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The Stability of Carotenogenic Food Colorants and Strategies to Prolong the Shelf Life in Process Cheese Spread

Ann Marie Doneski Craig
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Ann Marie Doneski Craig entitled "The Stability of Carotenogenic Food Colorants and Strategies to Prolong the Shelf Life in Process Cheese Spread." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

We have read this dissertation and recommend its acceptance:

John R. Mount, Federico M. Harte, Dean A. Kopsell

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Ann Marie Doneski Craig
December, 2007

Dedication

To the Furry Herd…..

Acknowledgements

I would like to start by thanking my research advisor, Dr. Svetlana Zivanovic. Her continued encouragement and unparalleled guidance truly made this research experience one of the best times in my life. I am proud to say I am a product of her teachings and wisdom and can only hope to make her proud of my achievements someday. Dr. Lana will always be an advisor to me professionally and personally.

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Abstract

Food quality is often times measured by the way one perceives the food, particularly with respect to color and texture. Color quality often pre-determines expectation making it an important parameter to understand. The same can be said for textural properties of food. Maintaining color and texture are just two ways in which the shelf-life of food can be measured. One particular product that has experienced problems in this area is processed cheese spread in which the US military uses as part of the Meal, Ready-to-Eat (MRE) rations. The cheese spread is one of the most highly accepted products in the MRE's; therefore, research was necessary to determine formulary changes that could be made in order to improve product quality and increase the parameters of its shelf life. Studies were done to determine the cheese-age effect and ingredient effects for the addition of vitamins, colorants, emulsifiers, and stabilizers. The greatest improvement for the problems of hardening and darkening over time was observed when vitamins were removed from the product. Colorants were studied in the cheese spread, as well as in model systems. Carotenoid pigments were selected to determine stability against the effects of light and oxygen, and to measure antioxidant capacity after exposure to ozone. These compounds are responsible for the yellow, orange, and reds observed in fruits, vegetables, and some algal species. Extraction from the natural source has made carotenoid pigments commercially available to the food industry. Environmental influences such as atmosphere and lighting do affect the stability of carotenogenic compounds by causing structural degradation which in turn causes changes in antioxidant abilities.

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1. Introduction

The term ‘shelf-life’ can be interpreted in many ways. A consumer thinks about the shelf-life as the length of time a product can be kept in the home before it can no longer be used, while a retailer defines it as how long the product can be safely stored prior to sale. There is no question the fundamental understanding of shelf-life entails how the product behaves over a set amount of time. During a product’s shelf-life, if properly handled and stored, few sensory, chemical, physical, or microbial changes occur. However, proper handling and storage is a difficult factor to control during distribution of food products, and it is necessary for food scientists to determine shelf-life ability for many scenarios. Every food product should be accepted as having separate, yet interdependent, chemical, microbial, and organoleptic shelf-lives since these characteristics deteriorate at different rates. The shelf-life of a food is intended to reflect the overall effect of these different attributes under a specified storage regime (Man, 2002).

Color is one of the first recognizable indicators of food quality. Whether naturally present or artificially added, colorants often pre-determine the desired expectation. Therefore color quality is an important consideration in prolonging the shelf-life of a product. Undesirable color will often lead the consumer to believe the food product is past its expiration. Understanding the behavior of colorants in different systems is the first step in determining ways to extend their stability. Proper handling and storage conditions minimize the effects of possible over exposure to light, oxygen or other degrading environments.

In addition to providing desirable appearance, some colorants add an antioxidant capacity to foods. Colorants can behave as internal antioxidants to the food system, thus reducing lipid oxidation, or they may add health benefits to the consumer. One group of colorants exhibiting antioxidant properties are the carotenoids. These naturally

occurring compounds are responsible for the yellow, orange, and red colorations in foods. Found naturally in many fruits and vegetables, these compounds are extracted or synthesized and used in the coloring of food. One of the most commonly used colorants is annatto (actually containing two carotenoids, bixin and norbixin). It has been used for over one hundred years to impart color to many foods, including cheese (Lauro et al., 2000).

The processing stability of carotenoids has long been a focus of research. Their wide application in the food industry has come from their ability to be incorporated in aqueous systems, emulsions, or even complexed with proteins. However, depending on the carotenoid, exposure to thermal processing, and storage conditions, degradation may occur. When this happens, the color diminishes and numerous secondary degradation products are formed. Understanding the colorant stability is just one consideration in overall possible shelf-life extension.

Color stability is one of many scenarios leading to shelf-life improvement. There is generally more than one factor affecting shelf-life making a single, conclusive test unattainable. Proper temperature of the food matrix throughout handling and storage, limited physical abuse during transportation and handling, and proper packaging all play an important role in shelf-life determination. Continued research in this area is crucial for safety, nutritional reasons, and consistent quality expectations on the part of the consumer.

The focus of this work entails: the study of carotenogenic food colorants and their stability to light, dark, nitrogen, air, and ozone environments; the ability to conclusively measure their antioxidant capacity; and their incorporation into a processed cheese spread. Also, as part of the shelf-life improvement of the cheese spread, several

physical parameters were measured over a timed-temperature storage period and individual ingredient effects were determined.

2. Review of Literature

2.1 Shelf Life

Food is undoubtedly one of the most complex systems studied by researchers, particularly the laborious task of shelf-life determination since physical, chemical, and microbial changes are constantly occurring. It is therefore crucial that all the different reaction possibilities be understood. Determination of shelf-life of a food product requires the utilization of storage tests. The most common tests involve storage under conditions that mimic manufacturing and consumer handing. The problem with this is when a product has a very long shelf-life and therefore changes in formulations would take a long, unpractical time to conclude. Accelerated shelf-life testing (ASLT) is a way to study changes in food under shorter, more stressed conditions in order to estimate a safe and reasonable shelf-life (Ragnarsson et al., 1977).

Physical Changes

Physical changes in food most commonly occur by incorrect handling during harvesting, processing, distribution, and consumer care. All of the possibilities for physical abuse affect the shelf-life of the food product, as well as consumer approval. For example, when dried foods are exposed to humid conditions the product may become moist and rendered unacceptable. A very common sign of mishandling lipid-containing candies, like chocolate, is the effect of fluctuating temperatures resulting in 'fat-bloom'. This is the visual separation of the butter from the chocolate when temperatures are altered between cold and hot. The white or grey residue on the chocolate surface is undesirable to most consumers, although there are no nutritional problems. Lastly, a widespread problem limiting a products shelf-life is through general handling. Fruits and vegetables can become easily bruised leading to color changes by

enzymatic browning. Mishandling of dry foods like crackers, chips, or cereals can result in a broken or cracked product.

Chemical Changes

Throughout the processing, handling, and storage of foods, chemical reactions are constantly occurring between the numerous components. All of these changes affect the shelf-life of the product, generally reducing it in some fashion. Typically, the reactions taking place involve enzymatic action, oxidative reactions, non-enzymatic browning, light-induced, and package interactions (Man et al. 2000). The rate at which these chemical reactions occur can be dependant on factors such as water activity or unwanted exposure to oxygen or light. Enzymes, for example, are proteins that act as catalysts in most chemical reactions like proteases, lipoxygenases, and phenol oxidase, just to name a few. Oxidative reactions can occur in meats resulting in myoglobin and oxymyoglobin being oxidized into metmyoglobin, causing a red to brown color change. Other lipid oxidation reactions tend to occur with unsaturated fatty acids, which lead to rancidity problems. A classic example of a chemical change in food is due to non-enzymatic browning and is commonly referred to as Maillard reactions. These reactions, between amino groups of proteins and carbonyl groups of reducing sugars, lead food through a series of transformations and polymerizations responsible for the degradative browning color and possible textural changes. Maillard browning can be related to nutritional losses in food especially when the amino acid lysine is involved. Related to Maillard browning is the loss of carbohydrates, especially those with reducing carbonyl groups. Carbohydrates also may undergo carmelization, although the higher temperatures that are needed for this reaction is not typically reached during normal distribution and storage of a carbohydrate-containing food product. (Singh et al., 2004)

Microbial Changes

Although microbiological effects are not in the scope of this project, it can not go without simple mention that microbial stability really is an important consideration in food preservation.

In general, microbial growth in a food product requires certain intrinsic parameters be optimized. These parameters are based on chemical principles which include pH, moisture content, re-dox capacity, and presence of any naturally occurring antimicrobials. Microbial survival, like many chemical reactions, is also reliant on certain extrinsic factors like temperature, relative humidity, and atmosphere (Jay et al., 2005). Although much research has been conducted, the need to continue bridging the gap between microbial and chemical parameters in regard to water activity, pH, and physical state of food, is an ever-growing field.

Shelf-life Evaluation

There is a constant desire to have a longer shelf-life of food products. It has become increasingly unrealistic for manufacturers to rely on long storage tests. Most companies want to get new products out as quickly as possible, so a faster means to determine shelf-life is necessary. Procedures have been developed to quickly estimate a shelf-life based on accelerated methods, or by using computer-based models (Man 2004). Accelerated testing is designed to mimic ambient storage processes using a stressed condition, usually based on a temperature increase to shorten the storage time necessary for changes in the product to occur. Shelf-life predictions using computer modeling is based on fundamental principles of food quality loss modeling and primary kinetic modeling on diverse deterioration mechanisms (Labuza 2000). Selection of a dependable and suitable approach to shelf-life determination is the important first step.

Reaction Kinetics

Chemical kinetics is the study of chemical systems whose composition changes with time and can involve gas, liquid or solid phase substances (Steinfeld et al., 1999). Many researchers have attempted ways in which to use this theory to predict shelf-life of food. Labuza (2000) employed the chemical kinetic theory with random variable temperature conditions to obtain an efficient temperature, which has simplified the calculations for real-time temperature fluctuation. A similar advance, along with the Arrhenius relationship, was suggested by Buera and Karel (1993) that describes the influence of temperature on reaction rate constants. Other studies have also looked at methods to determine the value of the loss of activation energy from shelf-life data at known temperatures (Singh 2000).

Rate Reactions

A general chemical reaction model can be obtained by the following reaction (*eq. 2.1*):

$$aA + bB \underset{k_b}{\overset{k_f}{\leftrightarrow}} cC + dD$$

where A and B are reactants, C and D are products, *a,b,c,* and *d* are reactant coefficients, and k_f and k_b are rates for the forward and backward reactions, respectively. The rate equation for reactant A as it changes can be written as (*eq 2.2*):

$$\frac{-d[A]}{dt} = k_f[A]^{\alpha}[B]^{\beta} - k_b[C]^{\gamma}[D]^{\delta}$$

where A, B, C, and D are concentrations with respective exponent representing reaction orders, and *t* is time. These two equations represent general cases and are unsolvable due to numerous unknowns. Simplification is necessary so either the forward or backward reaction is dominant, usually done by assuming one reactant is present in a

much higher amount compared to the other. In regards to [A], assuming [B] is high, the following can be written (*eq. 2.3*):

$$\frac{-d[A]}{dt} = k'_f[A]^n$$

where, *n* is the reaction order and k'_f is the pseudo forward reaction rate constant. Since foods are so complex, intermediate reactions leading to quality changes are hard to discern. With food, a general rate expression may be written as (*eq 2.4*):

$$\pm\frac{d[Q]}{dt} = k[Q]^n$$

In this case, the ± symbolizes the decreasing or increasing value of quality attribute *Q*, *k* is the rate constant, and *n* is the observed order. It is understood that temperature, relative humidity, light and concentrations of other components be kept constant.

Zero-Order Reactions

When shelf-life in food is being determined, a decrease in quality attributes is likely occurring at a given rate. The rate expression for the decrease of [*Q*] is written as (*eq. 2.5*):

$$-\frac{d[Q]}{dt} = k[Q]^n$$

In any reaction, there will be an initial and final parameter like $[Q]_0$ and $[Q]_t$, respectively. The integrated form of the rate expression can then be written as (*eq. 2.6*):

$$[Q]_t = [Q]_0 - kt$$

Zero-order reactions assume a linear plot. Such reactions as enzymatic degradation, non-enzymatic browning, and lipid oxidation follow zero-order kinetics (Singh 2000).

First-Order Reactions

It is assumed again that in shelf-life determination a decrease in quality is being measured. The rate expression in eq. 2.5 is a valid starting point in accessing first-order reactions. The difference comes from the fact that first-order reactions in food measure the loss of a quality attribute dependant upon the amount of the attribute that remains at a certain time point. This kind of exponential decay will have n= 1 thus changing eq. 2.5 to the following (*eq.2.6)* and separation of variables to integrate (*eq. 2.7)*:

$$-\frac{d[Q]}{dt} = k[Q] \qquad \text{and} \qquad -\frac{d[Q]}{[Q]} = k\,dt$$

After integrating (*eq 2.8*):

$$\ln\left(Q/Q_0\right) = -kt$$

The types of deterioration that occur via first-order reactions include vitamin and protein losses, and microbial growth.

(Equations 2.1 thru 2.8 are adapted from Singh 2000 and Steinfeld et al., 1999.)

Temperature Kinetic Model/ Arrhenius Relationship:

Environmental factors, such as temperature, can greatly affect the rates of the reactions and need to be closely monitored during kinetic experiments (Labuza 2000). The rates of chemical reactions increase sharply with the increase in temperature. This theory was best described by Arrhenius in 1889 where he proposed a kinetic model expressed as (*eq. 2.9*):

$$k = k_0 \exp(-Ea/RT)$$

where k_0 is a constant, *Ea* is activation energy, *R* is ideal gas constant, and *T* is temperature. Since temperature change is the important effect on reactions rates, the

Arrhenius model is broadly used. This model was theoretically designed for reversible, complex chemical reactions like viscosity, diffusion, and sorption. The quality loss seen in food can follow such a kinetic action, but has an alternate way of expression. The term Q_{10} is often used to describe the relationship between temperature and reaction rate constants. Q_{10} is defined as (*eq. 2.10*):

$$Q_{10} = \frac{\text{reaction rate at temperature (T + 10)°C}}{\text{reaction rate at T°C}}$$

This is a useful model for describing how much faster one reaction will go in a high abuse temperature versus the same reaction at another temperature. This basic approach of using Q_{10} has made it tantamount with ASLT and easy to understand. Essentially, a 10^0C increase will double reaction rate, while a 10^0C decrease will cut reaction rate in half (Franks 1994). If accelerated temperature factors are known, then extrapolation to lower temperatures may also be predicted. This is the fundamental principle behind ASLT (Mizrahi 2004; Lebuza 2000; Man 2002).

Accelerated Shelf life Test Limitations

Accelerated storage tests do have limitations and they tend to be product specific (Man 2002; Labuza 2000). Some of these limitations include: 1) temperature changes affecting physical state which alters the rates of other reactions; 2) elevated temperature storage with inappropriate relative humidity conditions; 3) short shelf-life refrigerated or frozen foods, since reactants may concentrated in liquid where Q_{10} value is unaccounted; 4) Arrhenius model is inappropriate for most foods because of their complexities (Cohen et al., 1985); and 5) gas solubility in fats or water decrease with

increase temperature, thus lowering rate and under prediction of shelf-life (Ragnarsson et al., 1977).

Food Compositional Differences

There are many parameters in a food system that can dictate what, and how reactions take place. Primarily, water has one of the greatest effects in food with regard to stability, palatability, and quality. Physiochemically water is related to water activity, $a_{w,}$ which is a measure of the water availability. Most deteriorative changes and microbial growth occur at a_w >0.6, however some chemical and enzymatic changes may occur at considerable low a_w activities (Roos, 2001). Water activity is a thermodynamic property of the water in food which in part acts as the food's solvent (Labuza 2000). Understanding and controlling a_w is the basis for preservation.

In more specific reactions, like Maillard and lipid oxidation, there are several factors that will regulate reaction rate, ultimately affecting shelf-life. The most important factors for Maillard reactions are the availability of free amino groups, reducing carbonyl groups, temperature, pH, and water activity. The effect of temperature is particularly important since increased reaction rates promote browning, changes in texture, and aroma profiles. The pH can affect production of volatiles and colored products, and browning occurs faster in neutral foods and slower in acidified food. Water activity for Maillard reactions is most desired around 0.65-0.75, known as a low-moisture environment (Arnoldi, 2004). Similarly, in lipid oxidation, temperature increases the rate of oxidation and as reaction rate increases, the a_w decreases (0.6-0.8 range) (Gordon 2004). Lipid oxidation is not governed by strict pH values; however, the presence of metals can also dictate reaction rates (Ragnarsson et al., 1977; Labuza 2000).

Advances in Shelf-Life Extension

To ensure that a final shelf-life is appropriate, at least four different types of considerations need to be noted. These include: 1) the initial shelf-life study typically done during product conception; 2) preliminary determination carried out later in product development; 3) confirmatory determination performed at the end of product development; and 4) routine determination executed at various times during normal production. (Man 2002) The precise shelf-life test is product-dependant and may include microbiological measurements, chemical analysis, and physical testing like rheology, color, texture, and emulsion stability.

2.2 Carotenoids

Color and Colorants

To fully understand the stability and application of colorants in food, some basics must be considered. Color refers to how one perceives an object - yellow, red, orange, etc. Colorants are chemicals that can be naturally occurring, or synthetically derived. Foods have color characteristics because of their ability to reflect light at wavelengths that stimulate the retina of the eye. Color perception depends on the type and intensity of light, chemical and physical characteristics of the food, and an individual's ability to distinguish color (Hutchings 1999; MacDougall 2002).

