metzger, 2010).

Vitamin Stability and Storage

Several studies have been conducted on the stability of vitamin D in milk and other dairy products (Banville et al., 2000; Kazmi et al., 2007; Wagner et al., 2008; Hanson and Metzger, 2010; Tippetts et al., 2012). These studies have all indicated that vitamin D is stable during processing and storage. Vitamin D in fortified homogenized whole milk is very stable and is not affected by pasteurization or other processing procedures (PMO, 2013). Vitamin D_3 appears to be stable in cheese during both short-term (Banville et al., 2000) and long-term storage (Kazmi et al., 2007; Wagner et al., 2008). Vitamin D was also stable over the shelf life of HTST-processed 2% fat milk, UHT-processed 2% fat chocolate milk and low-fat strawberry yogurt (Hanson and Metzger, 2010). Tippetts et al. (2012) suggested incorporation of vitamin D_3 as an emulsion using milk proteins as emulsifier to improve retention of vitamin D_3 in the cheese curd. Vitamin D_3 is also stable in yogurt and ice cream stored for 4 weeks, with high retention of 95% to 100% and 98% to 100% respectively (Kazmi et al., 2007).

Vitamin D itself is susceptible to degradation by oxygen and light once freed from the protection of food matrix. Disagreement with respect to vitamin D stability of other environmental factors has been reported. Cremin and Power (1985) stated that vitamin D was unstable to oxidation, light, and acid. Kutsky (1981) reported that vitamin D was unstable to oxidation and light but stable to acid and alkali, whereas Kreutler (1980) reported that vitamin D was remarkably stable to light, heat, and oxygen, these factors did not affect its activity.

As stated previously, vitamin A is generally added to fluid milk as retinyl palmitate (Public Health Service, 1965). Retinyl palmitate is the ester of retinol and palmitic acid. The stability of added retinyl palmitate may be affected by heat, light or the presence of acids which may cause degradation or isomerization to cis-isomers, resulting in lowered biological activity (Mousseron-Cadet, 1971). Ultraviolet light causes isomerization and degradation of retinoid compounds in solution. Under more intense light, other transformations can take place such as dimerization or chemical reaction between two monomers of retinyl esters (Mousseron-Cadet, 1971). Despite reported vitamin A off-flavor in fluid milk products, degradation products of vitamin A palmitate in fluid milk have not been previously reported in the literature. However in nonfat dry milk, a distinct hay-like flavor was detected from oxidation products of vitamin A palmitate, the major oxidation products found and attributed to the off flavor were B-ionone (Figure 7) and dihydroactinidiolide (Suyama et al. 1983). In another study conducted with corn flakes fortified with vitamin A palmitate, the loss of vitamin A was reduced in the presence of other vitamins including B_1, B_6, B_{12}, C and D (Kim et al. 2000). Different forms of vitamin premix also have an effect on vitamin A stability when exposed to light. Triangle test results indicated that light-induced off flavor could be distinguished in skim milks with water based vitamin A (2000 IU) after 6 hours of light exposure, while off flavor in skim milks with oil based vitamin A (2000 IU) could not be distinguish until after 24 hours of light exposure (Fellman et al., 1991). Furthermore, oil matrices have been shown to have protective effects on vitamin A stability by delaying its oxidative degradation (Dary and Mora, 2002).

In general, vitamin A has been found to be stable in most fortified foods. Hartman and Dryden (1974) reported that procedures such as pasteurization, sterilization, spray and roller drying or evaporation caused little loss of vitamin A in milk products. However, prolonged heating of milk, butter or butterfat at high temperatures in the presence of oxygen can decrease vitamin A activity.

It is also important to note that vitamin concentrate potency will degrade with time. Therefore concentrates should be stored in accordance with manufacturer's recommendation to maintain label potency (PMO, 2013). According to Sensory Effects and International Food Products (IFP), two commercial suppliers, vitamin premix product should be stored at room temperature (10 C to 27 C) in a dry, dark place. Product should be used within 1 year from date of manufacture. IFP does not make any natural claims for their vitamin premix products (van Deutekom, 2015). Sensory Effects has an organic label claim on some of their vitamin premix products that contain sunflower oil as the vitamin carrier (Payne, 2015).

CONCLUSION

Off-flavors in fluid milk can negatively impact milk consumption and consumer product acceptability. Possible sources of off-flavor in fluid milk are thermal degradation, microbial contamination, and exposure to light. The most common source of flavor problems encountered in milk is exposure to light. Milk exposure to light can result in vitamin destruction. Another possible source of off-flavor in fluid milk is vitamin fortification. Several studies have addressed the influence of light oxidation on fluid milk flavor and stability, and the effect of light exposure on vitamin stability. Few studies have addressed the effect of vitamin addition on flavor of fluid milk. The objective of this research is to evaluate the role of vitamin A and D fortification on the flavor of milk. This study will determine if vitamin premix increases off flavors in fluid milk and/or contributes off flavor(s) to fluid milk.

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TABLES

Table 1. Vitamin analysis methods

Author name	Extraction method	Detection method	Product evaluated	Compounds separated	Sensitivity
Salo-Vaananen et al. 2000 (Food Chemistry)	saponification follow by extraction with hexane-ethyl acetate (8:2), stream N2	HPLC-UV	Fluid milk and fish	Cholecalciferol (vitamin D3), tocopherols, beta-carotene, all-trans-retinol	Detection limits: 0.003 ug/100ml for vitamin D3, <1 ug/100ml for beta-carotene, and 2 ug/100ml for tocopherols and all-trans-retinol
Upreti et al. 2002 (Journal of Dairy Science)	Saponification followed by extraction with petroleum ether/diethyl ether, stream N2	HPLC-UV	Processed cheese	Cholecalciferol (vitamin D3)	Vitamin D3 content of 2.5 ug/100ml for processed cheese
Staffas and Nyman 2003 (Journal of AOAC International)	saponification followed by extraction with heptane, stream N2	HPLC-UV	Milk, liquid infant formula, cooking oil, margarine, infant formula, fish oil	Cholecalciferol (vitamin D3)	Vitamin D3 content reported in milk: 0.4-0.418 ug/100ml

Table 1 Continued

Kazmi et al. 2007 (International Dairy Journal)	Direct extraction with methanol/chloroform, stream N2	HPLC-UV	Cheese, yogurt, ice cream	Cholecalciferol (vitamin D3)	95-97% recovery for cheese, 99-102% for yogurt, 98-99% for ice cream
Phillips et al. 2008 (Journal of Food Composition and Analysis)	Extraction with ethyl ether/petroleum ether and separatory funnel, stream N2	HPLC-UV and MS	Canned salmon, vitamin D3 fortified skim milk, orange juice, ready-to-eat breakfast cereal, processed cheese	Cholecalciferol (vitamin D3)	Vitamin D content reported in skim milk: 1.03 ug/100ml - 1.15 ug/100ml
Byrdwell 2009 (Journal of Agricultural and Food Chemistry)	Extraction with ethyl ether/petroleum ether and separatory funnel, stream N2	HPLC-UV and MS	Skim milk, orange juice, cereal, salmon, spiked peanut oil, processed cheese	Cholecalciferol (vitamin D3)	Vitamin D content reported in skim milk: 1.08 ug/100ml - 1.14 ug/100ml
Chen and Reddy 2015 (Journal of AOAC International)	Saponification followed by extraction with diethyl ether in hexane and separating funnel, stream N2	HPLC-UV	Skim, 2% fat and whole milk	All-trans-retinyl palmitate and cholecalciferol (vitamin D3)	Detection limits: 0.0022 ug/ml for vitamin A and 0.0008 ug/ml for vitamin D3

Table 1 Continued

Chauveau-Duriot et al. 2010 (Analytical Bioanal Chem)	saponification follow by extraction with hexane-ethyl acetate (8:2), stream N2	Comparison between HPLC & UPLC	Forages, bovine plasma, and milk	Carotenoids, retinol, tocopherols	Sensitivity was higher for UPLC than for HPLC (i.e. for retinol: 0.8 and 1.4 ng per injection vs. 1.3 and 2.0 ng per injection, respectively)
AOAC Official method 2002.06	Extraction with hexane, stream N2	LC-UV	Skim, 1% fat milk, 2% fat milk and 1% fat chocolate milk	All-trans-retinyl palmitate	
AOAC Official method 2002.05	Saponification followed by extraction with heptane and separatory funnel, stream N2	LC-UV	Fluid milk, infant formula, gruel, margarine, cooking oil and fish oil	Cholecalciferol (vitamin D3)	

Table 2 Vitamin losses from light exposure (modified from Tetra Pak, 2003)

Losses of vitamins at an exposure of 1,500 Lux

	Opaque Carton		Transparent glass bottle	
Hours	**Vitamin C**	**Vitamin B_2**	**Vitamin C**	**Vitamin B_2**
2	-1.0%		-10%	-10%
3	-1.5%		-15%	-15%
4	-2.0%		-20%	-18%
5	-2.5%		-25%	-20%
6	-2.8%		-28%	-25%
8	-3.0%		-30%	-30%
12	-3.8%	no loss	-38%	-35%