The addition of color to food products is an important factor in consumer acceptance. Colors must be pleasing to the eye and safe for the body since it is ranked alongside freshness as one of the key criteria governing selection (Baker et al. 2004). A color additive is defined the Food and Drug Administration (FDA) as any dye, pigment or substance that can impart color when added or applied to food, drugs, and cosmetics

or to the body. Today, in the United States, all food color additives are regulated by the FDA to ensure safety of the products. All color additives used in food are divided into two categories, “certified” and “exempt from certification”. Certified colors are manmade, while those exempt from certification include pigments that are derived from natural sources such as minerals, animals, vegetables, and man-made counterparts from the natural derivatives. Certification has no bearing on their overall safety; however, exempt colors are perceived as less of a health hazard than those derived synthetically from petroleum sources. (Griffiths 2005)

The colors added to a food may offer biological benefits as well. Although most colorants are low in flavor, some have nutritional effects, like β-carotene. β-carotene belongs to a group of naturally occurring compounds that exhibit antioxidant capabilities. These compounds are known as carotenoids.

The carotenoids are one of the largest group of pigments and certainly the most well known. Carotenoids are one of the most important classes of plant pigments. They play a crucial role in defining quality parameters of many fruits and vegetables. There are approximately 600 isolated carotenoids, of which about 50 are present in a typical diet (Krinsky 1994). Chemically they are sorted by structure into two classes, the carotenes and the xanthophylls, each demonstrating unique biological activity. It is the structure of a carotenoid that determines its biological function and coloring capability. (Rodriguez-Amaya 2001; Delgado-Vargas et al. 2000)

Structural Importance

Food carotenoids are built from eight C_5 isoprenoid units resulting in the usual C_{40} backbone. This basic structure can be linear or cyclized at one or both ends with lateral methyl groups along the chain. Other modifications include hydrogenation,

dehydrogenation, double-bond migration, isomerization, chain shortening, and inclusion of oxygen. The carotenoids void of any oxygen species are referred to as the carotenes, while those containing oxygen are known as the xanthophylls. Examples are shown in Figure 2.1.

Sources of dietary carotenoids for humans come from fruits, vegetables, fish and other meats. It is thought that a diet high in these foods can protect against disease. Such diseases include atherosclerosis, certain types of cancers, and age-related macular degeneration (Hinds et al., 1997). Carotenoids are known for their pro-vitamin A activity. Vitamin A can be produced within the body from certain carotenoids, notably β-carotene. This activity serves many vital systemic functions in humans, for example, it is essential for vision; immune response; epithelial cell growth and repair; bone growth; reproduction; maintenance of the surface linings of the eyes; and epithelial integrity of respiratory, urinary, and intestinal tracts (Rodriguez-Amaya 1996). However, not all carotenoids have pro-vitamin A activity. Only those carotenes with an unsubstituted β-ring and a C_{11} polyene chain exhibit the ability to convert to retinol, or Vitamin A (Rodriguez-Amaya 1996 and 2001; Delgado-Vargas et al., 2000).

Aside from their brilliant pigment ability and potential health benefits, carotenoids also exhibit strong antioxidant properties (Paiva et al., 1999). They are considered as potential membrane antioxidants due to their strong interaction with reactive oxygen species and free radicals. By intercepting these damaging chemical reactants, carotenoids essentially save lipids from the destructive behavior of radicals. Their distinct chemical structure is what allows for energy absorption from the reactive species. This antioxidant behavior is what accounts for their important function in food and biological systems (El-Agamey et al., 2004; Krinsky 1994; van den Berg et al. 2000). Figure 2.2 depicts the many physical and chemical properties of carotenoids.

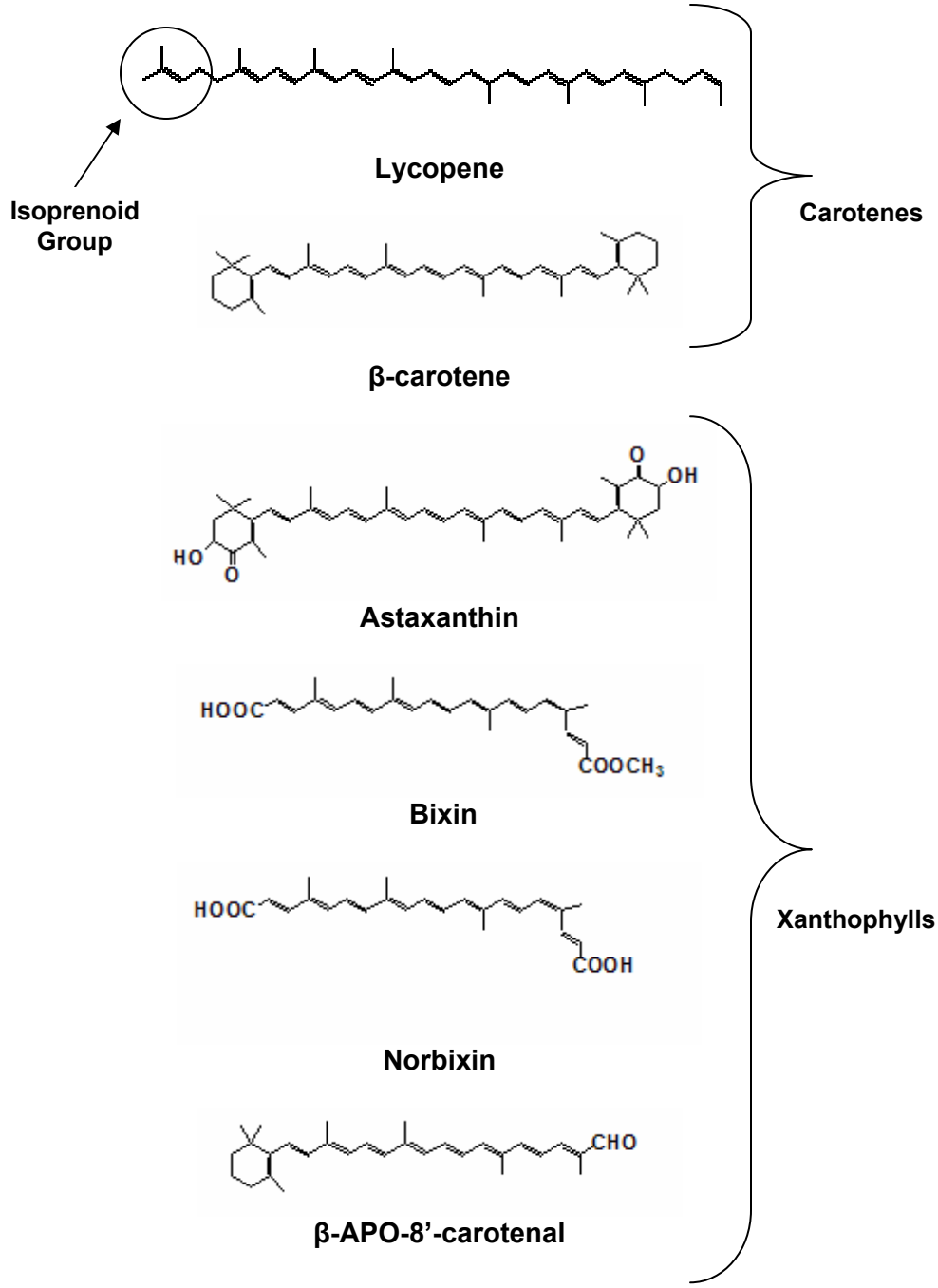


Figure 2.1 Structures of selected carotenes (lycopene and β-carotene) and xanthophylls (all others)

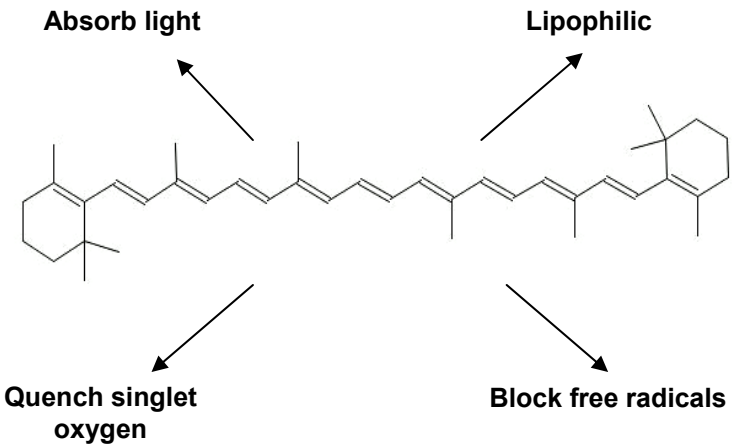
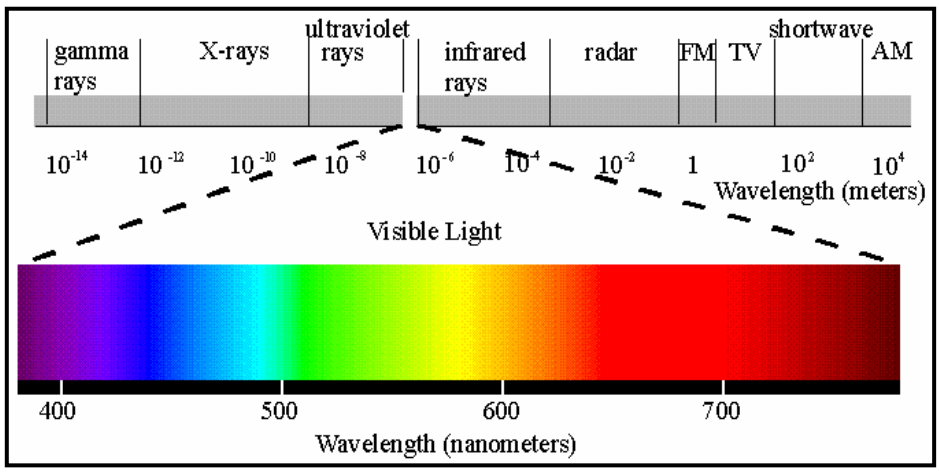


Figure 2.2 General physical and chemical properties of carotenoids

The colorant capacity of a carotenoid is imparted by its conjugated double bond system that resides mainly in a trans configuration. Any extensive conjugated system is highly delocalized about the pi-bonds, those forming the double bonds. As a result of delocalization, carotenoids exist in a low-energy excited state. Essentially, this is the reason we see the color that we do. When samples are exposed to energy that matches a possible electronic transition within a molecule, part of the light energy will be absorbed as the electron is promoted to a higher energy orbital. The transition energy is in the visible region of the electromagnetic spectrum ~380-800 nm, but more specifically are the carotenoids in the 400-500 nm range which is where yellow, orange, and red hues are observed. Figure 2.3 depicts the spectrum and colors associated with energy transitions.

A



B

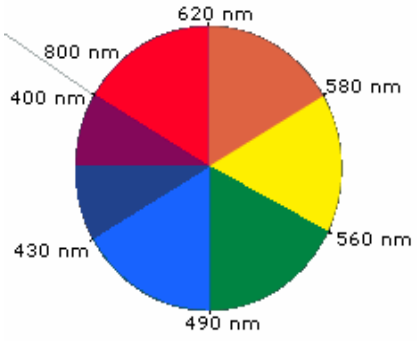


Figure 2.3 (A) Electromagnetic spectrum indicating the visible region of the spectrum. The colors shown are those of the absorbed energy.(B) A color wheel depicting complementary colors. (Adapted from cem.msu.edu/~ruesch/virtualtext/spectrpy/uv-vis/spectrum.htm)

In addition to the absorbing light energy, carotenoids are capable of energy transfers to the excited state of itself known as a triplet state species. This ability is what allows for the structure to accept the transfer of energy from highly reactive species. Most notably, singlet oxygen ($^1O_2$). The first reaction between carotenoid and singlet oxygen was determined using β-carotene (Foote et al. 1968) and the theory is now widely accepted. The ability to quench is based on the number of conjugated bonds with in the carotenoid structure, thus lycopene is more effective than β-carotene (Krinsky 1994). Research by Cantrell et al. (2003) suggests that singlet oxygen quenching is dependant on the type of carotenoid, xanthophylls or carotenes, and the environment in which they are incorporated, i.e. a lipid membrane. Their results still propose lycopene and β-carotene as the fastest to quench, while lutein was slowest and astaxanthin and canthaxanthin were intermediate quenchers. The quenching ability of the xanthophylls may be hindered by the longer delocalized pi-bond system present in their structure.

Carotenoids are a very diverse group of compounds given their physicochemical properties. The structure determines the unique functionality that each carotenoid possesses. Depending on the molecular arrangement and length of the conjugated bond system, carotenoids can act as a singlet oxygen quencher or free radical blockers. Current research has established the efficacy of carotenoids in intercepting such detrimental molecules, thus adding to the vital diversity of these compounds and also assigning them as antioxidants.

Dietary Antioxidant Capacity

Autoxidation of lipids and free-radical generation is a natural process. In food systems, the naturally present antioxidants can be lost during processing and storage, making it necessary for the further addition afterwards. Antioxidants successfully hinder

the onset of lipid oxidation in food systems. Because of this action they truly are an essential group of additives due to the enhancement of shelf-life stability and sustained sensory qualities (Madhavi et al., 1996).

Lipids are one of the major food constituents. The oxidative damage of lipids can cause rapid degradation leading to many undesirable effects. When oxidation in food occurs, often nutritional losses take place and production of off-flavors, off-colors, and potentially toxic compounds can occur (Min et al., 2002). All this essentially leads to a lack of acceptance by consumers. Lipids undergo such decline in a variety of ways during the handling, processing, and/or storage of a food product. In highly unsaturated fats, oxidation can lead to the formation of polymeric end products, while the rancidity, reversion, and odors occur more readily with oxidation of moderately unsaturated fats (Jadhav et al., 1996). Antioxidants are generally added to stop these effects. This is an example of an indirect benefit by antioxidants since the consumption of lipid oxidation products is reduced. A direct benefit would be antioxidant consumption to stop *in vivo* lipid oxidation.

Since carotenoids possess antioxidant capacity, it is natural to believe that the addition of these compounds to foods would either protect the food matrix itself or, by consumption, protect against possible disease. In foods, the processing effects, improper handing or storage, and even the environment can have detrimental effects to the antioxidant capacity by causing changes in its chemical structure (Lin et al. 2005; Delgado-Vargas et al. 2000; Martley et al., 2001; Carnevale et al., 1979; Minguez-Mosquera 1993). The study to determine antioxidant capacity in foods and their components has become a major focus of research.

There are different methods in which to determine antioxidant capacity. They include ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity

(TEAC), and oxygen radical antioxidant capacity (ORAC). The ORAC test shows the change in fluorescence intensity as a measurement of the degree of free radical damage against the Trolox standard. Trolox is a water-soluble vitamin E analog used such that differences between instrumentation and operators can be standardized. If an antioxidant is present, free radical damage will be repressed. ORAC values essentially indicate if a sample possesses any chemically active antioxidants, it is not an indication of bioavailability (Wu et al. 2004; Ou et al. 2001).

2.3 Process Cheese Spread

Functionality of Process Cheese Spread

Federal standards have been established for allowable ingredients, moisture content, and fat content of process cheese spread. Pasteurized process cheese is defined as a "food prepared by comminuting and mixing with the aid of heat, one or more natural cheeses of the same or two or more varieties", by the Food and Drug Administration. The fat content can be no less than 47%, and moisture content can be no more than 43%. Other ingredients allowed include emulsifying salts, acids, water, salt, coloring, and enzyme-modified cheese (EMC). From this basic definition for processed cheese, a few modifications are recognized for a process cheese spread product. Cheese spread follows all the same manufacturing guidelines, but is distinguished by the fact it must be spreadable at 70°F (21°C). Moisture cannot be greater than 44% and fat must be at least 20%. It must contain 51% cheese, excluding optional dairy ingredients like milk or EMC. Additional ingredients such as hydrocolloids, sweeteners, and acidifying agents can be utilized. These additional additives are usually

used in food to assist with the production, processing, packaging, or storing of the food product.

The proper manufacture of process cheese spread involves many variables that undoubtedly determine the quality of the finished product. Process cheese products begin by extruding and blending, if needed, a single type or a combination of natural cheeses. The blended cheese is then mixed with additional fat, water, emulsifiers, stabilizers, and colorants. The entire mix is then agitated to produce a homogeneous stable emulsion. Processing temperatures must reach 170°F (76.6°C) to ensure microbial growth is reduced; however, the heat applied can be higher if a “cooked” flavor is desired. The process may sound simple, but many attempts at making processed cheese were not successful due to the product drying out or separating. Food additives are necessary to develop a stable food product (Fox et al., 2000). Table 1 shows the non-cheese ingredients used in pasteurized processed cheese products. A review of ingredients will be discussed in more detail following the table.

Stabilizers

Stabilizers are often required to maintain texture, viscosity, and water-controlling properties in various food products. Chemically, stabilizers are polysaccharides and are commonly referred to as gums. The distinctive feature of gums is their strong affinity for water and how they maintain a high viscosity in aqueous solutions. Gums, in concentrations used as stabilizers, do not form gels, but rather they retain plasticity. The reason for this unique function is based on their molecular structure. Essentially the large chain structure can trap water molecules; this interaction ensures some level of viscosity. No matter the strength of the gum network, it still entraps liquid and takes on the properties of a viscoelastic solid.

Table 2.1. Non-cheese ingredients used in process cheese foods

Ingredient	Function	Example
Dairy:		
Milk fats	Composition and texture	Butter, plastic cream
Milk protein	Composition, texture, and meltability	Casein, caseinates
Stabilizers	Aids in physiochemical stability; provides texture	Carrageenans, guar gum, locust bean gum, xanthan gum, low-methoxypectin
Emulsifier	Ensures uniform dispersion	Mono- and Diglycerides
Acidulant	Assists in pH regulation	Food- grade acids: lactic, citric, and phosphoric
Colors	Visual appearance	Annatto, beta-carotene, beta-apo- carotene, astaxanthin
Flavoring	Enhancement	Sodium chloride
Preservatives	Prolong shelf-life	Potassium sorbate, calcium or sodium propionate
Vitamins	Product enrichment	Vitamins A, B6, B1 and C

The gums listed in Table 1 have similar properties; however, they do differ slightly by specific functionality. Knowing the characteristics of each allows for appropriate application in the food system. (Descriptions listed below have been compiled from Whistler et al., 1997, McClements 2005, Cui 2005).

- *Carageenan*: A linear sulfated galactan polysaccharide, stable over a wide pH range. They produce strong, rigid structures, especially in the presence of potassium and calcium ions. They are thermoreversible when cooled. Kappa-carrageenan, a specific variation in this group, has strong reactivity with the milk protein casein. It is able to stabilize casein by charge interaction, incorporating it into the gum's network.
- *Guar Gum*: This long, rigid polysaccharide made up of galctomannan and galactose subunits is stable over a wide pH range, but will degrade at certain pH levels when high temperatures are reached.
- *Locust Bean Gum:* This irregularly shaped molecule consists of branching galactomannan subunits. This polymer is only slightly soluble in cold water so it requires heat to achieve full hydration, thus maximum viscosity. This gum is used in many dairy products to help resist heat shock to the food product.
- *Xanthan Gum:* Structural backbone consisting of glucose subunits with branched groups that ensure a high affinity for water entrapment. It is soluble in hot and cold mediums, stable to a large pH range when in the presence of high salt concentrations. Xanthan gum exists synergistically with galactomannans such as locust bean and guar gum by increasing viscosity and/ or gel formation.
- *Low-methoxy Pectin:* This is a linear polysaccharide consisting mainly of galacturonic and galacturonic methyl ester subunits. There are high and low-methoxy equivalents, the low have less than 50% methyl ester subunits attached.

For these, the presence of calcium ions increases the gelling temperature and ultimately the strength.

Emulsifiers

Emulsions are composed of immiscible liquids. Mixture of oil and water is the classic example. Emulsifying is done by adding one ingredient to another then mixing. The agitation disperses and suspends minute droplets of one liquid throughout the other liquid. To stabilize such mixtures the use of emulsifiers, a surface-active ingredient, has to be added to reduce the interfacial energy between the droplets of each liquid.

There are two primary types of emulsion systems found in foods, oil-in-water (O/W) and a water-in-oil (W/O). The O/W has droplets of oil suspended in an aqueous continuous phase. An example would be mayonnaise or creamers. The second emulsion, W/O, consists of water droplets dispersed in an oil phase. Examples include butter and margarine. There is a third type of emulsion currently being researched called a multiple emulsion in which three components are present like W/O/W and O/W/O. However, these multiple emulsions are still being studied to determine if they can be produced economically and have a sufficiently long shelf-life (McClements 2005).

Emulsifiers are necessary in all types of emulsions to aid in uniform quality, texture and shelf-life. Emulsifiers are defined as any substance that can reduce surface tension between oil-water or air-water (Belitz et al., 2004). Reduced surface tension results in increased emulsions stability. Food emulsifiers do not interrupt the emulsification process; they simple enhance and stabilize it. A major factor in processed cheese stability is being able to control texture properties. The emulsification process, where the cheese is blended with emulsifying salts and stabilizers, is ultimately responsible for providing such control over texture and mouthfeel properties (Gupta et

al., 1984). Emulsifying salts are not really emulsifiers; however, they promote physicochemical changes such as fat emulsification and protein rehydration through ionic interactions (Fox et al., 2000). Essentially, they help the fat in the processed cheese to break down into smaller particles.

Emulsifiers and emulsifying particles tend to promote dispersion of the phase in which they do not dissolve. Emulsions typically have a cloudy appearance, because the phase interfaces that scatter light as it passes through the particles ranging is size from 0.2 to several micrometers (Fenemma 1996). Emulsions are part of a more general classification of two-phase systems called colloids. The terms 'emulsion' and colloid' are sometimes used interchangeably; however, 'emulsion' tends to imply that both the continuous and dispersed phases are liquid, although the separation of two media like liquid and gas is can also be considered an emulsion besides a typical oil and water system (Cui 2000).

Acidulants

Acids occur extensively in food systems and offer a variety of function. They provide acidity that contributes to flavor and preservation. Some acids can also act as chelating agents, buffers, and coagulation agents. Depending on the acid used, each has a particular function and can vary its role depending on the food environment. Also, the strength of the acid affects its functionality. A very low pH may impart a very tart or sour flavor, despite benefits of increased antimicrobial effect.

Another important consideration of some acidulants is their ability to form ring structures with metal ions. This is called chelation. Chelating agents prevent metal ions from reacting with other materials or catalyzing damaging reactions. Metal ions in food products catalyze oxidation reactions that lead to fat rancidity, flavor degradation and

browning. The sequestering ability of the acidulants can provide protection from degenerative reactions. The following list offers a brief description of some common food acids.

- *Citric Acid:* The most commonly used food acid. It can be extracted from citrus fruit or produced from sucrose (sugar) fermentation. Both products are identical. Citric acid is a strong chelating agent used in foods (pKa=3.13, 4.76, and 6.40). It also offers an effective range for buffering purposes, pH 2.5 - 6.5. It is odorless with a strong acid flavor. The FDA lists citric acid as GRAS (Generally Recognized as Safe).
- *Lactic acid:* It can be chemically synthesized or produced through lactose fermentation. Lactic acid is a natural product in cheese. It is capable of reasonable chelating ability. It is can be used in conjunction with other acidulants to be optimally effective in food preservation systems. Degradation of lactic acid in fermented products does not readily occur. This acid can impart a mild flavor, with no lingering results. The FDA considers lactic acid GRAS.
- *Phosphoric acid:* This is one inorganic acid used in food and it is chemically processed. The pH (~2) is lower than the aforementioned organic acids. Phosphoric acid is a reasonable metal ion chelator. It has a harsh, sour taste; therefore it is used minimally and in the presence of other flavoring agents. It is mainly used in soft drinks.

Vitamins

Vitamins are complex organic molecules that offer unique nutritious benefits. Chemically and biologically speaking, they are very different, yet they seem to have some common functionality. The commonality is a result of their essential necessity in

the human diet in small amounts. Their absence can certainly have detrimental dietary consequences. Some examples include Vitamins B1 and B6, A, and C. Vitamin B1, or thiamine, is needed to process carbohydrates, fat, and protein. Vitamin B6 is used for amino acid processing in proteins. These are both water-soluble vitamins. Vitamin A is a fat-soluble vitamin, which serves many purposes in the body. It is essential for cell reproduction and differentiation, and it is required for the transduction of light into nerves in the retina. Vitamin C, another water-soluble vitamin also known as ascorbic acid, is another major contributor to many biological functions. One function is as an antioxidant. As an antioxidant, it protects high-density cholesterol from undergoing oxidative damage, which can lead to heart disease (Joshipura et al., 2001). Although nutritionally desirable in food, when ascorbic acid is present in excess, non-enzymatic browning may result rendering a visually undesirable product (Hui et al., 2006).

The exact pathway to ascorbic acid browning is highly variable and dependant on the food system. A description of the chemical pathway of L-ascorbic acid and how it relates to food is presented by Liao et al. (1988). The exact process is complex since the formation of over one-hundred different intermediates and degradation products have been identified (Shultz et al., 2007). The basics of what is known is that if oxygen is present then L-ascorbic acid is oxidized primarily to L-dehydroascorbic acid; however, under anaerobic conditions this product is not formed, but rather other keto-acids ultimately leading to the formation of furfural. Furfural and other carbonyl containing reaction intermediates from either oxidative or non-oxidative pathways can then react with amino acids resulting in formation of brown polymers (Hui et al., 2006). Formation of brown polymers can also be explained in terms of the classic Maillard reaction between a carbonyl group of a reducing sugar and an amino acid (Fenemma 1996). These carbonyl-amino reactions are accelerated in the thermally processed environment. In the

cheese spread, there may be residual lactose, a reducing sugar, present from the initial cheddar cheese production (Fox et al., 2000). Also, due to the presence of thiamine and pyridoxine, any reducing sugar may be susceptible to nucleophilic attack by the amino moiety present from the vitamin (Doyon et al., 1983).

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3. Stability of carotenoids as affected by light and oxygen

Abstract

Carotenoids used as natural food colorants often undergo various processing and storage conditions that can induce isomerization or degradation of the molecule. These changes due to photochemical modification were studied using five different carotenoid colorants including β-Carotene, β-APO-8'-carotenal, astaxanthin, annatto, and a paprika oleoresin. Samples were prepared at an appropriate concentration to be followed by UV-Vis spectroscopy, and then stored at 29°C±2 in the dark or exposed to 6000 lux fluorescent lighting. All carotenoid samples were stored under air or purged with nitrogen gas to simulate an oxygen-free atmosphere. The colorants were analyzed over six-weeks by UV-Vis spectroscopy, a Hunter MiniScan Colorimeter, and High Performance Liquid Chromatography (HPLC). Overall trends indicate that sample degradation occurs more readily in the air and light environments as compared with those in the dark and nitrogen purged. Annatto was nearly diminished after only three weeks under air and light exposure as was similarly observed for β-Carotene in the same conditions. The astaxanthin, β-APO-8'-carotenal, and paprika oleoresin were all similar in air-dark, and nitrogen-light and nitrogen-dark environments.

Introduction

As consumers, we are presented with a kaleidoscope of colors when making a food selection. Color dictates ones perception of quality and flavor expectation. The intensity of color can also influence our perceived notion of flavor, for example, a deeply colored red beverage may have a strong flavor versus a faintly pink solution. However, with the constant technological advances in food science, this may not always be the

case. The action of adding colorants to food is also a manufacturing necessity for uniformity of samples under constant production.

Naturally occurring carotenoids pigments are often used as food colorants resulting in products that are appealing (Delgado-Vargas et al., 2002). Carotenoids are responsible for the yellow, orange, and reds that are observed in many fruits, vegetables, and even crustaceans. Carotenoids are certainly one of the more abundant pigments in biological systems (Krinsky 1994). There are many benefits to utilizing these colorants besides being observed for their beauty. For example, if astaxanthin, a red carotenoid, is added to salmon feed which in turn produces a more appealing flesh tone, people may be more inclined to consume the fish thus consuming more ω-3 fatty acids. (Baker et al., 2004)

For several years, carotenoids have been the focus of researchers for their antioxidant potential (Edge et al., 1997). They may act as antioxidants within a food system to protect lipids from undergoing oxidation or have dietary benefits. Sources of dietary carotenoids in humans come from fruits, vegetables, and fish. It is thought that a diet high in these foods can protect against disease. Such diseases include atherosclerosis, certain types of cancers, and age-related macular degeneration (Hinds et al., 1997). Carotenoids are also known for their pro-vitamin A activity (Rodriguez-Amaya 1996). Vitamin A can be produced within the body from certain carotenoids, notably β-carotene. This activity serves many vital systemic functions in humans, for example, it is essential for vision; immune response; epithelial cell growth and repair; bone growth; reproduction; maintenance of the surface linings of the eyes; and epithelial integrity of respiratory, urinary, and intestinal tracts (Bauernfiend 1981). However, not all carotenoids are pro-vitamin A active. Activity is based on the chemical structure of the

carotenoid. (Delgado-Vargas et al. 2000) This paper is focused on the colorant aspect of selected carotenoids and how their structure relates to this stability.

Carotenoids are colored because of their chemical structure. The colorant capacity of a carotenoid is imparted by its conjugated double bond system that resides mainly in a trans configuration. Any extensive conjugated system is highly delocalized about the pi-bonds, those forming the double bonds. As a result of delocalization, carotenoids exist in a low-energy excited state. When samples are exposed to energy that matches a possible electronic transition within a molecule, part of the light energy will be absorbed as the electron is promoted to a higher energy orbital. The transition energy is in the visible region of the electromagnetic spectrum ~380-800 nm, but more specifically are the carotenoids in the 400-500 nm range which is where yellow, orange, and red hues are observed. Spectrometric determinations based on structure are commonly measured as an indication of stability (Silverstein et al. 1991).

In addition to absorbing light energy, the conjugated bond system in carotenoids is capable of energy transfers to the excited state of itself known as a triplet state species (Polivka et al., 2004). This ability is what allows for the structure to accept the transfer of energy from highly reactive species. Source of energy capable of producing these transfers could be from heat, light, or other chemical interactions (Nielson et al., 1996). Most notably is singlet oxygen ($^1O_2$) (Min et al.,2002). One of the first reactions between carotenoid and singlet oxygen was determined using β-carotene (Foote et al. 1968) and the theory is now widely accepted. The ability to quench is based on the number of conjugated bonds with in the carotenoid structure so it is not surprising that lycopene is more effective than β-carotene (Krinsky 1994). Research by Cantrell et al. (2003) suggests that singlet oxygen quenching is dependant on the type of carotenoid, xanthophylls or carotenes, and the environment in which they are incorporated, i.e., a

lipid membrane. However, the structure can only take so much abuse before it breaks down. Carotenoid degradation is visually observed with loss of color and quantitatively measured by instrumentation. Structures of selected carotenoids are shown in Figure A.1. (Figures and Tables appear in Appendix A at the end of chapter.)

The objectives of this study include the determination of the photo-stability of selected carotenogenic food colorants and its relationship to environmental stresses (i.e., light and modified atmosphere) in a model system. The selected carotenoids include β-carotene, astaxanthin, β-APO-8'-carotenal, annatto, and paprika oleoresin. These were selected on the basis of structural classification and usage in the food industry. Fluorescent light will be the energy source chosen due to its common use in the food outlets. Previous studies have been done in real and model systems, however the carotenoids and system used here is much more inclusive (Colchin et al., 2001; Petersen et al., 1999; Carnevale et al., 1979).

Materials and Methods

Food Colorant Preparation

Five carotenoids were selected for observation. They include β-carotene (BC), astaxanthin (AX), β-APO-8'-carotenal (APO), annatto (ANT), and paprika oleoresin (PO). The BC, AX, and APO were purified standards from Sigma Aldrich (St. Louis, MO) and the ANT and APO were liquid food grade colorants obtained from Food Ingredient Solutions, LLC (Barnhart, MO). 100 ppm stock solutions were made for all colorants. BC, APO, and PO solutions were made by dissolving the weighed sample in ~5 mL acetone then diluting to 500 mL with hexane. Small fractions (~2mg) of AX were dissolved in 10 mL acetone before diluting with methanol. All solvents used were HPLC grade from Fisher Scientific (Waltham, MA). The ANT sample was dissolved in deionized water.

Since UV-Vis scans were to be obtained, solutions were made such that the absorbance would not exceed two absorbance units. All samples were diluted to 5 ppm except for AX which was diluted to 10 ppm.

Sample Preparation and Storage

Aliquots of 7 mL of carotenoid solution were pipetted into 10 mL crimp top sample vials and sealed. Half of the samples had nitrogen gas (N_2) bubbled throughout and over the headspace for 30 seconds before sealing. The remaining samples had no special treatment before sealing.

All samples were placed into a large white, wooden chamber equipped with two fluorescent bulbs attached to either side. The light intensity in the box was measured at 6000 lux. The temperature in the light box averaged 29°C ± 2°C. All nitrogen purged samples were placed inside additional glass chambers with an oxygen absorbing pack and oxygen indicator (RP system, Mitsubishi Chemicals, NY). The RP indicator colormetrically changed from pink to blue if greater than 0.1% oxygen was present. After being sealed in the chamber with the samples and oxygen scavenger, the tablets were completely pink after ~10 hours (after turning blue in the short time during transfer from the packaging to the chamber <15 seconds). Samples were removed from the light box and analyzed weekly. Samples devoid of nitrogen treatment were sealed in screw top borosilicate test tubes. Those samples for which light may be a factor were wrapped in aluminum foil and placed in the box (air) or in the glass chambers (N_2).

Instrumentation

Samples were analyzed spectrophotometrically, chromatographically (on chemical standards, not food-grade colorants), and with a colorimeter. The UV-Vis

scans were obtained on a Shimadzu 2101-PC spectrophotometer (Shimadzu, Columbia, MD) using a quartz cuvette, 1 cm path length and measure from 200-800 nm. Hexane, methanol, or water was used as the blank depending on the sample being scanned. The measured λ-max for each is as follows: BC 451 nm, AX 479 nm, APO 454 nm, PO 444 nm, and ANT 453 nm. The changes observed in the recorded absorbance at λ-max indicate that structural variations are occurring.