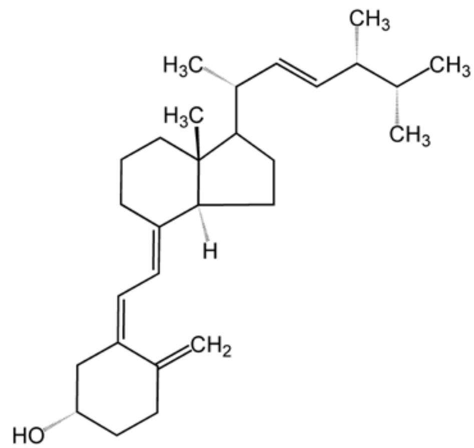

Figure 1. Chemical structure of ergocalciferol or vitamin D_2

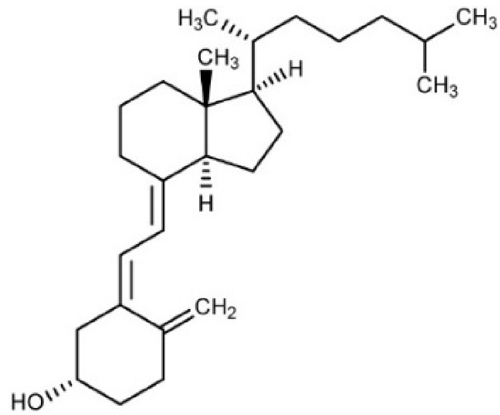

Figure 2. Chemical structure of cholecalciferol or vitamin D_3

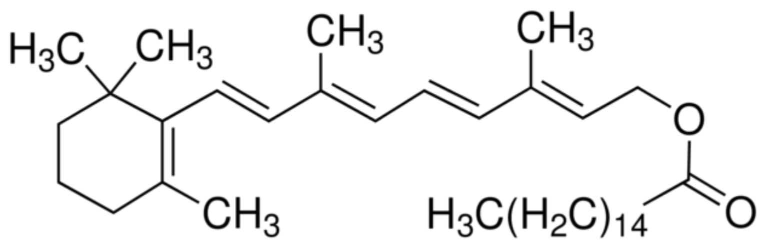

Figure 3. Chemical structure of retinyl palmitate

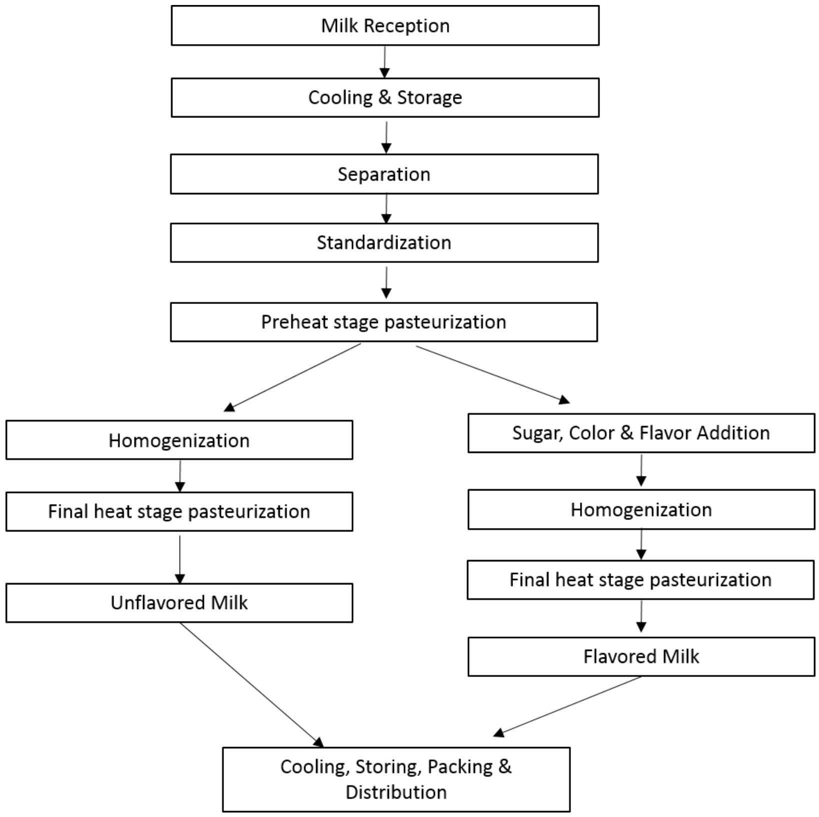

Figure 4. Fluid milk processing flow chart (adapted from Tetra Pak, 2003)

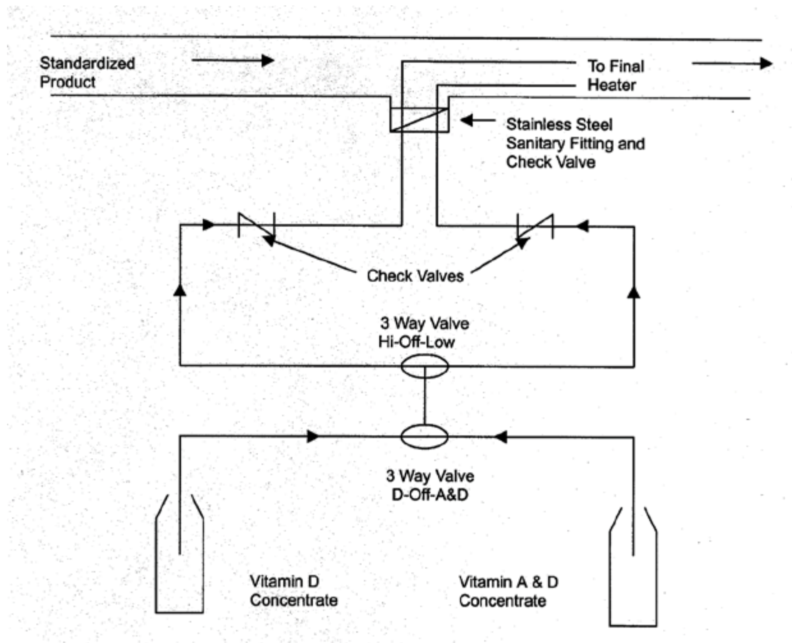

Figure 5. Vitamin Fortification Processing Diagram (Taken from PMO, 2013)

$$\text{Riboflavin} \rightarrow \text{Light} \rightarrow {}^{*}\text{Riboflavin}$$

$$^{*}\text{Riboflavin} + {}^{3}O_2 \rightarrow {}^{1}O_2 + \text{Riboflavin}$$

Figure 6. Riboflavin Excitation (modified from Frankel 1984)

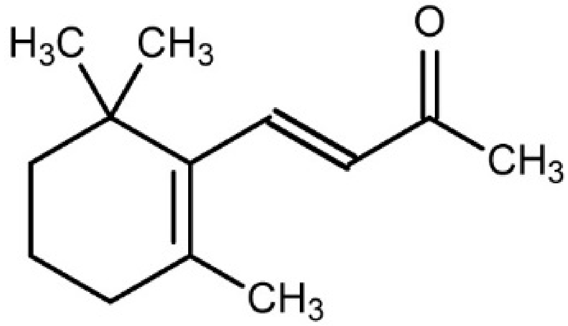

Figure 7. Chemical structure of beta-ionone

CHAPTER 2: THE IMPACT OF VITAMIN CONCENTRATES ON FLAVOR OF PASTEURIZED FLUID MILK

THE IMPACT OF VITAMIN CONCENTRATES ON FLAVOR OF PASTEURIZED FLUID MILK

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INTERPRETIVE SUMMARY

Vitamin premixes with vitamins A and D are used for fortification of fluid milk. Although many of the degradation components of vitamins A and D have an important role in flavor/fragrance applications, they may also be source(s) of off-flavor(s) in vitamin fortified milk due to their heat, oxygen, and the light sensitivity. This study characterized the aroma-active volatile compounds of commercially available vitamin concentrates, and established the role of vitamin A and D fortification on pasteurized fluid milk flavor using instrumental and sensory techniques.

ABSTRACT

Fluid milk consumption in the United States has continued to decline. As a result, the level of dietary vitamin D provided by fluid milk in the U.S. diet has also declined. Undesirable flavor(s)/off-flavor(s) in fluid milk can negatively impact milk consumption and consumer product acceptability. The objectives of this study were to identify aroma active compounds in vitamin concentrates used for fluid milk, and to determine the influence of vitamin A and D fortification on flavor of milk. The aroma profiles of fourteen (14) commercial vitamin concentrates (vitamins A and D), in both oil soluble and water dispersible forms, were evaluated by sensory and instrumental volatile compound analyses. Orthonasal thresholds were determined for 8 key aroma active compounds in skim and whole milk. Six (6) representative vitamin concentrates were selected to fortify skim and 2% fat high temperature short time (HTST) pasteurized milks (vitamin A at 1500-3000 IU/qt, vitamin D at 200-1200 IU/qt, vitamin A and D at 1000/200-6000/1200 IU/qt). Pasteurized milks were evaluated by sensory and instrumental volatile compound analyses and by consumers. Fat content, vitamin content and particle size were also analyzed. The entire experiment was replicated in duplicate. Water dispersible vitamin concentrates had overall higher aroma intensities and more odor active compounds detected than oil soluble vitamin concentrates. Trained panelists and consumers were able to detect flavor differences between vitamin A, and vitamin A and D water dispersible fortified skim milks compared to unfortified skim milks. Consumers were not able to detect flavor differences in oil soluble fortified milks but trained panelists documented faint carrot flavor in oil soluble fortified skim milks at the higher vitamin A concentrations (3000-6000 IU). No differences were detected in vitamin D fortified skim milks ($P > 0.05$) and no differences were detected in all

2% milks ($P > 0.05$). These results demonstrate that vitamin concentrates may contribute to off flavor(s) in fluid milk, especially in skim milk fortified with water dispersible vitamin concentrates.

INTRODUCTION

Vitamin fortification has a long history in fluid milk in the U.S., since the 1930s, to reduce rickets in children (Public Health Service, 1940). Vitamin D is essential for calcium absorption and is involved in the mineralization process required for bone growth. Deficiency of vitamin D causes rickets (softening of bones) in children and osteomalacia in adults (Ceglia, 2009). Recent studies also suggest that vitamin D plays a role in prevention of prostate, breast and colorectal cancers (Grant et al., 2007; Schwartz and Skinner, 2007; Garland et al., 2006; Bouillon et al., 2006). Vitamin A is needed for normal growth, vision, reproduction, and differentiation of epithelial cells. Vitamin A deficiency results in night blindness, xerophthalmia (progressive blindness caused by drying of the cornea of the eye), keratinization (accumulation of keratin in digestive, respiratory and urinary-genital tract tissues) and finally exhaustion and death (Zile and Cullum, 1983).