HPLC analysis was performed in determining possible isomerization or degradation products. The system was a Dionex LC20 (Dionex Corp. Sunnyvale, CA) equipped with a photodiode-array detector. Detection was carried out at 450 nm. A Prontosil C_{30} carotenoid column (Mac Mod Analytical, Chadds Ford, PA), 4.6 x 250 mm, with a mobile phase of methanol and methyl-t-buyl ether (89:11) and 0.1% triethylamine and a 1mL/min flow rate were used (Emenhiser et al., 1996). Before HPLC analysis, APO and AX samples were dried under nitrogen gas and re-dissolved in the HPLC mobile phase, BC was injected directly. HPLC data will be presented numerically within the text. Chromatograms will not be shown as consistent retention times could not be recorded due to ambient temperature changes in the laboratory. However, the absorbance spectra detected by the PDA did confirm that peaks were of the carotenoid being analyzed.

The color of the samples was measured by a Hunter MiniScan XE Plus Portable Colorimeter (Hunter Corporation, Reston, VA, USA), L, a, and b-values were recorded (only b-value data will be presented). The colorimeter was standardized with a white tile and black tile before samples were measured. The b-values obtained indicate yellow (positive) to blue (negative) color dimension. A stage was placed over the opening such that the sample vial could stand freely and equidistant for each scan; a black cloth covered the sample and the scanner to block any light during analysis.

Statistical Analysis Description

A factorial experimental design was used to evaluate the combination of treatments on the selected carotenoids. The treatments were atmosphere (air or nitrogen) and environment (light or dark). A Generalized Linear Model (GLM) was used to generate statistical ANOVA tables. In SAS 9.1 (Carey, NC) a PROC GLM analyzes data relating to one or several continuous dependant variables. The combined interactions of the atmosphere, environment, and time (atm-env-time) for each carotenoid are evaluated based on the ANOVA analysis; p-values will be reported for nteractions. Data for the initial and first sampling period were grouped and compared to data grouped from the last two sampling periods for the GLM analysis. The grouping of data was done so that changes over the storage time were more easily recognized. In addition to combined interaction effects, a simple Duncan's Multiple Range analysis was done for each carotenoid in each environment and atmosphere over time. The statistical mean separation data will be given in the data tables for absorbance and b-value. All statistical analyses were tested at $\alpha < 0.05$ level of confidence.

Results and Discussion

β-carotene

The UV-Vis spectra and Hunter b-values are shown in Figure A.2. The UV-Vis spectra indicate a decrease in absorbance in each of the four environment/ atmospheric combinations at six-weeks as compared to the initial spectrum. The Hunter graph illustrates the trend in b-values during the entire storage period. ANOVA analysis for absorbance and b-values indicated a significant difference in all main effects and combined interactions ($p<0.001$). Table A.1 lists the mean separations for BC over six-weeks for absorbance and b-values. Significant differences were found at each time

point within each environment/ atmosphere. The decreased absorbance is an indication of changes in the electronic structure of BC, ultimately suggesting structural degradation. The greatest decrease was observed in air-light, whereas after six-weeks almost no absorbance was measured at λ_{max}. In terms of visible color, the b-values present a similar trend in that the yellowness of the sample had diminished in air-light, while it remained only slightly changed in the other three environments. The significant differences observed in b-value and absorbance indicates that several factors play a role in stability of B-carotene. According to the HPLC, a single peak with an average retention time of 57 minutes for the initial sample was observed. By the end of six weeks no peak was present for air-light; peaks for both dark environments were reduced to about 50% from initial; N_2-light decreased by nearly two-thirds.

β-APO-8'-carotenal

The UV-Vis spectra and Hunter b-values are shown in Figure A.3. The UV-Vis spectra indicate that the greatest reduction in absorbance is observed after six-weeks in air-light. After six-weeks, the N_2 light and dark samples decreased from the initial but remained similar to each other, while air-dark experienced the least change in absorbance. The b-value graph shows the trend during the entire six-week sampling period. ANOVA analysis indicated that all main effects were significant ($p<0.0005$); combined interactions were significant for atmosphere/ time ($p=0.0143$) and atmosphere/time/environment ($p=0.0042$), but were not significant over time/ environment ($p=0.1310$). Table A.2 lists the mean separations for APO over six-weeks for absorbance and b-values. For absorbance, in both air and N_2 light conditions, significant differences were observed at each sampling time, but in both dark conditions, significant differences were not detected until the third week. Data indicates that for

APO, light and atmosphere both contribute to possible structural changes. Initial HPLC analysis indicated one peak with the average retention time of 20.18±1.2 minutes. After the third week, a second peak was detected at 15.57 minutes. At six weeks in air-light, the initial peak was reduced by half; peaks in the other three samples were minimally reduced; and the new peak observed in week three remained but had not increased.

Astaxanthin

The UV-Vis spectra and Hunter b-values for astaxanthin are shown in Figure A.4. The UV-Vis spectra indicate that the greatest reduction in absorbance is observed after six-weeks in air-light. A reduction was also observed in air-dark and N_2 light and dark, however, these three remained close to one another after six-weeks. The b-values gradually increased by then end of the six-week period indicating that yellowness of the samples had not degraded. ANOVA analysis indicates that the only significant factors contributing to changes in absorbance and b-value are atmosphere ($p=0.0020$) and time ($p<0.0001$). The combined interaction of atmosphere/ time/ environment was not significant to absorbance changes ($p=0.2052$) or b-values ($p=0.8735$). Table A.3 lists the mean separations for AX over six-weeks for absorbance and b-values. Significant differences were observed in each atmosphere/ environment throughout the sampling period. Although a decrease was observed in absorbance, b-values did not decrease overall for any of the samples, but rather increased. HPLC analysis initially indicated a single peak with a 10.45 ± 0.43 minute retention time. By week one, in all conditions, another peak was detected and appeared just prior to the initial sample peak at 8.8±0.21 minutes; as the initial peak decreased with time, this new peak slightly increased. It is possibly explained by products of free radical reactions or isomerization with in the samples.

Annatto

The UV-Vis spectra and Hunter b-values for annatto are shown in Figure A.5. The UV-Vis spectra indicate that after six-weeks absorbance at λ_{max} in air-light was no longer detectable. Absorbance in both air-dark and N_2 -light were appreciably reduced as well when compared to the initial spectrum. N_2 –dark also exhibited a reduction in absorbance. The b-value graph follows the same trend as observed in absorbance measurements; a colorless solution remained after six-weeks in air-light. ANOVA analysis indicated that all main and most combined effects were significant ($0<0.0001$) to b-value changes; the atmosphere/time/environment interaction was not significant ($p=0.5646$). Only time and atmosphere were significant to changes in absorbance ($p<0.0001$) while environment ($p=0.0793$) and combined effects where not. Table A.4 lists the mean separations for ANT over six-weeks for absorbance and b-values. Within each atmosphere/environment combination, significant changes were observed. The best scenario would be to keep this colorant in the dark and in a modified atmosphere to ensure color stability as indicated by b-value and absorbance trends. HPLC was not obtained for this colorant.

Paprika Oleoresin

The UV-Vis spectra and Hunter b-values for paprika oleoresin are shown in Figure A.6. As observed with the other colorants, the greatest reduction in absorbance was in air-light. After six-weeks of storage, the smallest absorbance decrease was in the N_2 environments. Minimal change was observed in b-value from initial to final sampling. ANOVA results indicate no significant differences in all main and combined effects ($p>0.0500$). For changes observed in absorbance, atmosphere ($p=0.2931$), environment ($p=0.1323$) and atmosphere/time ($p=0.2860$) were not significant, while

all other interactions were significant. Table A.5 lists the mean separations for PO over six-weeks for absorbance and b-values. When analyzed within each atmosphere/environment combination, significant differences were observed. Some of the stability in PO may come from the fact that this is an oleoresin. Food colorant oleoresins are essentially resin extracts of the natural source in oil. The paprika carotenoids present may be protected by the presence of natural antioxidants in the oil and the oil is degraded before the carotenoid. HPLC analysis was not performed on this colorant.

Photo-modification effects

Photo degradation can lead to isomerization of the carotenoid polyene chain causing molecular bending (cis and trans interaction) or cleavage (production of short chain products) (Hornback 1998). The results of the photodegradation may be different for each of the colorants tested; however, the differences in behavior are primarily based on structure and the length of the polyene chain present in each carotenoid (Deng et al., 2000). When the conjugated π-bonds are photochemically acted upon, excited energy states are produced. In the absence of oxygen, carotenoid degradation may occur by breaking a C-C or C=C bond in one of the excited states and produce a radical product. This is a direct result of molecular orbital theory where promotion to an anti-bonding orbital weakens other bonds and homolytic cleavage occurs (Hornback 1998). Termination of radical reactions happens then by coupling or disproportionation. In the presence of oxygen, an excited state carotenoid may be more likely for reaction with $^3O_2$, due to molecular orbital spin interactions (Nielson et al., 1996; Pan et al., 2005; He et al., 2000). These are the likely explanations to what was observed in the UV-Vis spectra. It is easy to speculate on what was occurring in the solution, however, for determining

radicals in solution or excited state carotenoids electron spin resonance spectroscopy would have to be employed.

Conclusion

Although the trends were varied for each of the colorants in the four different atmospheric and environmental conditions, a general order of stability can be established, particularly for air-light conditions. The general inclination for greatest stability was APO=PO>AX>BC>ANT. The results are interesting since they indicate that annatto was the least stable yet it is a popular colorant in the food industry. However, in this study, the colorants were analyzed in a model system which is not indicative of a typical food matrix (carbohydrates, proteins, other lipids, etc.). The model system only offers insight into possible behavior of a particular compound.

Based on our results, it can be concluded that a comparison of color degradation by UV-Vis spectroscopy and Hunter color analysis cannot be established despite the fact that decreased absorbance is suggestive of structural degradation and should therefore change the color perceived. What was determined is the fact that stability is affected differently due to individual effects of atmosphere, time, and environment and/ or their interaction. Unfortunately, no trend could be established based on carotenoid structural similarities or differences within the model system studied.

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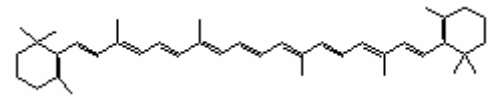
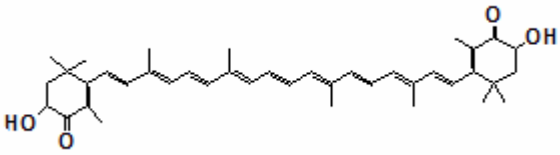
Appendix A

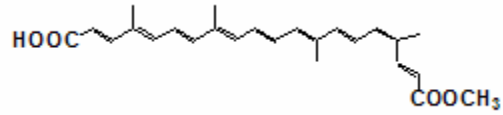
Lycopene



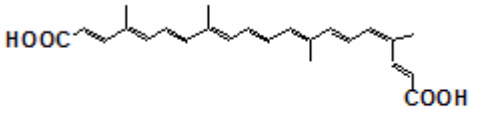
β-carotene



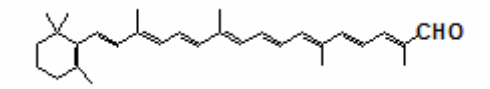
Astaxanthin



Bixin

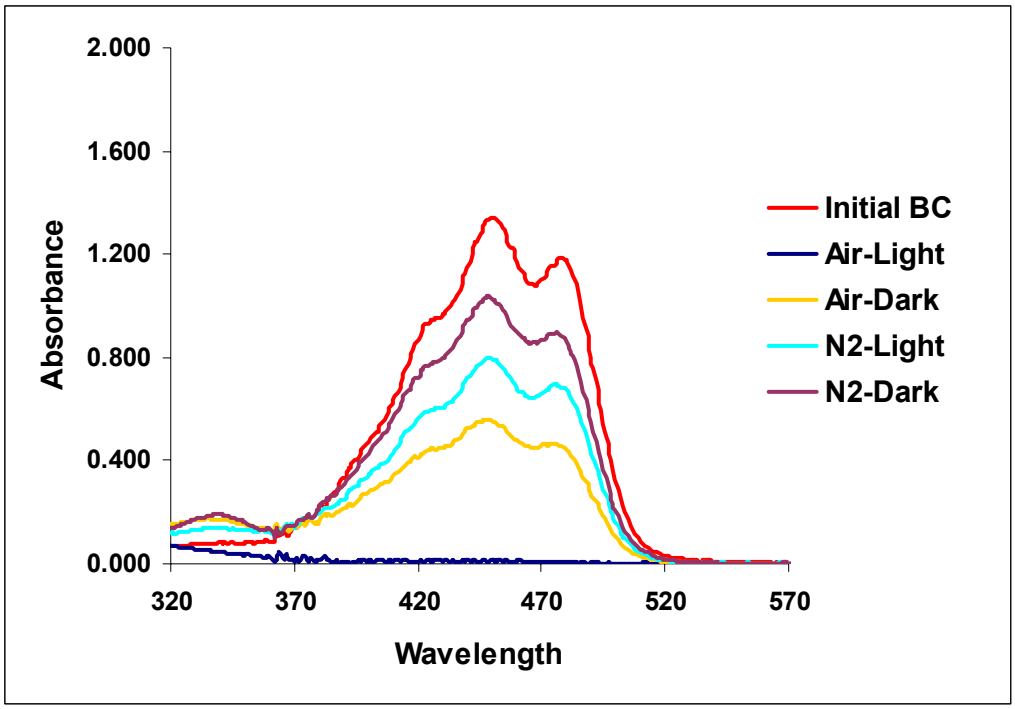


Norbixin

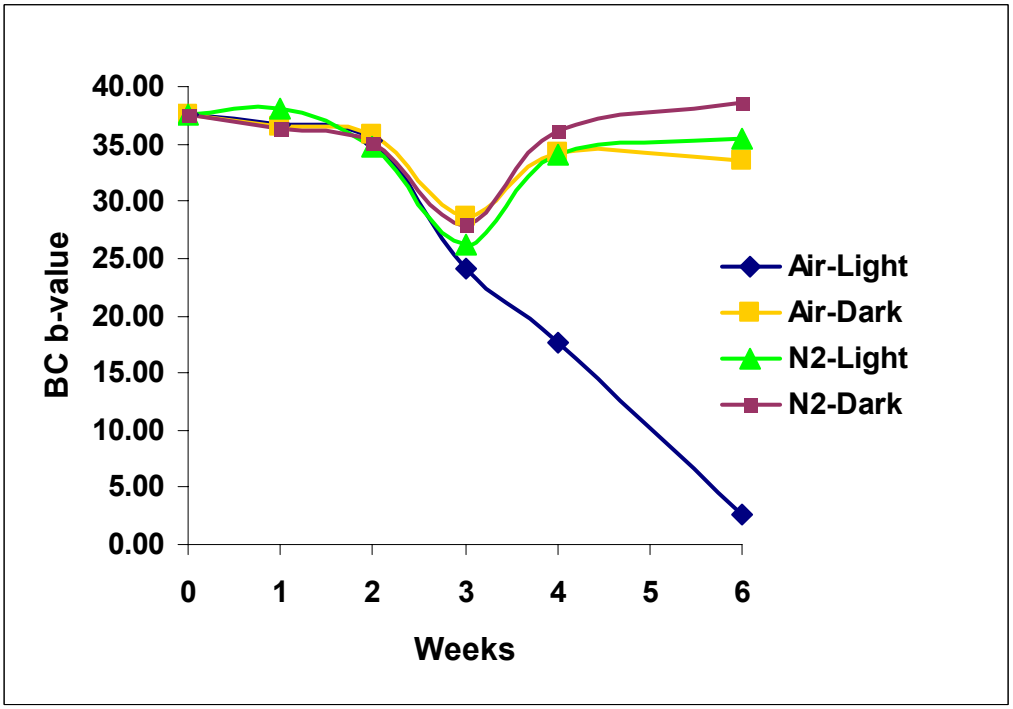


β-APO-8'-carotenal

Figure A.1 Structures of selected carotenes (lycopene and β-carotene) and xanthophylls (all others)



(A)



(B)

Figure A.2 (A) UV-Vis absorbance spectra of β-carotene (BC) after six-week storage; (B) Hunter colorimeter b-values of ß-carotene

Table A.1 (A) UV-Vis data (λ_{max}= 451nm) for β-carotene (BC); (B) Hunter colorimeter b-values for β –carotene. Mean separations determined by Duncan's Multiple Range (data in columns followed by different letters are significantly different at α=0.05)