The FDA mandated in the 1990's that fortified fluid milks must be within 100 to 150% of label claims to address documented variability in vitamin amounts (Public Health Service, 1940). Reduced fat and skim milks are required to be fortified with vitamin A at a minimum of 2000 International Units (IU) per quart (946 mL) and this is optional for whole milk. All fluid pasteurized milk must be fortified with vitamin D at a minimum of 400 IU per quart (946 mL). Vitamin fortification is a standard procedure for pasteurized fluid milks in the U.S., and vitamin concentrates are added to milk before pasteurization (PMO 2013). There are two types of vitamin concentrates: oil soluble and water dispersible formulations (Murphy and Newcomer, 2001). Vitamin A is sensitive to light exposure and rapidly degrades. Oil matrices have been shown to have protective effects on vitamin A stability by delaying oxidative degradation (Dary and Mora, 2002; Loveday and Singh, 2008).

The standard shelf life of commercial vitamin concentrates for fluid milk is one year at room temperature away from light. Recent work has addressed the stability of vitamin D in milk and other dairy products and has indicated that vitamin D is stable during processing and storage (Banville et al., 2000; Kazmi et al., 2007; Wagner et al., 2008; Hanson and Metzger 2010; Tippetts et al., 2012). Vitamin A is unstable to heat, light or the presence of acids, and prolonged heating of milk, butter or butterfat at high temperatures in the presence of oxygen can decrease vitamin A activity (Mousseron-Cadet, 1971; Hartman and Dryden, 1974). The specific role of vitamin concentrates on flavor of fluid milk has not been addressed. A few studies have suggested that added vitamin A concentrate imparted a detectable off-flavor, particularly in skim and low-fat milk (Weckel and Chicoye 1954; Whited et al., 2002). However, there are no published studies, to our knowledge, that have directly evaluated the specific role of the actual vitamin source and the carrier on flavor contributions to fluid milk. Understanding the role of vitamin sources on flavor contributions to fluid milk can help the dairy industry to strategically position vitamin fortification and enhance fluid milk quality. The objectives of this study were 1) to characterize aroma active compounds in vitamin concentrates used for fluid milk, and 2) to determine the influence of vitamin A and D fortification on flavor of 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 key aroma active volatile compounds in pure vitamin concentrates. To carry out this objective, descriptive analysis, headspace extraction and quantification of volatile compounds by gas chromatography-mass spectroscopy (GC-MS) and gas chromatography-olfactometry (GC-O) were performed on 14 different pure vitamin concentrates. Orthonasal threshold testing was then conducted on the 8 key aroma compounds identified via GC-O. The purpose of experiment 2 was to determine possible vitamin flavor contributions to vitamin A and D fortified fluid milk. To conduct this objective, representative vitamin concentrates from experiment 1 were selected to fortify skim and 2% fat milk to determine if trained panelists or consumers could detect flavor differences in fluid milks with and without vitamin fortification. Skim and 2% fat milk were evaluated since they represent what most consumers purchase, and fat plays a protective role on vitamin degradation, light oxidation, and sensory thresholds of off flavors. If vitamin fortification results in off flavor(s), it will be detected in reduced fat or skim milks. Descriptive analysis, volatile compounds, vitamin content and other proximate analyses were also measured.

Commercial Samples and chemical standards

Fourteen (14) commercial vitamin concentrates (vitamins A and D) in both oil and water matrices, were obtained in duplicate lots from multiple companies. All concentrates were within 45 days of manufacture with > 9 months remaining shelf life. Upon arrival, samples were stored at room temperature away from light until analysis. All chemical standards were obtained from Sigma Aldrich (Milwaukee, WI).

Vitamin Analysis

Vitamin analyses was conducted in a laboratory with UV light filters to prevent light oxidation during extraction and analysis. Vitamin analysis for pure vitamin concentrates were conducted in accordance with the Standard Method Performance Requirements for vitamin A

(AOAC SMPR 2012.003) and vitamin D (AOAC SMPR 2012.004) in pre-blends, pre-mixes, and pure-materials. The concentration of retinyl palmitate (vitamin A) and vitamin D_3 in the commercial vitamin premixes were confirmed by UPLC (Acquity H-Class, Waters Corporation, Milford, MA) with PDA detection (325 nm for retinyl palmitate and 265 nm for vitamin D_3). Vitamin A analysis for fortified fluid milk was conducted using the AOAC method 2002.06 (AOAC, 2007). Briefly, retinyl palmitate from a 2 ml test portion of milk was extracted into 5 ml hexane (Sigma Aldrich, Milwaukee, WI) containing retinyl acetate (1.25 ug/ml; Sigma Aldrich, St. Louis, MO) as internal standard. Vitamin A palmitate was measured by UPLC with PDA detection (325 nm). The concentration of retinyl palmitate (ug/ml) was calculated using a relative response factor determined with calibration standards (0.01-5 ug/ml). Vitamin D analysis was also conducted using the AOAC method for fortified fluid milk (AOAC method 2002.05). Briefly, vitamin D from a 15 ml test portion of milk were saponified by mixing with 10 ml of 50% (w/w) KOH (Sigma Aldrich) and 20 ml of ethanol (Sigma Aldrich) containing 2% (w/v) pyrogallol (Sigma Aldrich), followed by extraction with 20% (v/v) diethyl ether (Sigma Aldrich) in hexane containing vitamin D_2 (0.02 ug/ml; Sigma Aldrich) as internal standard. Vitamin D_3 was measured by UPLC with PDA detection (265 nm). The concentration of vitamin D_3 (ug/ml) was calculated using a relative response factor determined with calibration standards (0.001-0.05 ug/ml). Vitamin analyses of milks were conducted at day 0 and day 10 post processing.

Descriptive Analysis of Aroma of Vitamin Concentrates

All sensory testing was deemed exempt by the North Carolina State University Institutional Review Board for human subjects. Pure vitamin concentrates were dispensed (30 mL) directly into three-digit-coded 60 mL lidded soufflé cups (Solo Cup Company, Champaign, IL) for orthonasal aroma evaluation. Orthonasal aroma profiles of the concentrates were evaluated following a 30 min equilibration period, no samples were consumed or placed directly in the mouth. 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^{TM} descriptive analysis method (Meilgaard et al., 2007). For vitamin concentrates, panelists documented overall aroma impact and aroma intensity of pre-determined attributes (carrots, rancid oil, painty, fruity, and citrus). These attributes were selected based on three 30 min lexicon development sessions conducted prior to profiling.

Headspace Extraction of Volatile Compounds from Vitamin Concentrates

Gas Chromatography-Mass Spectrometry (GC-MS)

Volatile compounds were extracted from pure vitamin concentrates by headspace solid phase microextraction (HS SPME). All injections were made on an Agilent 7820 GC with 5975 MSD (Agilent Technologies Inc., Santa Clara, CA) with a ZB-5MS column (Zb-5ms 30 m length x 0.25 mm i.d. x 0.25 µm film thickness; Phenomenex, Torrance, CA). Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (Zwingen, Switzerland). Five (5) ml of vitamin concentrate and 20 ul internal standard solution (2-methyl-3-heptanone in ether at 81 mg/kg (Sigma Aldrich) were added to 20 ml SPME vials (Microliter Analytical, Suwanee, GA) in triplicate. Vials were equilibrated for 25 min at 40^{o}C with 4 second pulsed 250 rpm agitation. A 1 cm SPME fiber [divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS); Supelco, Bellefonte, PA] was used for all analyses. The SPME fiber was exposed to the samples for 40 min at depth 3.1 cm. The fiber was retracted and injected at 5.0 cm in the GC inlet for 5 min.

Scanning from 30-350 *m/*z was performed to identify compounds of interest. Each lot of each vitamin concentrate was evaluated in triplicate.

Gas Chromatography-Olfactometry (GC-O)

Headspace solid space microextraction (HS SPME) followed by gas chromatography-olfactometry (GC-O) was conducted for characterization of aroma active compounds extracted from vitamin concentrates. Ten (10) ml of vitamin concentrate was added to 40 ml amber vials (28 x 98 mm; Supelco Inc.) with a stir bar in duplicate. The vials were heated at 40^{o}C for 30 min with constant stirring. A SPME fiber (DVB/CAR/PDMS; Supelco Inc.) was exposed in each sample at 2 cm for 30 min. The fiber was then injected on an Agilent 6850 gas chromatography-flame ionization detector (FID) equipped with an olfactometer port (Agilent Technologies Inc.). The GC method used an initial temperature of 40^{o}C for 3 min. The temperature was then increased at a rate of 10^{o}C/min to 150^{o}C, followed by 30^{o}C/min to 200^{o}C, and held for 5 min. Samples were evaluated in duplicate on 2 different columns: polar ZB-Wax (30m x 0.25mm ID x 0.25µm; Zebron; Phenomenex Inc.) and a nonpolar ZB-5 (30m x 0.25mm ID x 0.25µm; Zebron; Phenomenex Inc.). The FID sniffing port was held at a temperature of 300^{o}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 on each column by 2 highly experienced sniffers (each with > 50 h previous experience with GCO) who recorded retention time, aroma character and perceived intensity.