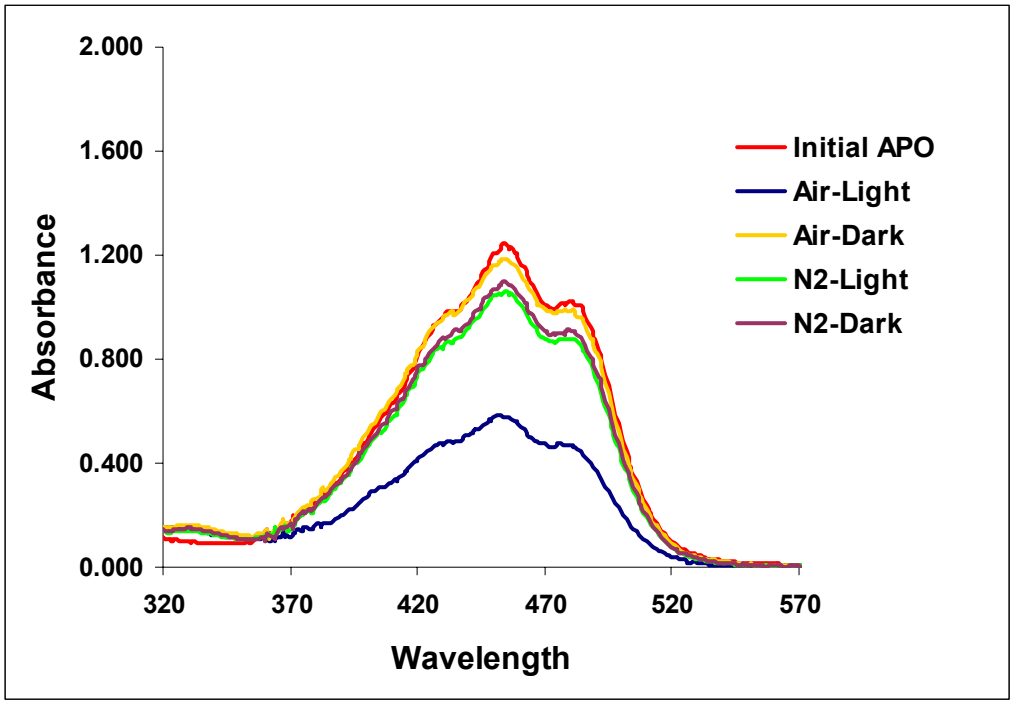
(A)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial BC	1.337 ± 0.005[a]	1.337 ± 0.005[a]	1.337 ± 0.005[a]	1.337 ± 0.005[a]
1w	1.076 ± 0.010[b]	1.324 ± 0.004[b]	1.308 ± 0.003[b]	1.363 ± 0.006[b]
2w	0.700 ± 0.002[c]	1.167 ± 0.005[c]	1.256 ± 0.010[c]	1.291 ± 0.002[c]
3w	0.414 ± 0.001[d]	1.187 ± 0.004[d]	1.115 ± 0.002[d]	1.179 ± 0.004[d]
4w	0.118 ± 0.002[e]	1.019 ± 0.002[e]	1.088 ± 0.006[e]	1.145 ± 0.003[e]
6w	0.010 ± 0.004[f]	0.552 ± 0.002[f]	0.791 ± 0.003[f]	1.022 ± 0.008[f]

(B)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial BC	37.59 ± 0.59[a]	37.59 ± 0.59[a]	37.59 ± 0.59[b]	37.59 ± 0.59[b]
1w	36.74 ± 0.98[b]	36.53 ± 0.18[b]	38.16 ± 0.11[a]	36.42 ± 0.23[c]
2w	35.25 ± 0.08[c]	35.83 ± 0.14[c]	34.68 ± 0.10[d]	35.08 ± 0.14[d]
3w	24.02 ± 0.09[d]	28.71 ± 0.09[f]	26.25 ± 0.05[f]	27.88 ± 0.08[e]
4w	17.60 ± 0.09[e]	34.20 ± 0.15[d]	34.11 ± 0.08[e]	36.07 ± 0.11[c]
6w	2.66 ± 0.08[f]	33.61 ± 0.02[e]	35.54 ± 0.20[c]	38.58 ± 0.04[a]

(A)



(B)

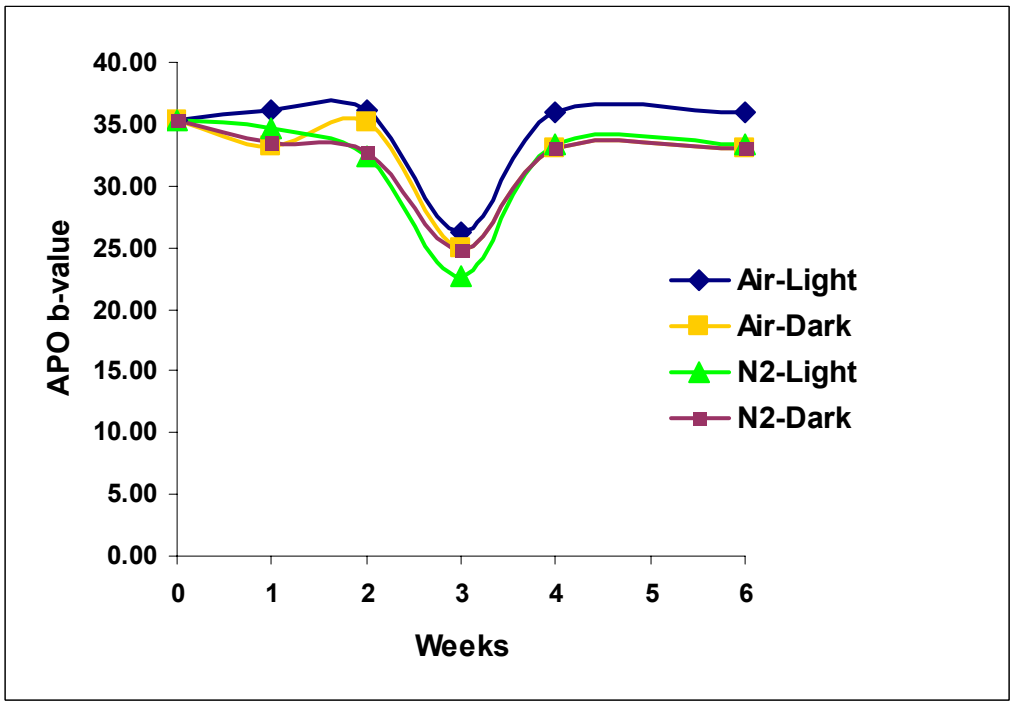


Figure A.3 Six-week sampling of (A) UV-Vis absorbance spectra of β-APO-8'-carotenal (APO); (B) Hunter colorimeter b-values of APO

Table A.2 (A) UV-Vis data (λ_{max}= 454nm) for β-APO-8'-carotenal (APO); (B) Hunter colorimeter b-values for APO. Mean separations determined by Duncan's Multiple Range (data in columns followed by different letters are significantly different at α=0.05)

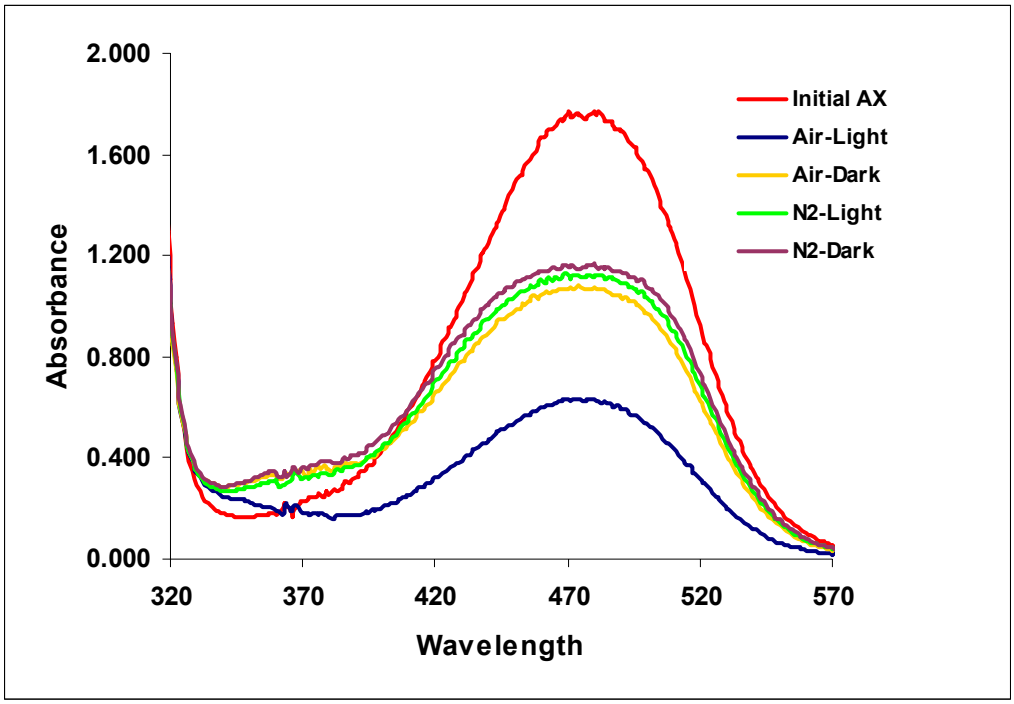
(A)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial APO	1.244 ± 0.002[a]	1.244 ± 0.002[a]	1.244 ± 0.002[a]	1.244 ± 0.002[a]
1w	1.167 ± 0.001[b]	1.260 ± 0.004[a]	1.223 ± 0.002[b]	1.247 ± 0.008[a]
2w	1.094 ± 0.004[c]	1.232 ± 0.018[a]	1.201 ± 0.009[c]	1.240 ± 0.005[a]
3w	1.036 ± 0.007[d]	1.177 ± 0.036[b]	1.171 ± 0.013[d]	1.203 ± 0.005[b]
4w	0.884 ± 0.006[e]	1.187 ± 0.008[b]	1.154 ± 0.004[e]	1.191 ± 0.004[c]
6w	0.577 ± 0.004[f]	1.181 ± 0.007[b]	1.055 ± 0.008[f]	1.097 ± 0.003[d]

(B)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial APO	35.35 ± 0.95[b]	35.35 ± 0.95[a]	35.35 ± 0.95[a]	35.35 ± 0.95[a]
1w	36.07 ± 0.05[a,b]	33.12 ± 0.05[b]	34.66 ± 0.15[a]	33.49 ± 0.23[b]
2w	36.19 ± 0.13[a]	35.10 ± 0.13[a]	32.36 ± 0.08[c]	32.68 ± 0.02[c]
3w	26.24 ± 0.13[c]	24.91 ± 0.21[c]	22.61 ± 0.09[d]	24.79 ± 0.12[d]
4w	36.03 ± 0.13[a,b]	33.01 ± 0.03[b]	33.34 ± 0.04[b]	32.99 ± 0.02[b,c]
6w	35.77 ± 0.06[a,b]	33.57 ± 0.04[b]	33.98 ± 0.03[b]	32.82 ± 0.17[b,c]

(A)



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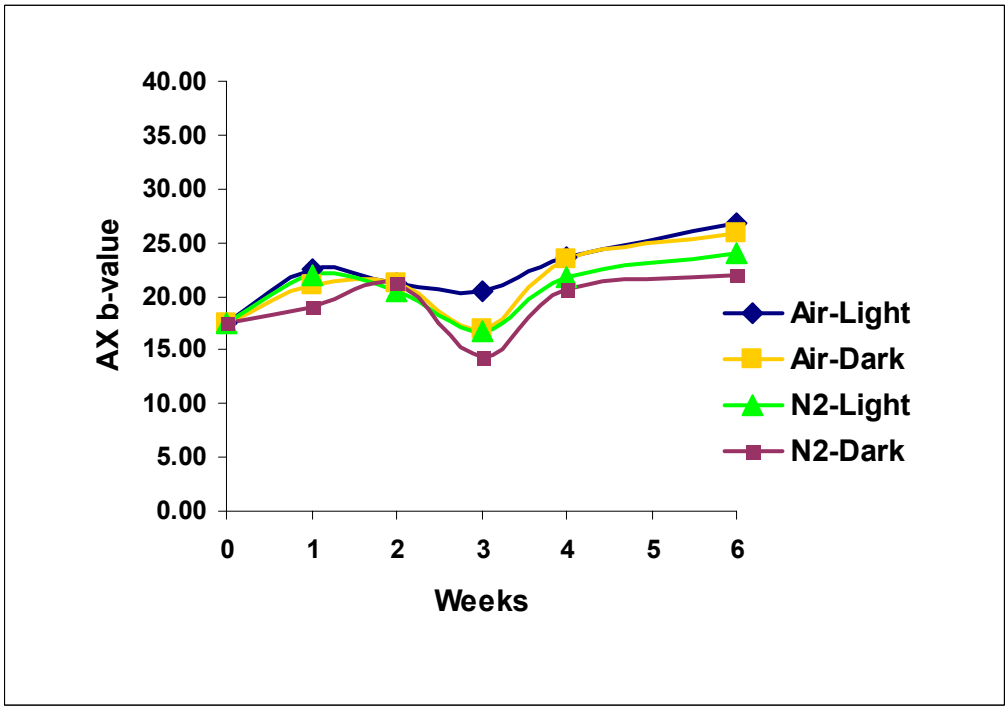


Figure A.4 Six-week sampling of (A) UV-Vis absorbance spectra of astaxanthin (AX); (B) Hunter colorimeter b-values of astaxanthin

Table A.3 ((A) UV-Vis data (λ_{max}= 479nm) for astaxanthin (AX); (B) Hunter colorimeter b-values for astaxanthin. Mean separations determined by Duncan's Multiple Range (data in columns followed by different letters are significantly different at α=0.05)

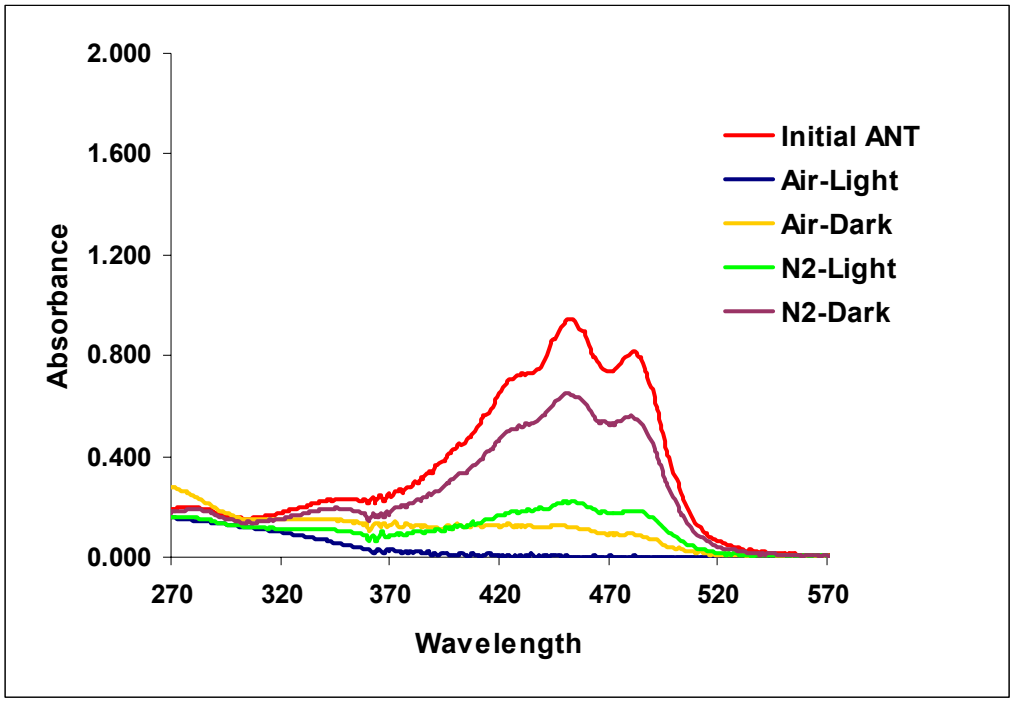
(A)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial AX	1.769 ± 0.003[a]	1.769 ± 0.003[a]	1.769 ± 0.003[b]	1.769 ± 0.003[d]
1w	1.777 ± 0.001[a]	1.752 ± 0.007[b]	1.879 ± 0.002[a]	1.879 ± 0.007[a]
2w	1.711 ± 0.009[b]	1.712 ± 0.002[c]	1.814 ± 0.009[c]	1.816 ± 0.008[c]
3w	0.863 ± 0.007[c]	1.714 ± 0.003[c]	1.748 ± 0.006[c]	1.848 ± 0.003[b]
4w	1.284 ± 0.013[d]	1.553 ± 0.006[d]	1.878 ± 0.010[a]	1.815 ± 0.005[c]
6w	0.626 ± 0.004[e]	1.070 ± 0.006[e]	1.117 ± 0.001[d]	1.161 ± 0.003[e]

(B)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial AX	17.40 ± 1.10[e]	17.40 ± 1.10[d]	17.40 ± 1.10[d]	17.40 ± 1.10[d]
1w	22.59 ± 0.14[c]	21.10 ± 0.13[c]	21.88 ± 0.17[b]	19.07 ± 0.16[c]
2w	21.15 ± 0.16[d]	21.28 ± 0.10[c]	20.43 ± 0.10[c]	21.29 ± 0.20[a,b]
3w	20.47 ± 0.05[d]	16.98 ± 0.09[d]	16.79 ± 0.07[d]	14.34 ± 0.12[e]
4w	23.62 ± 0.06[b]	23.45 ± 0.05[b]	21.81 ± 0.05[b]	20.73 ± 0.23[b]
6w	26.87 ± 0.09[a]	25.91 ± 0.10[a]	23.95 ± 0.02[a]	21.89 ± 0.06[a]

(A)



(B)