Compound Identification and Quantitation

For positive identification of aroma active compounds, retention indices (RI), and mass spectra of unknowns as well as odor properties were compared with those of authentic standard compounds analyzed under identical conditions on the GC-MS and GC-O. Tentative identifications were based on comparing mass spectra of unknown compounds with those in the Natl. Inst. of Standards and Technology (NIST MS Search 2.0, NIST/EPA/NIH Mass Spectral Library) mass spectral database or on matching the RI values of unknowns against those of authentic standards. For the calculation of retention indices, an alkane series (C_8-C_{20} Fluka Chemie Sigma Aldrich, Switzerland) was used (Van den Dool and Kratz, 1963). The area ratio (area of internal standard/area of compound) was multiplied by the concentration of the internal standard to determine the relative abundance of each compound.

Selected Odor Active Compounds

Eight key volatile compounds identified in vitamin premixes via gas chromatography-olfactometry (GCO) were selected based on highest frequency detected and previously reported literature as vitamin degradation products. The compounds selected were trans, trans-2,4-hexadienal, trans,trans-2,4-heptadienal, isoamyl acetate, beta-cyclocitral, alpha-irone, beta-ionone, beta-phellandrene, and beta-damascone. All chemicals were obtained from Sigma Aldrich Chemical Company (St. Louis, MO).

Orthonasal Threshold Analysis

A modification of the ASTM procedure E679-9 (American Society for Testing and Materials [ASTM] 1992), an ascending forced choice method of limits, was used to determine orthonasal threshold values for each compound. Three separate media were tested: deionized water, unfortified skim milk, and unfortified whole milk. These three matrices were selected to compare the impact of a polar matrix, a nonpolar matrix and fat on sensory threshold of each compound. Unfortified skim and whole milks were HTST processed in the pilot plant (described later) and stored at 4°C a week prior to testing. Deionized water and processed milks were screened for off-odors prior to testing.

Stock solution of key compounds were prepared in 95% methanol (Sigma Aldrich). Methanol was chosen as a solvent instead of ethanol due to its lower odor activity. Aliquots of these stock solutions were placed into each media (deionized water, unfortified skim milk, and unfortified whole milk). The orthonasal detection thresholds of each of these compounds were evaluated individually in each of the three different matrices. These solutions were serially diluted (factor of 3), and 30 mL of each was poured into clean, three-digit-coded 60 mL lidded soufflé cups (Solo Cup Company, Champaign, IL). Seven ascending series were tested. For each series, the blank samples of the appropriate matrix were prepared with the same amount of methanol. The samples for each threshold test were prepared 3 h before the test to achieve equilibrium for each compound in the cup (Leksrisompong et al., 2010). Subjects (n=35) were instructed prior to testing (Leksrisompong et al., 2010). The individual best estimate threshold (BET) was taken as the geometric mean of the last concentration with an incorrect response and the first concentration with a correct response except for the following sequence: if the subject indicated a “not sure” response for the correct choice, that concentration was increased by a factor of 1.41 to adjust for the possibility of a chance correct response (Leksrisompong et al., 2010). The estimate group BET was taken as the geometric mean of the individual BET values. Orthonasal threshold tests were conducted in duplicate on different days for each compound in each different matrix.

Pilot Plant Manufacture of Vitamin Fortified Milk

Six (6) vitamin premixes (vitamin A and D) were selected to fortify milk. Vitamin premixes were selected from each category (oil or water) based on number of aroma active compounds and sensory aroma profile such that these represented the worst case scenario (intense sensory aromas and frequency and intensity of aroma active compounds by GC-O). Raw skim and raw cream were obtained from the NCSU Dairy Enterprise System. Raw milk samples were sent to Cornell University for somatic cell count. Additional proximate analysis on raw milk included coliforms, aerobic plate count, fat content and total solids content. Coliform count (CC) and aerobic plate count (APC) were conducted using standard methods (Wehr and Frank, 2004; method number 7.070 and 6.040 respectively) with Petrifilm plates (Coliform Count Plates and Aerobic Count Plates, 3M, St. Paul, MN). CC and APC plates were incubated at 32°C for 24 h before being counted. Fat and total solids content were measured by a Fourier-transform mid-infrared milk analyzer (LactoScope FTIR; Delta Instruments BV, Drachten, the Netherlands). Raw skim and raw cream were standardized to 2% fat. Fat content was measured again by the mid-infrared milk analyzer (calibrated monthly) to confirm proper standardization of 2% fat milk.

Vitamin concentrates were added to the raw skim or raw 2% milk. Raw milks were batched as follows: skim control with no vitamin addition, skim with vitamin addition to be used as stock solution; repeat with 2% milk. 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 sanitized milk cans and then placed at 4°*C* and tested for complete pasteurization by the alkaline phosphatase test (Wehr and Frank, 2004; method 14.060). Fat content, vitamin content, and microbial quality were evaluated using the same methods described previously. Milk fat globule size distribution was measured (Smith et al., 1995; Marzo et al., 2016) using a Malvern Mastersizer 3000 (Malvern Instruments, Westborough, MA) for the 2% fat milks.

Four (4) different concentrations (1500 IU, 2000 IU, 2500 IU, 3000 IU per quart) of vitamin fortified milk were made for premixes containing just vitamin A. These concentrations were chosen since 2000 IU to 3000 IU per quart of vitamin A is the target concentration levels in retail milk products and it was necessary to test the level just below the target due to reported underfortification of retail milks. Four (4) different concentrations (200 IU, 400 IU, 800 IU, 1200 IU per quart) were made for premixes containing just vitamin D. Retail milk products are usually fortified with vitamin D at 400 IU per quart. The chosen concentrations were at 50%, 100%, 200% and 300% of the recommended fortification level (400 IU per quart) for vitamin D. Due to increasing interests in fortifying vitamin D at higher concentration in fluid milk, it was necessary to test higher fortification levels. Seven (7) different concentrations (1000/200 IU, 1500/300 IU, 2000/400 IU, 2500/500 IU, 3000/600 IU, 4000/800 IU, 6000/1200 IU per quart with A/D concentrations respectively) were made for the premixes containing both vitamin A and D. These concentrations were chosen because the premixes contain a set potency ratio of vitamin A and D, therefore fortifying at these levels were necessary to cover the ranges for both vitamin A and D that were tested before. Different concentrations of vitamin fortified milk were made by diluting the stock vitamin pasteurized milk solution with no vitamin added pasteurized milk and bottled into half gallon, light shielded milk jugs (Upstate Niagara Cooperative, Inc., Buffalo, NY) and stored in the dark at $4°C$. Vitamin analysis was conducted again to ensure correct target vitamin concentrations in the final products. Skim and 2% fat milk for a given vitamin premix replication were processed from the same lot of milk. This process was repeated on different weeks for each premix. The entire experiment was replicated in duplicate.

Descriptive Analysis

Descriptive sensory analysis of milk flavor was conducted at days 3 and 10 post processing on fortified fluid milks with vitamin A and D, and control milks without vitamins using a trained descriptive sensory panel and an established milk flavor language (Croissant et al., 2007; McCarthy et al., 2016). 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^{TM} descriptive analysis method (Meilgaard et al., 2007). Fortified milks with and without vitamins were dispensed (60 mL) directly into three-digit-coded 120 mL lidded soufflé cups for evaluation. Preparations were conducted with overhead lights off to avoid exposure to light. Samples were evaluated by each panelist in duplicate. Sensory data were collected using paper ballots. All sensory testing was conducted in accordance with North Carolina State University Institutional Review Board for Human Subjects guidelines.

Consumer Difference Tests

Triangle difference tests (ASTM E1885) for vitamin concentrates (vitamin A oil/water, vitamin D oil/water, vitamin A and D oil/water) with at least 50 milk consumers were conducted at day 10 post processing. All 4 vitamin levels were evaluated for vitamin A concentrate and vitamin D concentrate alone. Four selected vitamin levels were evaluated for premixes containing both vitamin A and D, 4 difference tests (4 vitamin levels at 2000/400 IU, 3000/600 IU, 4000/800 IU, and 6000/1200 IU with A/D concentrations respectively) were conducted. These vitamin levels were chosen based on consumer results from vitamin

A only premixes and vitamin D only premixes. Consumers were not recruited before the panel and consisted of walk-ins from the NCSU campus. Consumers were informed about the testing via flyers posted around campus and emails sent out to the online database maintained by the Sensory Service Center (Raleigh, NC). Each consumer was verbally instructed on how a triangle difference test was performed and that they were to pick the coded sample which they believed was different from the other two samples. In addition to verbal training, an instruction text describing how the test was to be performed was also provided at each station.

Milks (88 mL) were served at 4°*C* in 177 mL Styrofoam cups (Dart Container Corp.) with random three digit blinding codes. Each panelist was given all 3 samples at once and instructed to taste the samples from left to right (randomized order). Within each set of 3 samples, one vitamin level was compared to the control, so a total of 4 difference tests (4 vitamin levels) for each fat content (skim and 2%) was conducted. A 5 min rest was enforced between each difference test within a fat content. Consumers returned in the afternoon to complete the other series of samples within a fat content. Upon completion of each test, panelists were compensated with a $5 Target gift card. Compusense Cloud version 7.8 (Compusense, Guelph, Canada) was used for data collection. All consumer testing was conducted in accordance with North Carolina State University Institutional Review Board for Human Subjects guidelines.

Quantitation of Selected Volatile Compounds in Fortified Fluid Milk

The eight volatile compounds previously identified in vitamin premixes via GCO were quantified via GC-MS. Standards for all eight compounds were provided by Sigma (St. Louis, MO). Samples of skim milk fortified at the highest concentrations (3000 IU Vitamin

A) used for consumer testing were run on an Agilent 7820 Series GC/Agilent inert 5975 mass selective detector (MSD) equipped with a ZB-5MS 30m x 0.25mm 0.25 μm film column (Phenomenex, Torrence, CA) using a three phase solid-phase microextraction (SPME) fiber (DVB/CAR/PDMS; Supelco Inc.). This configuration failed to provide adequate resolution for quantification of these compounds even when a SIM method was utilized. A ZB-SemiVolatiles 30m x 0.25mm 0.25 μm film column (Phenomenex, Torrence, CA) was then used in an attempt to improve resolution considering the high molecular weights of the target compounds. Samples of pure premixes were run on this column in an attempt to locate the eight target compounds; only beta-ionone showed up in the chromatogram so this approach was not used.

Volatile compounds were then extracted using a novel modified stir bar sorptive extraction (SBSE) approach, which involved swelling PDMS stir bars directly in solvent prior to extraction (Ochiai et al., 2016). Stir bar sorptive extraction (SBSE), has been shown to further improve resolution and limits of detection for gas chromatography when compared to SPME in foods, including in milk powders (Prieto et al., 2010; Park et al., 2016). Volatiles in skim milk fortified with 3,000 IU vitamin A were extracted using PDMS stir bars swollen in four solvents (dichloromethane, diethyl ether, hexane, and cyclohexane). One PDMS coated with solvent stir bar (10 mm x 0.5 mm thickness, Gerstel, Inc.) was placed in the vial, sealed, and stirred for one hour at 800 rpm. After 1 h, the stir bar was briefly rinsed in HPLC grade water, dried, and placed in a TDU autosampler tube (Gerstel, Inc.). Compounds were desorbed on to the GC TDU inlet using an autosampler (MPS Autosampler, Gerstel, Inc.). The stir bars were desorbed at 250°C for 10 min (TDU, Gerstel, Inc.) and the volatile compounds were cryogenically trapped at -120°C (CIS 4, Gerstel, Inc.). Cyclohexane provided the best resolution of the solvents evaluated, allowing identification of all compounds and thus was the solvent and conditions of choice for SBSE in this study.

Volatile compounds were analyzed by gas chromatography mass spectrometry (GC-MS). An Agilent 7890B GC (Agilent Technologies Inc., Santa Clara, CA) with an inert mass selective detector (model 5970A, Agilent) with a ZB-Semivolatiles column (30 m x 0.25 mm x 0.25µm) (Phenomenex, Torrance, CA) was used to identify and quantify volatile compounds of interest. 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. Compounds were identified by comparison with the 2014 NIST mass spectral library (NIST, 2014), retention index, and retention time of authentic standards injected under identical conditions. Relative abundance of selected compounds was calculated using recovery of the internal standard.

Statistical Analysis

Statistical analysis was conducted using XLSTAT Version 2015.1.01 (Addinsoft, New York, NY). For threshold BET values, the delta method was used for the pair-wise comparison for group BET values among different matrices using the formula $Z = (BET_2 - BET_1)/ \sqrt{(SE_1^2 + SE_2^2)}$ (Leksrisompong et al. 2010). Consumer difference data were determined using the minimum correct judgement for significance at $\alpha = 0.05$ (ASTM E1885). Descriptive analysis and volatile compound analysis data were analyzed by a one-way analysis of variance (ANOVA) using a general linear model with Fisher's least significant difference for means separation. All data were analyzed at a significance level of 0.05.

RESULTS AND DISCUSSION

Experiment 1

Instrumental Volatile and Descriptive Analysis

The concentration of retinyl palmitate (vitamin A) and vitamin D_3 in the commercial vitamin premixes were confirmed to have a purity of 99.1% and 98.9% respectively by UPLC/PDA detection (325 nm for retinyl palmitate and 265 nm for vitamin D_3). Trained panel profiling of aroma of vitamin concentrates demonstrated that aroma attributes of vitamin concentrates were distinct and characterized by carrot, fruity, citrus, rancid oil and painty aromas. Vitamin A and vitamin A and D concentrates were associated with carroty aromas, while vitamin D concentrates were associated with rancid oil and painty aromas. Vitamin concentrates in water matrices had higher overall aroma intensities compared to vitamin concentrates in oil matrices ($P < 0.05$) (Table 1).

Instrumental results were consistent with sensory results, the vitamin concentrates with more odor active compounds also were associated with higher overall aroma intensity (Table 1). More odor active compounds were also detected in water dispersible vitamin concentrates, which was also consistent with higher overall aroma intensities in water dispersible vitamin concentrates by sensory analysis (Table 1). A total of forty three odor active compounds were detected in the vitamin concentrates by HS-SPME GC-O. Of the 43, 32 were identified by aroma, retention index, and mass spectra, with 7 tentatively identified by retention index and odor quality, and 4 unknowns (Table 2).

Among the odor active compounds were acids, alcohols, aldehydes, ketones, esters and terpenes. Alcohols such as Z-3-hexen-1-ol, as well as the aldehydes hexanal, octanal, heptanal, E, Z-2,4-heptadienal, 2,4-nonadienal, and E-2-octenal have been previously identified as aroma odor compounds of sunflower oil and oxidized sunflower oil (Keszler et al., 1998, 2000; van Ruth et al., 2000; Doleschall et al., 2003), and in other plant oil oxidative degradation products (Snyder et al., 1985; Solinas et al., 1987; Angelo, 1996; Vichi et al., 2003). These volatiles are products of linoleic acid oxidation which are the major unsaturated fatty acids of sunflower oil (Grosch, 1987; Frankel, 1982, 1985). These volatiles were detected in oil soluble vitamin concentrates, which suggested that the source of these compounds could be from the carrier of the vitamin concentrate, sunflower oil or corn oil.

The ketones, beta-ionone, alpha-irone, and beta-damascone, as well as terpenes, beta-phellandrene, beta-cyclocitral, beta-cyclohomocitral, and alpha-copaene were previously reported as odor active aroma compounds in essential oils, tobacco, tea, fruits, vegetables, and wine (Winterhalter and Rouseff, 2002; Friedrich and Acree, 2002; Nielsen and Poll, 2006), and as vitamin A thermal oxidation products (Kanasawud and Crouzet, 1990; Crouzet and Kanasawud, 1992; Suyama et al., 1983; Kim et al., 2000; Enzell, 1985), and vitamin A light oxidation products (Gaylord et al., 1986; Budowski and Bondi, 1960; Litzenburger and Bernhardt, 2016). These volatiles were detected mostly in vitamin A concentrates, and in vitamin A and D concentrates, suggesting that these volatiles are degradation products from vitamin A in the premixes. Some of the aldehydes that were detected in vitamin D concentrates were heptanal, E-2-heptenal, trans, trans-2,4-hexadienal and trans, trans-2,4-heptadienal, and these volatiles have been reported as byproducts of vitamin D oxidation (Kutsky, 1981; King and Min, 2002; DeMan, 1999).

Isoamyl acetate was detected in vitamin concentrates containing ascorbic acid as antioxidant. Isoamyl acetate is an enzymatic degradation product of ascorbic acid (Hooper and Ayres, 1950; Obretenove et al., 2002). This ester may come directly from the ascorbic acid added in the vitamin premixes as antioxidant. Other odor compounds including toluene, styrene, and dodecane have been previously reported in food packaging material, specifically in polyethylene (Bravo et al., 1992; Karatapanis et al., 2006). These volatiles may be sourced from the packaging of vitamin premixes, which are commercially packaged in plastic material.

Thirty eight compounds were detected in water dispersible vitamin concentrates while 23 compounds were detected in oil soluble vitamin concentrates. Previous studies have reported that oil matrices have protective effects on vitamin A stability by delaying oxidative degradation and degradation components (Dary and Mora, 2002; Loveday and Singh, 2008). Vitamin D in plant oils are resistant at high temperatures and during long term storage. In contrast, vitamin D in non-lipid environments is susceptible to oxidation agents and form 5,6-trans-vitamin D, which has only 10% to 30% of vitamin activity (King and Min, 2002; DeMan, 1999). Fewer aroma compounds were detected in oil soluble vitamin concentrates due to the protective effects of oil matrices in these concentrates by delaying or preventing oxidative degradation of vitamin A and/or D. The key volatile compounds found in these vitamin concentrates based on highest frequency detected and previously reported as vitamin degradation products were beta-damascone, beta-ionone, beta-phellandrene, isoamyl acetate, beta-cyclocitral, alpha-irone, trans, trans-2,4-hexadienal, and trans, trans-2,4-heptadienal.

Orthonasal Threshold Analysis

The BET values of the 8 key volatile compounds from vitamin concentrates were impacted by matrix (Table 3). All eight compounds are hydrophobic and had higher BET values in whole milk than in water and skim milk ($P < 0.05$), this is expected since hydrophobic compounds are more attracted to the fat in the milk matrix and thus less compound is driven into the headspace, and increases the threshold value. Milk also contains protein, which binds organic compounds and forces the equilibrium more into solution, further increasing the threshold. Previous studies have demonstrated that the aroma intensity and headspace concentration of hydrophobic compounds were more affected by the presence of fat than that of hydrophilic compounds (Roberts et al., 2003; Leksrisompong et al., 2010; Kim et al., 2011). Threshold results suggested that fat reduction has an influence on threshold and possibly flavor release and perception of these compounds.

Experiment 2

Raw milk aerobic plate counts averaged 190 cfu/mL and 31 cfu/mL coliforms. HTST skim and 2% milks averaged <20 cfu/mL aerobic plate counts, and <1 cfu/mL coliform counts. Skim milk composition was 0.061% fat and 9.11% solids. Two percent fat milks averaged 1.99% fat and 10.93% solids. Milk composition was not different between treatments in each fat level ($P > 0.05$). The D90 (the maximum diameter at which 90% of the particles are below) for milk fat particle size was <1.5 um for all milks, indicating appropriate homogenization. Milkfat particle sizes were not different ($P > 0.05$) among 2% milks (data not shown). All milks were negative for alkaline phosphatase, indicating complete pasteurization. Vitamin A and D analysis for each target concentration at each time point was consistent ($P > 0.05$), no degradation over storage was detected (data not shown). Previous studies have also reported no significant change in vitamin D during 21 d storage of HTST 2% milk, UHT 2% chocolate milk and low-fat yogurt (Hanson and Metzger, 2010). Vitamin D was not affected by HTST processing (Wagner et al., 2008) and was stable during storage of milk at 4°C (Renken and Warthesen, 1993). No significant change was found in vitamin A content during 24 h storage of fortified milk in the absence of light (Brothersen et al., 2016), and vitamin A in unexposed skim milk was more stable than the light-exposed milk (Fellman et al., 1991; Whited et al., 2002).