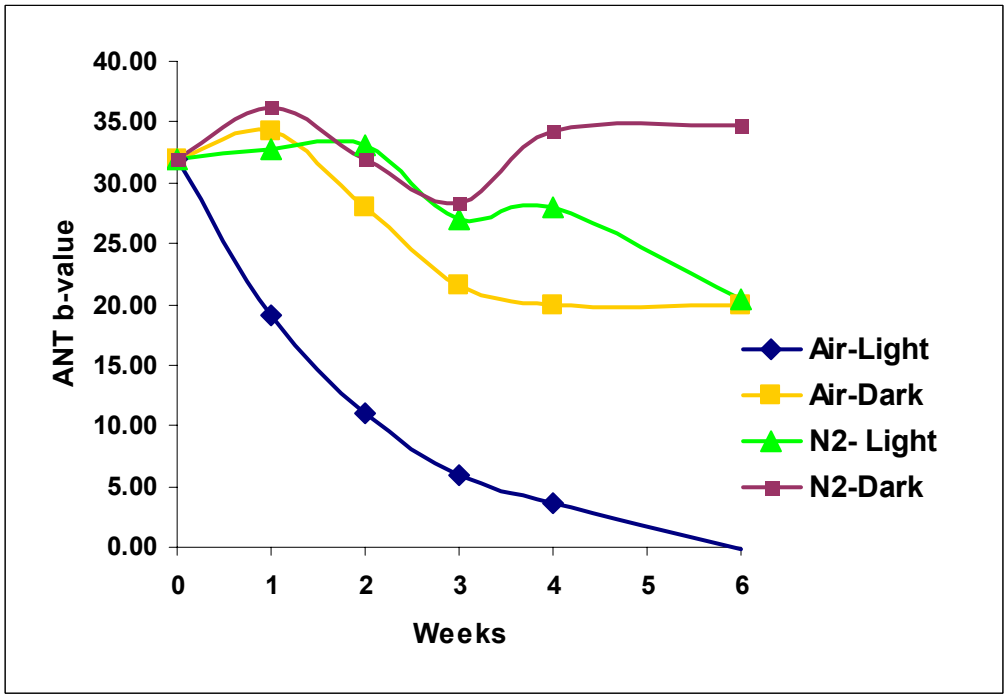


Figure A.5 Six-week sampling of (A) UV-Vis absorbance spectra of Annatto (ANT) ; (B) Hunter colorimeter b-values of Annatto

Table A.4 (A) UV-Vis data (λ_{max}= 453nm) for annatto (ANT); (B) Hunter colorimeter b-values for annatto. Mean separations determined by Duncan's Multiple Range (data in columns followed by different letters are significantly different at α=0.05)

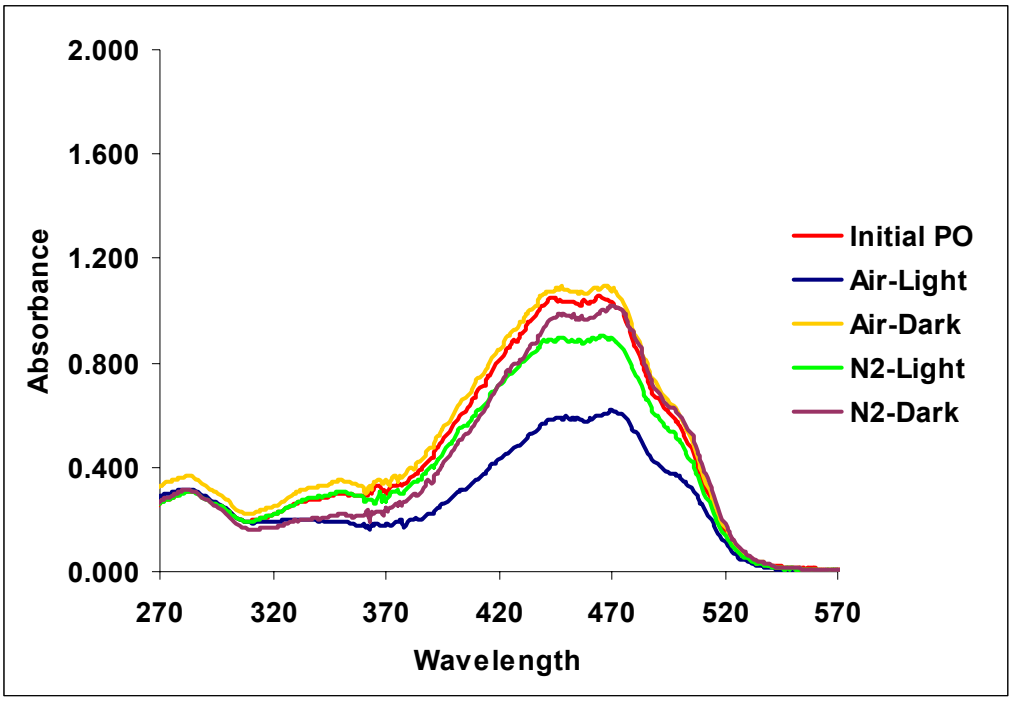
(A)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial ANT	0.939 ± 0.009[a]	0.939 ± 0.009[a]	0.939 ± 0.009[a]	0.939 ± 0.009[a]
1w	0.121 ± 0.001[b]	0.358 ± 0.001[b]	0.821 ± 0.023[b]	0.846 ± 0.003[b]
2w	0.061 ± 0.002[c]	0.244 ± 0.001[c]	0.649 ± 0.021[c]	0.785 ± 0.004[c]
3w	0.028 ± 0.004[d]	0.208 ± 0.002[d]	0.564 ± 0.003[d]	0.696 ± 0.002[d]
4w	0.011 ± 0.003[e]	0.125 ± 0.002[e]	0.489 ± 0.002[e]	0.496 ± 0.003[e]
6w	0.001 ± 0.000[f]	0.121 ± 0.002[f]	0.218 ± 0.004[e]	0.645 ± 0.001[d]

(B)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial ANT	31.97 ± 0.53[a]	31.97 ± 0.53[b]	31.97 ± 0.53[b]	31.97 ± 0.53[d]
1w	19.07 ± 0.03[b]	34.16 ± 0.04[a]	32.81 ± 0.04[a]	36.23 ± 0.05[a]
2w	10.97 ± 0.05[c]	27.92 ± 0.06[c]	33.14 ± 0.07[a]	31.92 ± 0.11[d]
3w	5.98 ± 0.05[d]	21.59 ± 0.04[d]	27.08 ± 0.10[d]	28.35 ± 0.10[e]
4w	3.56 ± 0.02[e]	19.87 ± 0.04[e]	28.01 ± 0.12[c]	34.18 ± 0.05[c]
6w	-0.12 ± 0.02[f]	19.92 ± 0.06[e]	20.39 ± 0.08[e]	34.69 ± 0.09[b]

(A)



(B)

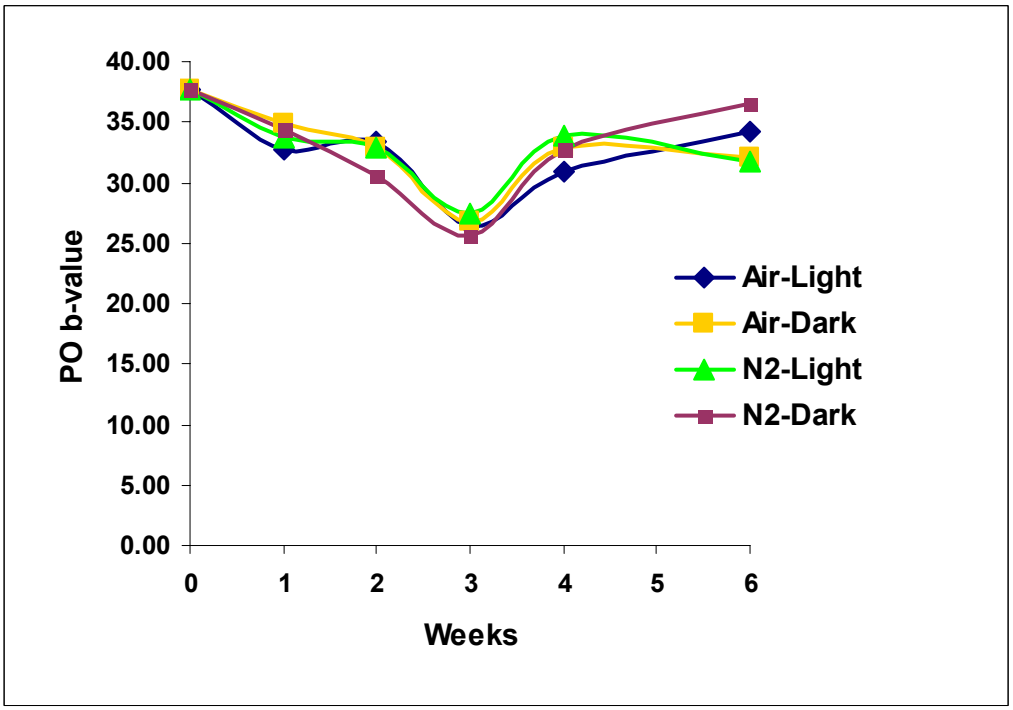


Figure A.6 Six-week sampling of (A) UV-Vis absorbance spectra of paprika oleoresin (PO); (B) Hunter colorimeter b-values of paprika oleoresin

Table A.5 (A) UV-Vis data (λ_{max}= 444nm) for paprika oleoresin (PO); (B) Hunter colorimeter b-values for paprika oleoresin. Mean separations determined by Duncan's Multiple Range (data in columns followed by different letters are significantly different at α=0.05)

(A)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial PO	1.051 ± 0.006^{b}	1.051 ± 0.006^{c,b}	1.051 ± 0.006^{c}	1.051 ± 0.006^{a}
1w	1.092 ± 0.008^{a}	1.038 ± 0.003^{c}	1.077 ± 0.009^{b}	1.051 ± 0.005^{a}
2w	0.941 ± 0.009^{d}	1.068 ± 0.026^{a,b}	1.098 ± 0.002^{a}	1.059 ± 0.002^{a}
3w	1.055 ± 0.027^{b}	1.080 ± 0.008^{a}	1.020 ± 0.003^{d}	1.003 ± 0.005^{c}
4w	1.016 ± 0.005^{c}	1.029 ± 0.004^{c}	1.043 ± 0.004^{c}	1.021 ± 0.003^{b}
6w	0.582 ± 0.004^{e}	1.070 ± 0.004^{a,b}	0.968 ± 0.005^{e}	0.888 ± 0.004^{d}

(B)

	Air		N_2	
	Light	**Dark**	**Light**	**Dark**
Initial PO	31.76 ± 1.22^{c}	31.76 ± 1.22^{c}	31.76 ± 1.22^{c}	31.76 ± 1.22^{d}
1w	32.75 ± 0.10^{b}	34.85 ± 0.10^{a}	33.80 ± 0.02^{a}	34.30 ± 0.09^{b}
2w	33.47 ± 0.24^{a,b}	32.96 ± 0.09^{b}	32.82 ± 0.11^{b}	30.63 ± 0.13^{e}
3w	26.41 ± 0.02^{d}	26.73 ± 0.06^{d}	27.50 ± 0.15^{d}	25.63 ± 0.10^{f}
4w	30.88 ± 0.15^{c}	32.96 ± 0.20^{b}	33.93 ± 0.03^{a}	32.74 ± 0.09^{c}
6w	34.16 ± 0.06^{a}	32.11 ± 0.07^{b,c}	31.68 ± 0.08^{c}	36.61 ± 0.04^{a}

4. Antioxidant capacity of carotenoids as affected by ozone

Abstract

Carotenoids are naturally occurring pigments responsible for the colors in many fruits and vegetables. Discoloration of such compounds after exposure to environmental effects, like UV-light and ozone, may indicate a loss in quality. Carotenoids are also known as potential antioxidants because of their chemical structure. When extensive changes in structure occur, visible defects include fading or discoloration is observed. If color parameter changes then it is likely that antioxidant capacity changes too. Oxygen Radical Absorbance Capacity (ORAC) was use to measure antioxidant capacity of β-carotene, astaxanthin, and β-APO-8'-carotenal. Carotenoids were exposed to ozone for two hours then solutions were combined with either a 0.7 or7.0% cyclodextrin solution to for soluble inclusion complexes which are required for use in lipophilic ORAC determination. The trends in ORAC values obtained for the three colorants after ozone exposure were not conclusive regardless of cyclodextrin concentration.

Introduction

Carotenoids are a class of naturally occurring compounds known to impart the yellow, orange, and red colors to fruits, vegetables, and several crustaceans. The length of the conjugated π-bond system of carotenoids is what produces the color; however, it also contributes to the antioxidant capabilities of the molecule. For example, astaxanthin, with 13 conjugated bonds, is considered a better antioxidant than β-carotene that possesses 11 conjugated bonds (Delgado-Vargas et al., 2000). This is due to the ability of the polyene chain to disperse the energy transferred from the free radical by resonance stabilization. In effect, the delocalized π-bond system is actually in a

lower, more stabilized energy state (Hornback 1998). It is believed that carotenoids are potential antioxidants because of this structural trait (Edge et al., 1997).

Carotenoids tend to be sensitive to extensive heat, light, and oxygen. Therefore methods need to be established for increasing bioavailability and stability towards irradiation, reactive oxygen, and other free radicals. Cyclodextrins (CD) have become popular molecules in which stable carotenoid inclusion complexes are formed (Polyakov et al., 2004).

Aside from carotenoid degradation by oxygen, ozone (O_3) is also of concern. Ozone is used in the food industry as a strong antimicrobial agent; however, it offers many other benefits besides reducing microbial load. Ozone can decrease biological oxygen, oxidize toxic organic compounds, and reduce inorganic waste. The most common use of ozone is as a disinfectant for drinking water, although it has been tested for the preservation of fruits and vegetables. Surface discoloration is often reported as a side effect to ozonation. (Henry et al., 2000; Kim et al., 1999) If discoloration of fruits and vegetables is observed, then degradation of the polyene chain of the carotenoids is occurring (MacDougall 2002), thus reducing the potential antioxidant abilities.

Cyclodextrins are enzyme modified starch derivatives. They are non-toxic and completely digestible in the colon. Cyclodextrins are used extensively in the food industry to improve stability of flavors, vitamins, colorants, and unsaturated fats by encapsulation into their cage-like structure (Del Valle 2004; Szente et al., 2004). Figure B.1 shows the chemical structure of a β-cyclodextrin molecule. (Figures and Tables appear in Appendix B at the end of the chapter.)

Oxygen Radical Absorbance Capacity (ORAC) is a method used to assess the antioxidant capabilities of a compound and can be applied to hydrophilic or lipophilic systems like that of the carotenoids (Cao et al., 1992). ORAC assays measure the

peroxy radical scavenging ability of an antioxidant. 2,2'-azobis(2-amidino propane)dihydrochloric acid (AAPH) is used as the peroxy radical generator. The inhibition of free peroxy radicals is measured and reported as the "ORAC value". The antioxidant capacity is determined by the area under the curve (AUC) of fluorescence decay of the sample versus a blank solution in which no antioxidant is present. The fluorescent probe more recently utilized is fluorescein (FL) (Ou et al., 2001). Samples are also measured along with an antioxidant standard, Trolox. The final value is then expressed with its reference to Trolox since it helps correct for differences in instrumentation, reagent, or assay conditions (Prior et al. 2003).

ORAC methods have been established for determining the antioxidant capacity of carotenoids by using randomly methylated β-cyclodextrins (Bangalore et al. 2005; Huang et al. 2002). However, the effect of ozone specifically on ORAC values of carotenoids has not been reported. Therefore, the objective of this study was determination of ORAC values for three carotenoids after being subjected to an ozonated environment.

Materials and Methods

Sample Preparation

Three carotenoids were selected for observation. They include β-carotene (BC), astaxanthin (AX), β-APO-8'-carotenal (APO), obtained from Sigma Aldrich (St. Louis, MO, USA). One-hundred ppm stock solutions were made for all colorants. BC, and APO were made by dissolving the weighed sample in ~5 mL acetone then diluting with hexane. AX had to be dissolved in small fractions (~2mg) in 10 mL acetone before

diluting with methanol. All solvents used were HPLC grade from Fisher Scientific (Waltham, MA, USA). Stock solutions were stored at -30°C until ready to use.

Two milliliter aliquots were used for ozone exposure. Samples were dried under nitrogen gas then placed into the ozonolysis chamber. An ozone generator with a continuous air flow was connected and produced a flow rate of 2 ppm/sec ozone. Ozone detection was determined by use of MSA Ozone Detection Tubes (Fisher Scientific (Waltham, MA, USA) prior to analysis. The ozone apparatus and sample chamber was set up in the laboratory hood to ensure proper ventilation due to ozone venting from the chamber. Carotenoids were exposed up to 2 hrs, with an intermediate sampling point of 1 hr. ORAC analysis was begun immediately after ozone exposure. Samples from 1 hr time point were stored at -30°C until 2 hr completion.

Lipophilic ORAC Assay

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich Milwaukee, WI, USA) was used as the antioxidant standard. A 500 μM stock solution was made by dissolving Trolox with a potassium phosphate buffer (pH 7.4). This stock was divided up into several 2 mL eppendorf tubes and stored at -80°C until needed. One tube was used per analysis. Standard concentrations of 6.25, 12.5, 25, and 50 μM Trolox solutions were made fresh for each ORAC analysis.

The fluoroscein solution was made by dissolving 22.5 mg fluoroscein (Fisher Scientific) into 50 mL of phosphate buffer (pH 7.0). 50μL of this solution was then diluted to 10mL with additional phosphate buffer. This stock was divided up into several 2 mL eppendorf tubes and stored at -80°C until needed. One tube was used per analysis.

The microplate assay as outlined in Prior et al. (2003) was conducted for analyses. The fluorescence microplate reader was the FLUOstar OPTIMA (BMG Technologies, Duraham, NC), equipped with two automated injectors and internal incubator set to 37°C. Two-hundred μL of Fluoroscein and 150 μL of AAPH were added by the injectors to each of the wells during the 35 cycles to be measured. Analysis time was 2 hrs.