Descriptive Analysis

Faint carrot flavor was detected by trained panelists in the highest vitamin A skim milk treatments (3000 IU) from oil based vitamin concentrate ($\bar{x}$ = 0.8 on a 0 to 15 point scale), and a higher intensity ($P < 0.05$) of carrot flavor were detected in skim milk with water dispersible vitamin A concentrate (3000 IU) ($\bar{x}$ = 1.5 on a 0 to 15 point scale). Faint carrot flavor were detected skim milk with vitamin A and D water based concentrate at 2000/400 IU and 2500/500 IU ($\bar{x}$ = 0.8), and a higher intensity ($P < 0.05$) of carrot flavor were detected at 3000/600 IU, 4000/800 IU and 6000/1200 IU ($\bar{x}$ = 1.3, 1.6 and 2.0). Faint carrot flavor was also detected in skim milks containing vitamin A and D oil based concentrate at 6000/1200 IU ($\bar{x}$ = 0.9). None of the carrot flavors that were detected in skim milks were detected in 2% milk (data not shown). No distinct flavor(s) associated with vitamin D were detected in skim or 2% milk with oil based or water dispersible vitamin D concentrate added ($P > 0.05$) (data not shown).

Consumer Difference Tests

Consistent with trained panel results, consumers were able to detect differences in skim milk fortified with vitamin A water dispersible at 2000, 2500 and 3000 IU levels compared to control unfortified skim milks (Table 4). No differences were detected in vitamin D fortified skim milks (Table 5). Several studies have been conducted on the stability of vitamin D in milk and other dairy products, and these studies have all indicated that vitamin D is stable during processing and storage (Banville and others 2000; Kazmi and others 2007; Wagner and others 2008; Hanson and Metzger 2010). Vitamin A on the other hand, is unstable to heat, light or the presence of acids (Mousseron-Cadet, 1971), and prolonged heating of milk, butter or butterfat at high temperatures in the presence of oxygen can decrease vitamin A activity (Hartman and Dryden, 1974). These breakdown products may contribute to perceptible flavors. Numerous studies have reported that vitamin A and D is stable during HTST processing and storage in the absence of light and acid (Banville and others 2000; Kazmi and others 2007; Wagner and others 2008; Hanson and Metzger 2010; Hartman and Dryden, 1974). In other words, HTST processing has little effect on vitamin A and D degradation, thus off flavor(s) detected in skim milk may come from the vitamin concentrates directly.

Consumers were able to detect the difference in vitamin A and D water dispersible fortified skim milk at 2000/400 IU, 3000/600 IU, 4000/800 IU and 6000/1200 IU levels compared to control unfortified skim milk (Table 6). No differences were detected among 2% milks ($P > 0.05$) (data not shown). This is also consistent with descriptive analysis results. This is likely due to the protective effects of milkfat on sensory thresholds of off flavors, where orthonasal thresholds of nonpolar compounds are reduced compared to whole milk or higher fat products (Leksrisompong et al., 2010; Kim et al., 2011). Previous studies have also suggested that added vitamin A concentrate imparted a detectable off-flavor, particularly in skim and low-fat milk, and occasionally in whole milk products (Weckel and Chicoye, 1954; Whited et al., 2002). No differences were detected in oil soluble vitamin concentrate treatments. This may be due to flavor protection from oil matrices as oil matrices have been shown to have protective effects on vitamin A stability by delaying its oxidative degradation and degradation components (Dary and Mora 2002; Loveday and Singh 2008). A previous study with triangle tests indicated that light induced off flavor was distinguished in skim milks with water based vitamin A at 2000 IU after 6 hours of light exposure, while off flavor in skim milks with oil based vitamin A at 2000 IU could not be distinguished until after 24 hours of light exposure (Fellman et al., 1991). The results from the previous study suggest that vitamin A degradation in the presence of light may also accelerate light oxidized flavor in milk and should be addressed in future studies.

Quantitation of Selected Volatile Compounds in Fortified Fluid Milk

Quantitation of the 8 selected volatile compounds in fortified fluid milk were consistent with sensory and consumer results (Table 7). Consumers and trained panelists were able to detect flavor differences in skim milks fortified with water soluble vitamin A at 2000, 2500 and 3000 IU levels. Trained panelists documented the flavor as sweet, perfumey, and carrot-like. Beta-ionone (fruit, floral) was found in the same milks above threshold, as well as E, E-2,4-heptadienal (fatty). Consumers and trained panelists also detected flavor differences in skim milks fortified with water soluble vitamin A and D at 2000/400 IU, 3000/600 IU, 4000/800 IU, and 6000/1200 IU levels. Beta-ionone, E, E-2,4-hexadienal (green) and E, E-2,4-heptadienal were found above threshold in skim milks fortified with water soluble vitamin A and D at the highest concentration (6000/1200 IU). Trained panelists were able to detect faint carrot flavor in skim milks fortified with oil soluble vitamin A at the highest concentration (3000 IU) and skim milks fortified with oil soluble vitamin A and D at the highest concentration (6000/1200 IU). Beta-ionone was also found in these milks above threshold, as well as E, E-2,4-hexadienal and E, E-2,4-heptadienal.

Consumers and trained panelists did not detect flavor differences in 2% fat milks, and the selected volatile compounds were not above threshold in these 2% fat milks except for E, E-2,4-hexadienal, E, E-2,4-heptadienal and beta-damascone. E, E-2,4-hexadienal was detected above threshold in 2% fat milks fortified with water and oil soluble vitamin A and D at the highest concentration (6000/1200 IU). E, E-2,4-heptadienal was detected above threshold in 2% fat milks fortified with water soluble vitamin A at the highest concentration (3000 IU), water soluble vitamin D at the highest concentration (1200 IU), and water and oil soluble vitamin A and D at the highest concentration (6000/1200 IU). Beta-damascone was detected above threshold in 2% fat milks fortified with water soluble vitamin A and D at the highest concentration (6000/1200 IU). Although trained panelists and consumers did not detect any flavor differences in vitamin D fortified milks, E, E-2, 4-heptadienal was found above threshold in skim and 2% fat milks fortified with water soluble vitamin D at the highest concentration (1200 IU). Beta-damascone and beta-ionone were previously reported as vitamin A thermal degradation products (Kanasawud and Crouzet, 1990; Crouzet and Kanasawud, 1992; Suyama et al., 1983; Kim et al., 2000; Enzell, 1985), and vitamin A light oxidation products (Gaylord et al., 1986; Budowski and Bondi, 1960; Litzenburger and Bernhardt, 2016). E, E-2,4-hexadienal and E, E-heptadienal have been reported as byproducts of vitamin D oxidation (Kutsky, 1981; King and Min, 2002; DeMan, 1999). This is consistent with the data that these compounds were found in vitamin A, vitamin D and vitamin A and D fortified milks. Trained panelists and consumers did not detect any flavor differences in vitamin D fortified milks even at the highest concentration (1200 IU). This is at about 300% of the recommended fortification level (400 IU per quart) for vitamin D. This result suggests that it is possible to increase vitamin D fortification levels in fluid milk products to improve dietary vitamin D intake in the U.S. diet without impacting fluid milk flavors.

CONCLUSIONS

Vitamin concentrates used for fluid milk fortification have very distinct aromas and aroma active compounds within sensory detection range at concentrations applied for milk fortification. Vitamin concentrates were characterized by carrot, fruity, citrus, rancid oil and painty aromas. Key aroma active compounds in vitamin concentrates were beta-damascone, beta-ionone, beta-phellandrene, isoamyl acetate, beta-cyclocitral, alpha-irone, trans, trans-2,4-hexadienal, and trans, trans-2,4-heptadienal. Four (4) of these compounds were above sensory threshold in the fortified milks. Water dispersible vitamin premixes had higher sensory aroma intensities and a greater number of aroma active compounds. Consumers were also able to detect flavor differences in skim milk fortified with water dispersible vitamin premixes. These findings suggest that vitamin premix may contribute off flavors in fluid milk, especially in skim milk fortified with water dispersible form vitamin premix. The dairy industry should reduce the use of water dispersible vitamin premixes, especially vitamin A, to improve fluid milk quality.

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TABLES AND FIGURES

Table 1. Sensory aroma profiles and frequency of aroma active events detected by GC-O of commercial vitamin concentrates from multiple companies.

Type	Sample code	Frequency of aroma active events[1]	Overall aroma	Carrots	Rancid oil	Painty	Fruity	Citrus
D in water	549	26	4.5a	ND	3.0a	4.5a	1.8a	1.5b
D in water	124	17	3.0b	ND	3.0a	4.5a	1.5b	1.5b
D in water	097	28	3.0b	ND	3.0a	4.0b	1.5b	2.0a
D in oil	054	4	1.5e	ND	1.5b	1.0d	1.0c	1.0c
D in oil	832	6	1.5e	ND	1.3b	1.0d	1.0c	1.0c
A in water	007	26	4.5a	4.5a	ND	ND	2.0a	2.0a
A in oil	617	11	2.5c	2.5c	ND	ND	1.0c	1.0c
A in oil	588	6	2.5c	2.5c	ND	ND	1.0c	1.0c
A&D in water	762	15	4.5a	4.5a	1.0c	1.0d	2.0a	2.0a
A&D in oil	817	9	3.0b	3.0b	1.0c	1.0d	1.3bc	1.3bc
A&D in oil	428	10	2.5c	2.5c	1.0c	1.1d	1.0c	1.1c
A&D in oil	611	12	3.0b	3.0b	1.3b	1.0d	1.8a	1.0c
A&D oil	399	9	3.0b	3.0b	1.1c	1.0d	1.5b	1.0c
A&D oil	450	8	2.0d	2.0d	1.1c	1.8c	1.0c	1.5b

[1]Frequency of odor-active compounds detected in pure vitamin concentrates by GC-O using HS-SPME

[a-d] Means in a column not sharing a common letter are different ($P<0.05$). Aroma intensities were scored on a 0 to 15 point universal intensity scale (Meilgaard et al., 2007)

ND = not detected

Table 2. Odor-active compounds detected in pure vitamin concentrates by gas chromatography-olfactometry using HS-SPME (head space-solid phase microextraction).

RI (ZB5)[a]	RI (WAX)[b]	Method of ID[c]	Compound	Aroma description	549	124	097	054	832	007	617	588	762	817	428	611	399	450
<600	nd	O	**unknown**	rust/rancid butter		+				+			+	+	+	+	+	+
619	1483	O, RI, MS	acetic acid	vinegar/sour										+	+			
650	1628	O, RI, MS	3-butenoic acid	vinegar/sour		+	+							+		+		
652	1154	O, RI	1-butanol	burnt/sweet			+						+	+		+	+	
760	1595	O, RI, MS	propylene glycol	chemical/ethanol	+	+	+			+			+					
764	1207	O, RI, MS	3-methyl-1-butanol	paint/rubber	+						+			+				
764	1076	O, RI, MS	toluene	paint		+					+						+	
800	1079	O, RI, MS	hexanal	grass, floral	+	+	+	+	+	+	+	+	+	+	+	+	+	+
807	1075	O, RI, MS	butyl acetate	floral, sweet			+		+	+		+	+			+		+
849	1495	O, RI, MS	furfural	nutty	+				+									
850	1365	O, RI, MS	Z-3-hexen-1-ol	citrus	+			+	+		+							
861	nd	O	**unknown**	carrot				+			+					+		
879	1126	O, RI, MS	Isoamyl acetate	fruity	+					+				+		+	+	+
880	1177	O, RI, MS	2-heptanone	plastic	+		+											
896	1409	O, RI, MS	E, E-2,4-hexadienal	Green	+		+	+					+		+	+	+	
896	nd	O, RI, MS	Styrene	chemical	+		+			+								
899	1180	O, RI, MS	heptanal	fruity	+	+	+			+			+					
905	1525	O, RI, MS	2-acetylfuran	plastic/nutty	+	+	+			+			+	+				
915	1012	O, RI, MS	alpha-pinene	dry/woody		+					+			+	+	+		
957	1173	O, RI, MS	cumene	acetone/nail polish			+											
965	1307	O, RI, MS	E-2-heptenal	metallic/fruity	+	+	+			+	+	+	+	+	+	+	+	+
985	1055	O, RI	1-decene	plastic/chemical	+	+	+	+	+	+	+				+	+		+
987	1451	O, RI, MS	E,Z-2,4-heptadienal	Grainy/fatty	+		+			+	+							
1007	1456	O, RI, MS	E,E-2,4-heptadienal	Fatty	+		+	+	+	+	+		+		+	+	+	+
1012	1283	O, RI, MS	octanal	fruity/citrus	+	+	+			+			+					
1022	1196	O, RI, MS	limonene	citrus/fruity		+	+			+			+					
1029	nd	O, RI, MS	m-cymene	fruity						+							+	
1073	1433	O, RI, MS	E-2-octenal	roast, fatty	+		+			+					+			
1084	nd	O, RI, MS	beta-Phellandrene	carroty/toasted						+	+	+	+					
1108	1864	O, RI	alpha-irone	carroty/toasted						+	+		+		+	+		
1133	1325	O, RI	1,6-heptadien-4-ol	popcorn	+		+				+	+						+
1156	1575	O, RI, MS	isopulegol	minty	+		+					+						

Table 2 Continued

1162	nd	O	**unknown**	leafy	+					+								
1189	1244	O, RI	1-dodecene	plastic		+	+			+								
1203	1202	O, RI, MS	dodecane	plastic/chemical	+		+			+								
1220	1667	O, RI, MS	2,4-nonadienal	oily		+	+	+	+		+		+	+		+	+	
1233	1619	O, RI, MS	β-Cyclocitral	carrot						+	+	+	+				+	
1264	1244	O, RI, MS	β-Cyclohomocitral	oil, woody						+	+							
1288	nd	O	**unknown**	greesy, oily/rubber tire			+								+			
1487	1849	O, RI, MS	beta-ionone	floral, fruity						+	+	+						+
nd	1472	O, RI	α-copaene	carrot/greasy						+	+							
nd	1806	O, RI	beta-damascone	carrot/green/floral						+	+	+	+					

[a]Retention Indices (RI) from GC-O data on the ZB-5 column
[b]Retention Indices (RI) from GC-O data on the WAX column
[c]Method of identification by RI (retention indices), O (olfactometry), and MS (mass spectrometry) compared with authentic standards
[d] + indicates the presence of compounds detected by 2 experienced sniffers; blank indicates the absence of the compounds
[e]ND = not detected

Table 3. Best Estimate Threshold (BET) values for each odorant in water, in unfortified skim milk and in unfortified whole milk

	BET (mg/kg)		
Compounds	Water	Skim Milk	Whole Milk
Ester			
Isoamyl acetate	$0.006^{b} \pm 0.003$	$0.001^{c} \pm 0.0003$	$0.030^{a} \pm 0.009$
Ketones			
α-irone	$0.002^{c} \pm 0.001$	$0.116^{b} \pm 0.034$	$3.160^{a} \pm 0.954$
β-ionone	$0.004^{c} \pm 0.002$	$0.044^{b} \pm 0.020$	$1.915^{a} \pm 0.963$
β-damascone	$0.031^{b} \pm 0.055$	$0.012^{b} \pm 0.006$	$0.199^{a} \pm 0.008$
Terpenes			
β-phellandrene	$0.027^{b} \pm 0.006$	$0.033^{b} \pm 0.009$	$0.944^{a} \pm 0.126$
β-cyclocitral	$0.032^{c} \pm 0.011$	$0.108^{b} \pm 0.025$	$0.846^{a} \pm 0.251$
Aldehydes			
E, E-2,4-hexadienal	$0.178^{b} \pm 0.027$	$0.114^{b} \pm 0.093$	$0.284^{a} \pm 0.054$
E,E-2,4-heptadienal	$0.066^{b} \pm 0.023$	$0.250^{a} \pm 0.072$	$0.344^{a} \pm 0.109$

Means are the geometric means of group BET values from 35 panelists
Units are in ppm (mg/kg)
[a,b,c] Letters above the means indicate significant differences ($P < 0.05$) within the same compound in different matrices

Table 4. Consumer difference test results for Skim milk with vitamin A

Premix concentrate		Control vs. 1500 IU No. Correct	Control vs. 1500 IU Significant at α=0.05	Control vs. 2000 IU No. Correct	Control vs. 2000 IU Significant at α=0.05	Control vs. 2500 IU No. Correct	Control vs. 2500 IU Significant at α=0.05	Control vs. 3000 IU No. Correct	Control vs. 3000 IU Significant at α=0.05
Vitamin A Water Dispersible	(n=61)	22	No	27	Yes	32	Yes	37	Yes
Vitamin A Water Dispersible Rep 2	(n=50)	17	No	23	Yes	24	Yes	28	Yes
Vitamin A Sunflower Oil	(n=60)	22	No	20	No	23	No	20	No
Vitamin A Sunflower Oil Rep 2	(n=60)	22	No	21	No	16	No	20	No

Table 5. Consumer difference test results for Skim milk with vitamin D

Premix concentrate		Control vs. 200 IU		Control vs. 400 IU		Control vs. 800 IU		Control vs. 1200 IU	
		No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05
Vitamin D Sunflower Oil	(n=60)	24	No	23	No	22	No	23	No
Vitamin D Sunflower Oil Rep 2	(n=54)	23	No	18	No	23	No	13	No
Vitamin D Water Dispersible	(n=64)	21	No	24	No	24	No	22	No
Vitamin D Water Dispersible Rep 2	(n=64)	19	No	24	No	27	No	24	No

Table 6. Consumer difference test results for Skim milk with vitamin A and D

Premix concentrate		Control vs. 2000/400 IU		Control vs. 3000/600 IU		Control vs. 4000/800 IU		Control vs. 6000/1200 IU	
		No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05
Vitamin A&D Sunflower Oil	(n=64)	28	No	21	No	23	No	24	No
Vitamin A&D Sunflower Oil Rep 2	(n=57)	20	No	18	No	14	No	19	No
Vitamin A&D Water Dispersible	(n=61)	33	Yes	33	Yes	36	Yes	37	Yes
Vitamin A&D Water Dispersible Rep 2	(n=61)	32	Yes	37	Yes	37	Yes	39	Yes

Table 7. Volatile compound analysis of vitamin fortified milk (ug/kg)