The ozonated samples were dissolved in 500 μL of acetone and then diluted with 1.5 mL of 0.7 or 7.0% randomly methylated β-cyclodextrin (RMCD) solution (1:1 acetone:water) (pharmacy grade, Cyclodextrin Technologies Development, High Springs, FL). The RMCD solution was used as the blank for all analyses. Twenty μL of each sample, including the blank, and the four Trolox standards were placed into designated wells on a 48-count wellplate. The wellplates were analyzed in the FLUOstar OPTIMA. The average and standard deviation of the ORAC values are reported.

Results and Discussion

The initial study was planned to follow lipophilic ORAC as outline by Prior et al. (2003) where a 7% RMCD solution was used to assist in solubility of the carotenoid for analysis. After several failed attempts, the results were not reproducible. It was determined that the possibility existed of using an inappropriate concentration of the RMCD solution. This decision was based on the work of Bangalore et al. (2005) that reported the use of a RMCD solution as the solubility enhancer with a commercially available lycopene colorant. They tested the RMCD concentrations for effectiveness; however, they were using solutions of 0, 0.4, 0.8, and 1.6% RMCD. It was then decided that possible discrepancies with our results may really be a problem of RMCD

concentration. It was therefore determined that a 0.7% solution would be compared to the reported 7% used elsewhere.

The results for ORAC values for the comparison of the two RMCD solutions for each of the three carotenoids are presented in Table B.1. For BC, the 0.7% solution had little change from initial and after two hours of ozone exposure, while the 7% solution decreased over analysis time. For APO, 0.7% showed a significant decrease in ORAC value as compared to the initial. This was not the case for the 7% solution. This sample is difficult to justify since both initial solutions came from the same stock solution. For AX, the trend remained the same for both solutions showing an increased ORAC value at both time points when compared to initial values. Unfortunately, the use of the other RMCD for comparison purposes did not clarify nor justify the results. Carotenoid-free trials were performed to determine the stability of the RMCD solution and they proved to be consistent trial after trial (results not shown). Inconsistencies are probably due to the lipophilic carotenoid system.

Conclusion

The determination of ORAC values for the ozonated carotenoid model systems is reported. However, trends are difficult to establish. That being said, there is obviously plenty of research that can continue in the determination of ORAC values for lipophilic compounds. It may be that a different fluorescent probe could be utilized or the RMCD concentration needs to be optimized for a given system.

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Appendix B

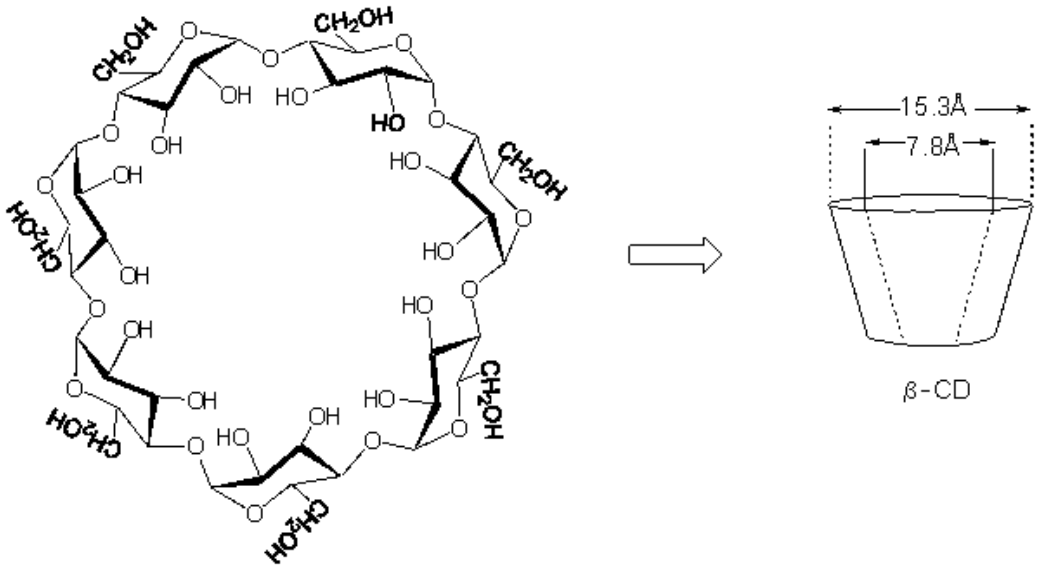

Figure B.1 Chemical structure of β-cyclodextrin

Table B.1 ORAC values for carotenoids using two different concentrations of randomly methylated cyclodextrin solution.

Carotenoid	Time (O_3 exposure)	ORAC values from RMCD Solution	
		0.7%	7.0%
B-carotene	Initial	27.30 ± 1.10	42.31 ± 2..92
	1 hr	38.16 ± 7.07	25.89 ± 3.49
	2 hr	36.70 ± 6.20	28.28 ± 8.92
APO-8'-carotenal	Initial	1143.60 ± 643.41	149.94 ± 15.00
	1 hr	124.68 ± 41.02	126.08 ± 16.70
	2 hr	56.46 ± 9.35	117.20 ± 31.32
Astaxanthin	Initial	5.79 ± 2.66	7.36 ± 2.79
	1 hr	21.65 ± 0.52	18.58 ± 5.25
	2 hr	22.47 ± 0.87	19.52 ± 2.35

5. Quality Improvement of Process Cheese Spread

Abstract

Process cheese spread is a commercially available product that is consumed by many people. Cheese spread is also a popular component used in the U.S. military feeding program for their Meal, Ready-to-Eat (MRE) rations. Currently, the military requires a 3-yr shelf life of their MRE's if stored at 80°F. However, due to product deployment into very warm climates, it would be beneficial to improve shelf life stability to 3-yrs at 100°F. In order to provide good quality at elevated temperatures, a systematic approach to ingredient evaluation was needed, making that the objective of this study. Problems with the existing cheese spread include darkening, hardening, and emulsion instability during storage at elevated temperatures. These parameters were studied for each of four main additive classifications: vitamins (due to required fortification with C, A, B1 and B6), colorants, emulsifying salts, and stabilizers. Results indicate that the greatest improvement of the product would be removal of vitamins due to the increased effect of non-enzymatic browning and potential textural changes in fortified product. No significant differences that would warrant a change in colorant or emulsifying salt were found. However, partial substitution of current stabilizer with carrageenan was suggested as it maintained a softer texture through out analysis with less overall hardening.

Introduction

Process cheese spreads are produced by the comminuting and melting of cheese with the aid of heat and the additions of emulsifying agents, stabilizers, acidulants, salt and colorants (FDA 21CFR133.179). The proper manufacture of process cheese spread involves many variables that undoubtedly determine the quality of the

finished product. Manufacturers are constantly working on formulary changes in an effort to increase product stability, functionality, and popularity (Fox 2000). Problems frequently encountered with cheese spreads include phase separation, hardening (Gupta et al., 1984), and/or darkening. Quality of the product is reduced when these changes occur which necessitates a manufacturer to improve a formulation. However, shelf-life stability tests must be performed for every alteration.

The cheese spread is one of the most highly accepted components in the U.S. Military Meals, Ready-to-Eat (MRE) feeding program according to military reports. Often times it is added to other entrees to improve overall acceptability, and to add variety. Although the cheese spread currently used has met the 3-yr shelf-life requirement if stored at 26°C (MIL-C-595E), it has shown darkening, hardening, or oiling-off due to prolonged storage at higher temperatures, and when the temperature fluctuates (DPSC 1997). The cheese spread currently used in the military is also vitamin fortified, which may influence product stability.

A systematic approach to proposing an improved formulation must be established. A general classification of additives can be divided as vitamins, colorants, emulsifying agents, and stabilizers. The need for research exists to explore formulation variables that may assist in prolonging the shelf-life from its currently assigned shelf-life to that of three years at 38°C. Therefore, the overall objective of this study was to develop a cheese spread formulation that maintains a desired quality at temperatures >26°C In the process of suggesting formulary changes, the establishment of methods to consistently evaluate physical parameters such as texture, color, pH, water activity and emulsion stability was also performed.

Materials and Methods

Samples

Individually packaged cheese spread (42.5 g) was analyzed. Samples were obtained through a commercial supplier or produced in a pilot plant. Initially, commercial samples were used for method development and to study cheese-age effect, while a basic standard cheese spread formulation was optimized and produced in the University of Tennessee pilot plant to study individual ingredient effects.

In order to determine the best formulary improvements, the effect of cheese-age was determined. Three batches of cheese spread, produced by the commercial supplier, were provided to the lab for age effect determination. A young cheese, 118 days old; a middle-aged cheese, 162 days old; and an older cheese, 207 days old were used (military requires cheese between 90-210 day old cheddar be used). Based on results of the cheese-age effects, a cheddar cheese aged between 145-155 days old was used for all UT produced spreads.

Optimized formulations based on results of ingredient effects were later produced at the commercial facility. Formulation guidelines and requirements, as prescribed by the military (MIL-C-595E), were followed in the creation of an in-house cheese spread. The ingredients that were evaluated with new formulations included vitamins, colorants, stabilizers, and emulsifiers. Table C.1 shows the individual formulary changes that were used in the production of the in-house blend of cheese spread. The flow chart outlining the general production scheme is shown in Figure C.1 (All tables and figures can be found in Appendix C at the end of the chapter.)

The cheese spread produced in the pilot plant was filled into 4 oz. Whirlpak Retain Bag (Nasco, Fort Atkinson, WI). The pouches were sealed and held at 77°C until ready for retorting. Approximately 90 pouches were packaged for each 10 lb cheese spread. The pouches were placed into the retort and steam applied until 110°C inside a test pouch of cheese spread was obtained (approx. 5 mins), then held for one minute. Steam was turned off and the pouches were allowed to cool under 7 psi air pressure until the test pouch cooled to 98°C. Pouches were removed and placed in a room temperature water bath to cool. Pouches were placed into three different storage temperatures: 52 °C for six-weeks (stressed shelf-life study); 4 and 38 °C for six-months (refrigerated and accelerated shelf-life study, respectively). The high temperature treatments were chosen based on military reports that major defects occur after storage at temperatures exceeding 38°C (DPSC 1997). A shelf-life of 3-yrs at 26°C is required for MRE's and government verification may include storage testing using the shorter, higher temperature schemes to obtain stability results. Refrigerated samples are observed to offer a more complete profile, although cold storage lengthens shelf-life. Approximately 10 pouches were kept at room temperature. Cheese spread samples were studied for time-temperature effects.

For the first six weeks of storage, three pouches from each temperature were taken weekly and analyzed for texture, color, water activity, emulsion stability, pH and moisture. Cheese spread pouches were acclimated to room temperature for approximately 2 hrs prior to analysis. All analyses were performed on non-kneaded samples. Portions of each pouch were divided and used in the obtaining the physical parameters. Remaining cheese spread was stored at -40°C for future chemical analysis.

Texture Measurements

Three 7.5 g replications were cut from each packet and gently spread into small plastic cups, also used for water activity. Samples were analyzed on the TA.XT*Plus* Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) using Texture Exponent 32 software version 2.06. The CHE3/P1S application for firmness and stickiness of cheese spread was loaded, using a test speed of 2.5 mm/sec with compression at 50%. A 25-mm diameter cylinder probe was used.

Color Analysis

One 7.5 g sample from each packet was measured for color using the Hunter Lab MiniScan XE Plus (Hunter Associates Laboratory, Reston, VA) after calibration using black and white tiles. L*, a*, and b* values were recorded using the "*Colorant Strength D/65"* setting. L-values measure lightness to darkness on a scale of 0 (black) to 100 (white); a* values are positive (redness) to negative (green); b* values are positive (yellow) to negative (blue).

Water Activity

One 7.5 g sample from each packet was measured using the Aqualab Series 3 meter (Decagon Devices Inc., Pullman, WA) after calibration with factory standards of 0.760 and 0.984.

Emulsion Stability

A single 5 g sample was cut from the middle of each cheese spread pouch and placed in a 50-ml polypropylene copolymer centrifuge tube. The Sorvall RC 5B Plus

centrifuge (ThermoElectron Corp., Asheville, NC) was set at 10,000 rpm (~11,900x*g*) for one hour at room temperature. If phase separation occurred, the upper liquid phase was removed and the percent weight loss was recorded.

pH

A 5 g sample from each packet was cut from the remaining cheese spread, placed in a plastic tube with 10 mL water and homogenized for ~30 sec. The pH of the sample was measured with the Accumet Basic AB15 meter (Fisher Scientific).

Moisture Analysis

Three 2.5 g samples from three packets were measured gravimetrically after 5 hrs at 100°C.

Statistical Analysis

A factorial experimental design was used to evaluate the combination of treatments on the cheese spread. Time, temperature, cheese age (for one section), ingredient (or formulations) are the treatment effects that were analyzed in the determination of significant differences for L-values and firmness measurements. A General Linear Model (GLM) was used to generate ANOVA tables using SAS 9.1 (Carey, NC). Significant differences for main effects and combined interactions are reported. Least-square means and the probability values for the differences observed in the temperature-ingredient interaction are given for cheese spread batches where ingredient substitutions occurred and for optimized formulas. Significance of cheese-age on firmness and L-values was analyzed by Duncan's Multiple Range for mean separation. Data are supplied in Appendix B.

Sensory Evaluation

Sensory evaluation was performed on the optimized formulations and the currently used standard formulations. Sensory testing involved consumer panelists. There were 100 panelists that sampled each of the three cheese spreads. Majority of panelists lacked familiarity with the product and only a few associated it to a "snack pack" type cheese product. The majority of the panelists were between 20-59 years old and not involved in the armed services. The samples were presented in succession with several questions pertaining to appearance, color, flavor, and overall liking. The responses were based on a nine-point hedonic scale where a 1=dislike extremely and a 9= liked extremely. Responses were averaged and a score between one and nine assigned. At the end of the sensory test and after all samples had been returned, the panelists were asked to rank the three samples in order of preference. A sum-ranking test was performed where 3=liked extremely and 1=strongly dislike. A score between 100 and 300 can be obtained.

Results and Discussion

Cheese-age Determination

The military feeding program reports the greatest defects of this product during storage are hardening and darkening, thus the discussion will be focused on the textural (firmness) and color (L-value) measurements. Figure C.2 shows the changes observed in firmness and L-value during storage for six-weeks at 52°C. Table C.2 lists the overall mean separation for the three ages. There were no significant differences in all ages of cheese spread in regard to L-value. However, for firmness, there were significant differences in all ages of cheese spread used. Table C.3 lists the mean separation data obtained during the six-month storage at 4°C and 38°C. Temperature storage for six

month was significant to the firming or darkening of the spreads. At each temperature, differences between firmness and L-values were observed. At 4°C the middle-aged cheese darkened least and had similar firmness to the young cheese. At 38°C there was no significant difference in L-value for the young and middle cheeses, however, the oldest cheese darkened significantly more. Although in both temperatures the oldest cheese hardened the least, it did show emulsion instability throughout analyses. Data for emulsion stability, pH, and water activity for all three cheese ages at all temperatures is listed in Table C.4. pH and water activity experience little to no change during analysis from initial to final sampling. Also, separation was either not observed or the separated liquid was too minimal to separate from the centrifuged sample for the young and middle-aged cheeses. However, measurable separation occurred during analysis for the 207 day old cheese.

The differences in cheese age are likely due to proteolysis during the ripening phases of cheddar cheese production. Proteolysis is one the most complex biochemical processes that occurs in the aging of cheese. There is no one proteolytic agent responsible for the differences in age effects. Sources of the proteolytic compounds may include the proteinase chymosin from the rennet, endogenous milk proteinases (i.e., plasmin), or proteolytic enzymes introduced from the starter. Researchers have shown that as ripening advances, there are detectable changes in the amount of casein or other peptides, indicating proteolytic activity (Fox et al., 2000; Chin et al., 1998). Changes in the protein structure are directly related to differences observed in textural and color properties. If the darkening is potentially the result of Maillard reactions, then more available amino groups, residual lactose, and presence of vitamins will promote the non-enzymatic browning process. Based on changes in firmness, darkening, and

emulsion stability, a middle age cheese was selected for use in the laboratory production of cheese spread as it showed greatest stability of those tested.

Ingredient Evaluation

An optimized formulation was developed in our laboratories based on the aforementioned military guidelines using cheddar cheese obtained from a commercial supplier. The types of ingredients were broken down into groups including vitamins, colorants, emulsifiers, and stabilizers. The vitamins group was the first selected for ingredient analysis to determine overall effect of the vitamin fortification that is required by military specifications.

Vitamins

The guidelines for cheese spread fortification include the addition of retinol (vitamin A), thiamine (vitamin B1), pyridoxine (vitamin B6), and ascorbic acid (vitamin C). There were four batches produced which include the following: (1) a control (4CON), which satisfies minimum requirements for all required vitamins; (2) excess ascorbic acid (CON40), which included 10 times more ascorbic acid than control with other vitamins in required quantities; (3) no ascorbic acid added (VNC) but vitamins added in required quantities; and (4) no vitamins added (NV). Firmness and L-value data are presented in Figure C.3 and least square means for temperature and formulation interactions are presented in Table C.5. Based on the ANOVA analysis, there were significant differences for main effects (time, temperature, and formulation) and combined interactions ($p<0.0001$) at the $p=0.05$ level of confidence. Mean separations indicate that no significant differences were found within the 4°C temperature storage for L-value and

firmness; however, at higher temperatures the formulations containing ascorbic acid did darken significantly more than those batches not containing it.