	E, E-2,4-hexadienal	E,E-2,4-heptadienal	β-cyclocitral	β-phellandrene	α-irone	β-ionone	β-damascone	Isoamyl acetate
A water sk 1500	ND	ND	ND	ND	ND	108.8	ND	ND
A water sk 2000	ND	ND	ND	ND	ND	217.9	ND	ND
A water sk 2500	ND	1407.4	ND	8.6	ND	90.2	ND	ND
A water sk 3000	ND	1390.6	ND	6.4	26.8	123.7	ND	ND
A water 2% 1500	ND	ND	ND	ND	ND	70.8	ND	ND
A water 2% 2000	ND	ND	ND	ND	ND	31.1	ND	ND
A water 2% 2500	ND	ND	ND	ND	ND	31.9	ND	ND
A water 2% 3000	ND	1834.2	ND	3.4	ND	148.9	ND	ND
A oil sk 1500	ND	ND	ND	ND	ND	66.8	ND	ND
A oil sk 2000	ND	ND	ND	ND	ND	89.6	ND	ND
A oil sk 2500	ND	378.5	ND	1.9	ND	61.2	ND	ND
A oil sk 3000	ND	913.6	ND	0.8	ND	81.6	ND	ND
A oil 2% 1500	ND	ND	ND	ND	ND	34.5	ND	ND
A oil 2% 2000	ND	ND	ND	ND	ND	71.8	ND	ND
A oil 2% 2500	ND	ND	ND	ND	ND	43.4	ND	ND
A oil 2% 3000	ND	ND	ND	ND	ND	39.0	ND	ND
D water sk 200	ND	ND	ND	ND	ND	ND	ND	ND
D water sk 400	ND	ND	ND	ND	ND	ND	ND	ND
D water sk 800	ND	ND	ND	ND	ND	ND	ND	ND
D water sk 1200	153.3	286.0	ND	ND	ND	ND	ND	ND
D water 2% 200	ND	ND	ND	ND	ND	ND	ND	ND
D water 2% 400	ND	ND	ND	ND	ND	ND	ND	ND
D water 2% 800	ND	ND	ND	ND	ND	ND	ND	ND
D water 2% 1200	ND	350.0	ND	ND	ND	ND	ND	ND
D oil sk 200	ND	ND	ND	ND	ND	ND	ND	ND
D oil sk 400	ND	ND	ND	ND	ND	ND	ND	ND

Table 7 Continued

D oil sk 800	ND	ND	ND	ND	ND	ND	ND	ND
D oil sk 1200	ND	ND	ND	ND	ND	ND	ND	ND
D oil 2% 200	ND	ND	ND	ND	ND	ND	ND	ND
D oil 2% 400	ND	ND	ND	ND	ND	ND	ND	ND
D oil 2% 800	ND	ND	ND	ND	ND	ND	ND	ND
D oil 2% 1200	ND	ND	ND	ND	ND	ND	ND	ND
A+D water sk 2000	ND	ND	ND	ND	ND	42.7	ND	ND
A+D water sk 3000	ND	ND	ND	ND	ND	23.2	ND	ND
A+D water sk 4000	ND	174.3	ND	ND	ND	32.9	ND	ND
A+D water sk 6000	266.7	1433.3	66.6	ND	ND	55.7	ND	ND
A+D water 2% 2000	ND	ND	ND	ND	ND	57.3	ND	ND
A+D water 2% 3000	ND	ND	ND	ND	ND	12.2	ND	ND
A+D water 2% 4000	ND	ND	ND	ND	240.4	61.6	ND	ND
A+D water 2% 6000	491.0	631.3	573.6	ND	657.1	49.3	260.1	ND
A+D oil sk 2000	ND	ND	ND	ND	ND	7.3	ND	ND
A+D oil sk 3000	ND	ND	ND	ND	ND	44.9	ND	ND
A+D oil sk 4000	ND	ND	ND	ND	ND	60.6	ND	ND
A+D oil sk 6000	1505.6	2018.6	ND	2.0	ND	79.3	ND	ND
A+D oil 2% 2000	ND	ND	ND	ND	ND	33.1	ND	ND
A+D oil 2% 3000	ND	ND	ND	ND	ND	50.3	ND	ND
A+D oil 2% 4000	ND	ND	ND	ND	ND	47.5	ND	ND
A+D oil 2% 6000	1091.5	1913.2	ND	1.5	ND	33.7	ND	ND
LSD	67.8	412.9	32.8	4.8	105.2	42.0	41.2	NA

Units are in ppb (ug/kg)
LSD=Fisher's least significant difference
ND = not detected
Means in a column that differ by ≥ LSD are different ($P < 0.05$)

APPENDIX

Appendix A- Vitamin content (mean of 3 replicates) of HTST-processed skim and 2% fat milk.

		SKIM				2%			
		Day 0		Day 10		Day 0		Day 10	
Premix concentrate	Treatment	Mean, IU/qt	SD	Mean, IU/qt	SD	Mean, IU/qt	SD	Mean, IU/qt	SD
Vitamin A sunflower oil	Control	<200	-	<200	-	299	2.00	289	7.21
	1500 IU	1521	1.71	1590	5.29	1585	4.76	1571	8.67
	2000 IU	2104	6.62	2194	8.74	2120	4.95	1920	9.18
	2500 IU	2559	10.81	2577	8.67	2517	9.99	2540	7.11
	3000 IU	3000	9.54	3001	9.05	3039	8.10	3109	5.75
Vitamin A water dispersible	Control	<200	-	<200	-	312	2.65	284	3.61
	1500 IU	1459	9.85	1500	7.21	1516	10.65	1528	8.93
	2000 IU	2029	4.28	1928	9.72	2003	4.54	2001	3.58
	2500 IU	2599	7.57	2582	6.49	2612	2.27	2536	7.52
	3000 IU	3045	6.81	3136	7.65	3007	5.46	3131	9.06
Vitamin D sunflower oil	Control	<10	-	<10	-	<10	-	<10	-
	200 IU	209	4.37	190	1.33	201	9.20	199	3.90
	400 IU	400	6.59	399	7.92	409	4.79	412	8.63
	800 IU	795	8.51	802	8.53	830	3.51	793	9.33
	1200 IU	1212	5.43	1223	7.99	1260	7.10	1197	5.29
Vitamin D water dispersible	Control	<10	-	<10	-	<10	-	<10	-
	200 IU	209	5.55	211	3.06	192	7.46	202	2.56
	400 IU	438	10.79	404	5.09	443	9.60	414	9.12
	800 IU	843	10.37	823	6.32	813	5.48	840	6.20
	1200 IU	1214	7.97	1233	8.00	1221	5.91	1216	3.99
Vitamin A and D sunflower oil displayed as A/D	Control	<200/<10	-	<200/<10	-	302/<10	2.65/-	285/<10	4.58/-
	2000/400 IU	2036/410	7.00/5.57	1999/409	5.56/5.29	2005/411	4.58/5.29	2008/402	5.00/9.85
	3000/600 IU	3031/614	5.29/3.61	3028/600	8.89/7.81	2995/603	10.15/8.72	3022/607	4.36/5.29
	4000/800 IU	4019/811	12.49/6.56	4014/810	5.57/5.56	4015/816	9.85/5.57	3989/804	9.17/6.08

Appendix A Continued

	6000/1200 IU	6101/1221	7.94/8.19	6102/1222	6.08/7.94	6003/1206	6.08/4.36	6032/1211	9.84/8.19
Vitamin A and D water dispersible displayed as A/D	Control	<200/<10	-	<200/<10	-	321/<10	7.00/-	322 /<10	2.00/-
	2000/400 IU	2035/408	4.04/4.58	2001/413	8.89/10.15	2004/399	5.29/6.56	2007/400	5.29/7.00
	3000/600 IU	3041/611	4.58/1.00	3029/606	6.08/10.54	3002/605	9.85/3.46	3034/616	4.36/2.65
	4000/800 IU	4021/815	3.00/5.57	4019/814	5.29/4.00	4025/821	5.29/3.61	4011/811	8.72/6.93
	6000/1200 IU	6103/1220	5.29/7.94	6102/1220	6.24/5.57	6107/1218	9.85/7.55	6084/1216	7.00/5.57

Values reported as international units (IU) of vitamin per quart

Appendix B- Consumer difference test results for 2% milk with vitamin A

Premix concentrate		Control vs. 1500 IU		Control vs. 2000 IU		Control vs. 2500 IU		Control vs. 3000 IU	
		No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05
Vitamin A Water Dispersible	(n=60)	16	No	18	No	20	No	20	No
Vitamin A Water Dispersible Rep 2	(n=50)	19	No	21	No	21	No	20	No
Vitamin A Sunflower Oil	(n=59)	21	No	21	No	22	No	21	No
Vitamin A Sunflower Oil Rep 2	(n=56)	19	No	16	No	14	No	24	No

Appendix C- Consumer difference test results for 2% milk with vitamin D

		Control vs. 200 IU		Control vs. 400 IU		Control vs. 800 IU		Control vs. 1200 IU	
Premix concentrate		No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05
Vitamin D Sunflower Oil	(n=61)	26	No	24	No	25	No	20	No
Vitamin D Sunflower Oil Rep 2	(n=54)	18	No	18	No	20	No	17	No
Vitamin D Water Dispersible	(n=64)	19	No	25	No	16	No	20	No
Vitamin D Water Dispersible Rep 2	(n=63)	21	No	25	No	20	No	25	No

Appendix D- Consumer difference test results for 2% milk with vitamin A and D

		Control vs. 2000/400 IU		Control vs. 3000/600 IU		Control vs. 4000/800 IU		Control vs. 6000/1200 IU	
Premix concentrate		No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05	No. Correct	Significant at α=0.05
Vitamin A&D Sunflower Oil	(n=64)	26	No	20	No	26	No	26	No
Vitamin A&D Sunflower Oil Rep 2	(n=56)	16	No	17	No	16	No	15	No
Vitamin A&D Water Dispersible	(n=64)	23	No	26	No	22	No	25	No
Vitamin A&D Water Dispersible Rep 2	(n=61)	14	No	18	No	22	No	19	No