Some of the darkening observed in the formulations may be due to changes in the carotenoid based colorant, or due to non-enzymatic browning. Thermal processing conditions can affect the stability of the colorant by causing structural isomerization or producing degradation products due to autooxidation. Colorants will be discussed further in the next section. However, since the greatest effect of darkening was observed in the presence of ascorbic acid, it is believed that the browning is likely due to its degradation. Chemical structures of the vitamins are shown in Figure C.4. A general reaction scheme for ascorbic acid catalyzed non-enzymatic browning is outlined in Figure C.5.

The exact pathway to ascorbic acid browning is highly variable and dependant on the food system. The exact process is complex since the formation of over one-hundred different intermediates and degradation products have been identified (Shultz et al., 2007). The basics of what is known is that if oxygen is present then ascorbic acid is broken down primarily to L-dehydroascorbic acid; however, under anaerobic conditions this product is not formed, but rather other keto-acids ultimately leading to the formation of furfural. Furfural and other carbonyl containing reaction intermediates from either oxidative or non-oxidative pathways can then react with amino acids resulting in formation of brown polymers. Formation of brown polymers can also be explained in terms of the classic Maillard reaction between a carbonyl group of a reducing sugar and an amino acid. These carbonyl-amino reactions are accelerated during the thermally processing (Hui 2006). In the cheese spread, there may be residual lactose, a reducing sugar, present from the initial cheddar cheese production. Also, due to the presence of thiamine and pyridoxine, any reducing sugar may be susceptible to nucleophilic attack by the amino moiety present.

Other physical parameters are shown for initial analysis and the six-week or six-month time point at all storage temperatures are listed in Table C.6. pH remained within the required range and water activity varied slightly. Also, there was no separation observed in these analyses. Therefore, it may be suggested that the greatest effect in product stability under these conditions may be the removal of vitamins or, at a minimum, vitamin C. However, since the assessment of other ingredients will be studied, the optimized control formulation will include the minimum requirement of all vitamins as specified by the military. This new control batch (4BCON) was produced and used in comparison analysis with the colorants, emulsifiers, and stabilizers.

Colorants

Figure C.6 shows the overall trends in both firmness and L-value changes at 52, 38, and 4°C over six-week or six-month storage. Least square means are listed in Table C.7. Based on the ANOVA analysis, there were significant differences for main effects (time, temperature, and formulation) and combined interactions ($p<0.0001$) at the $p=0.05$ level of confidence, except there was no significant difference ($p=0.3036$) for time-ingredient interactions. Means separations indicated that NCL differed at each temperature; however, this was expected for L-value given the lack of colorant added. Firmness measurements were most significantly affected by temperature storage

The maintenance of the cheese color was a primary indicator for stability. The military requirement for color suggests an L-value between 69.00 and 76.80 based on a national cheese color standard. For all the colored samples, L-values were unacceptable after just two weeks at 52°C and one month at 38°C. All refrigerated samples would be deemed acceptable.

Table C.8 lists data for pH, water activity, and emulsion stability initially and at the end of the storage per given temperature. Values were maintained throughout analysis. Phase separation was observed in refrigerated samples; the control showed no separation.

Emulsifying Salts

The results in Figure C.7 indicate an increased firmness in CIT as compared to LP and 4BCON at 52°C while observed weekly. According to least squares analysis (Table C.9) LP differed significantly from 4BCON and CIT in firmness at all stored temperatures. 4BCON and CIT were not significantly different from each other. ANOVA analysis indicated that significant differences did exist for temperature, time, and their interaction ($p<0.0001$), but no significant differences were observed for temperature-ingredient ($p=0.2220$) or time-ingredient ($p=0.2265$) interactions. For color, no significant difference was found between ingredient, temperature, and time interactions. L-value means indicate that 4°C stored samples were different from those stored at elevated temperatures. Table C.10 lists pH, water activity, and emulsion stability. The emulsion was stable for the control after storage in all temperatures but separation was observed after six weeks at 4°C for CIT and LP, not 4BCON. The effect of different salts in promoting various physicochemical changes has been studied; however, the differences are likely due to initial product formulation regarding initial protein content (Fox et al., 2000). The emulsifying salts most commonly used in process cheese spread are citrates and phosphates. Many studies have measured textural changes observed with the use of such salts (Gupta et al.,1984; Awad et al., 2002; Dimitreli et al., 2005; Joshi et al., 2004; Pastorino et al., 2003). Emulsifying salts are added to enhance meltability, sliceability, and spreadability. Since a requirement of process cheese is to be

spreadable at room temperature it is important to use the proper emulsifying salt, or combination of salts.

In both formulary substitutions, the emulsifying salts that were used consist of a monovalent cation, sodium, and a polyvalent anion, phosphate. Through ionic interactions several changes occurred resulting in calcium sequestration, buffering of pH, paracasein dispersal, and emulsification (Dimitreli et al., 2005). Although the citrates and phosphates have similar ionic components, the attractive forces between the surface of the cheese and other components will affect the rate of protein aggregation (Awad 2002) and emulsification. In general, for processed cheeses, phosphates have greater calcium sequestration than do the citrates; however, phosphates possess greater buffering capacity in the range of cheese spread (pH=5.5-6.0) and the more acidic citrates tend to produce crumbly spreads (Fox et al., 2000). This can potentially explain the behavior observed after six weeks in the stressed environment of 52°C. The effect of the emulsifying salts is difficult to discern based on the complex environment present in the cheese spread.

Stabilizers

Data presented in Figure C.8 shows the trend for firmness and L-value throughout the six-week (52°C) and six-month (4 and 38°C) storage periods. In the elevated temperatures, firmness increased over storage time while minimal changes are observed in the refrigerated temperature. L-values decreased, or darkened, in the elevated temperatures and remained relatively unchanged in cold storage. ANOVA analysis for L-value indicated time, temperature, ingredient, and temp-time interaction did significantly (<0.0001) effect the behavior of the stabilizers; however, there were no significant differences between time-ingredient interactions (0.3790). A similar trend was

observed by ANOVA for firmness as well. Least square means are given in Table C.11. The data supports that temperature had an effect on mean separation, while ingredients varied minimally within each temperature for both firmness and L-values. As seen in previous instances, the pH and water activity remained constant in initial and final values (Table C.12) while phase separation was observed at 4°C for all ingredients except that of the control. CAR did exhibit minimal emulsion instability after six-months at 38°C and 52°C.

Suggested Formulary Changes

To summarize the individual analysis of the four classifications of additives studied are: (1) vitamin removal will lessen the amount of darkening and hardening observed during storage; (2) minimal effect on firmness or darkening was observed with the colorants and emulsifying agents; and (3) the use of carrageenan as the stabilizer resulted in a less firm product as compared with the other stabilizers. From these conclusions, two major formulary changes were suggested.

The current commercial formulation was used as the base recipe and our suggested changes were applied. A total of three batches were produced, the first being a batch of the presently used commercial formulation. This was designated as the standard formulation (STD). The second formulation was produced with no added vitamins (NV); and the third formulation was with no added vitamins, and standard gums were partially substituted with carrageenan (GN). All analyses was performed as described previous, however, due to concern by military inspectors that the fluctuation of temperature on a daily basis may have an effect on product stability, an additional temperature scheme was observed. This involved the ramping up and down of temperature from 30 to 50°C over 24 hrs.

Figure C.9 shows the data for STD, NV, and GN at 52°C and temperature cycled for six weeks. In Figure C.10 data for all three batches is shown at 4 and 38°C for six-month storage. At all the temperatures, STD increased in firmness more than NV and GN. A greater reduction in L-value, or darkening, was also observed for STD in comparison to the other two formulations. However, less darkening occurred at 4°C. Table C.13 lists the least square means and their separations. Within each temperature parameter, STD darkened or became more firm significantly when compared to NV and GN. There was no significant difference between GN and NV within temperatures based on mean separation. ANOVA analysis indicated that all main effects (temperature, time, formula) and combined interactions were significant at α=0.05. Also, all batches remained in an acceptable L-value range for the temperature cycled, 4 and 38°C storage conditions, while the STD formula was the only one to drop below an acceptable L-value at the final time point of the 52°C storage.

In all three batches, no emulsion separation occurred and pH and water activity were maintained from initial to final analyses (Table C.14).

Sensory Evaluation

Sensory evaluation was performed on STD, NV, and GN. A sum ranking test was done to determine liking. Table C.15 shows the results. No significant difference was found between STD and NV, nor was there a difference between NV and GN. The other sensory test performed was based on a nine-point hedonic scale. Results in Table C.16 indicate that NV and GN scored higher in appearance and flavor and that no significant difference was found between them. NV and STD did not differ for flavor and overall liking, however GN scored higher in these two categories.

Conclusion

The systematic approach to formulary improvement began with the determination of cheese-age effect on the currently used formulation. Once it was established that a middle-aged cheese deemed was appropriate based on emulsion stability and comparatively less resistance to darkening or hardening. The greatest impact from the individual ingredient effects was seen in the vitamin studies. The removal of vitamins from the processed cheese formulation reduced the amount of darkening and hardening that was previously occurring. Although the military requires fortification of the cheese spread, possible solutions would be to encapsulate the vitamins and keep them present in the spread, or simply remove them and use another means of subjection. The suggestions we proposed for colorants and emulsifying salts based on the UT formulation turned out to be similar to what is currently used in industry. The final suggestion was made in regards to the stabilizer. Our results indicated that carrageenan offered a less firm product during time and temperature studies, so a partial gum substitution was suggested to minimally affect texture. The proposed formulary changes were made and cheese spread batches were produced commercially. Final results still support that the removal of vitamins will improve the product stability against hardening and darkening so rapidly, especially when stored at higher temperatures.

This study also shows that formulary changes must be carefully thought out and executed in order to occur. It is too costly in the food industry to produce something large scale only to discover the change did not work. A small pilot plant operation, like that performed in our laboratory, shows the symbiotic relationship that collaborative efforts (military, commercial supplier and the University of TN) can produce. It is up to the military if they want to pursue further studies based on these formulary suggestions.

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Appendix C

Table C.1 Codes and descriptions of cheese spread produced at the University of Tennessee

Batch Code	Description
4CON	Possible control batch, meets military specs, uses 4g ascorbic acid
CON40	Possible control batch, exceeds minimum ascorbic acid by 10x (40 g added)
VNC	Vitamins A, B1, B6 added, no vitamin C (as ascorbic acid)
NV	No vitamins A, B1, B6, or C added
4BCON	Control batch using UT formulation
NCL	Standard formula used, no colorants added
APO	Only APO-8'-carotenal used as colorant in standard formulation
ANT	Only Annatto used as colorant in standard formulation
PO	Only Paprika Oleoresin used as colorant in standard formulation
LMG	Lesser amount of gums used vs. standard formulation
XAN	Xanthan gum used as stabilizer substitute
LMP	Low-methoxy pectin used as stabilizer substitute
CAR	Carrageenan used as stabilizer substitute
LP	Altered ratio of trisodium and disodium phosphates used vs. standard formulation
CIT	Sodium citrate used as emulsifying salt substitute

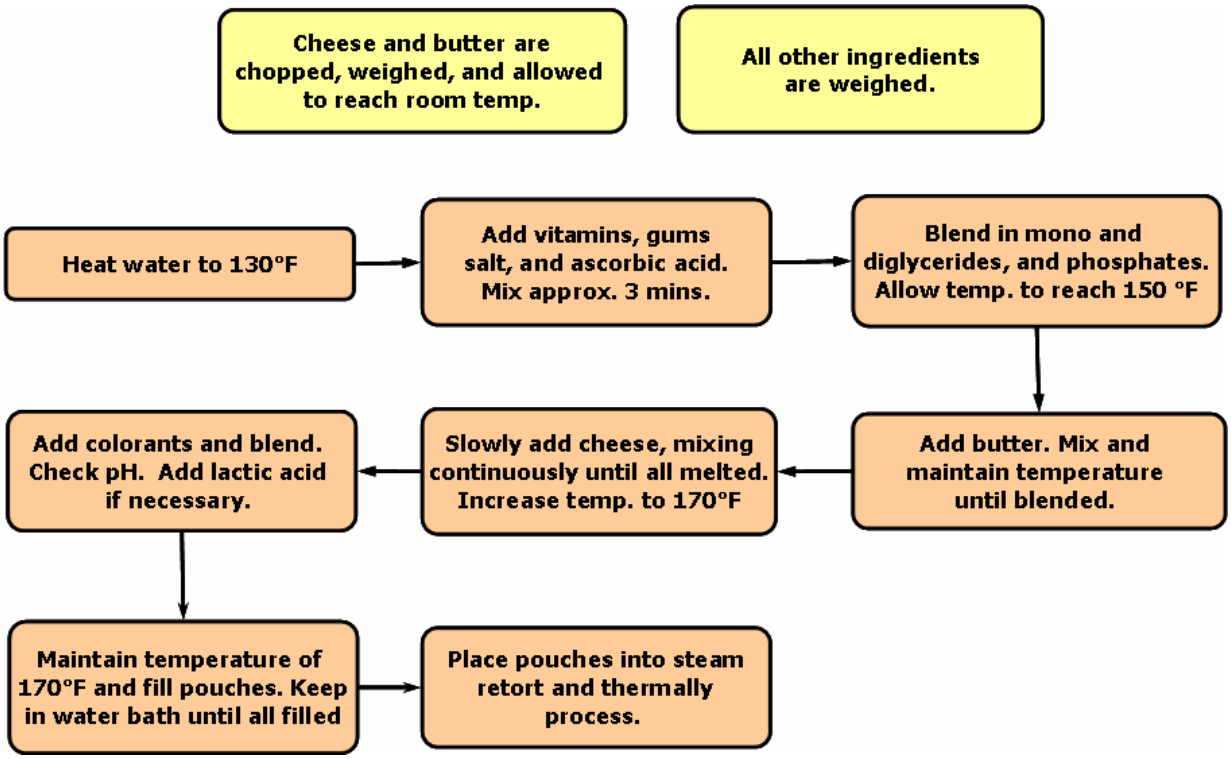


Figure C.1 General production scheme used for cheese spread produced at University of Tennessee.

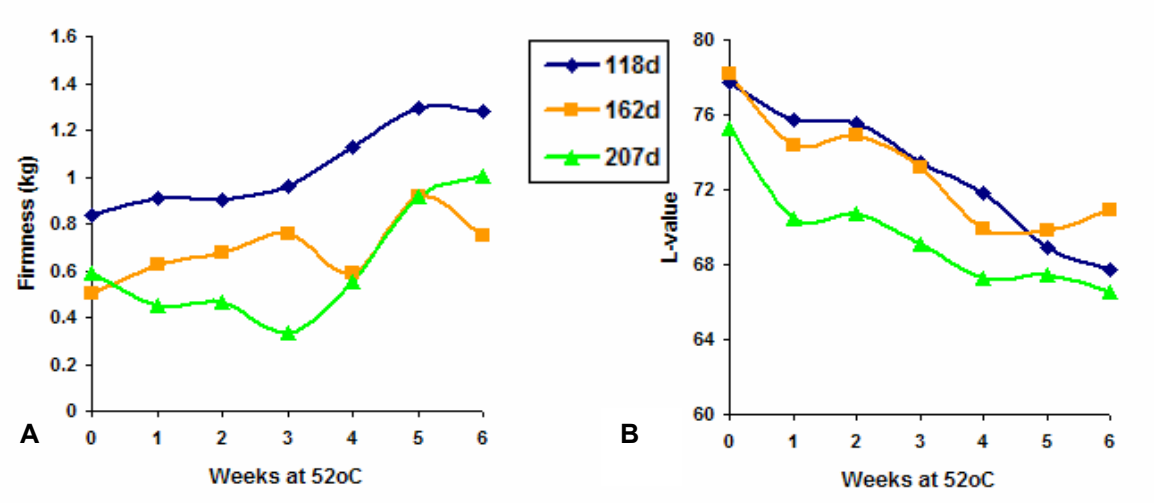


Figure C.2 Measurements of cheese spread made with 118 day (d), 162d, or 207d old cheddar during storage for six weeks at 52°C. (A) Texture measured as firmness (kg); (B) Darkening observations measured as L-value

Table C.2 Mean separation data for L-value and firmness of the three cheese ages during six week storage at 52°C. A Duncan's test was performed to determine if difference in cheese age was significant to quality.

Age (days)	L-value	Firmness (kg)
118	72.69[a]	1.056[a]
162	73.03[a]	0.688[b]
207	69.55[a]	0.616[c]

Data in columns followed by different letters are significant at ($p<0.05$).

Table C.3 Mean separation data for L-value and firmness for all three ages of cheese spread during six-month storage at refrigerated and stressed temperatures. A Duncan's test was performed to determine if difference in cheese age was significant to quality.

Temperature (C)	Age (days)	L-value	Firmness (kg)
4[A]	118	75.85[b]	1.084[a]
	162	77.18[a]	1.040[a]
	207	74.71[b]	0.829[b]
38[B]	118	72.65[a]	1.109[a]
	162	72.66[a]	0.987[a]
	207	69.25[b]	0.907[a]

Data in columns for each temperature followed by different letters are significant at $p<0.05$

Table C.4 pH, water activity and emulsion stability data are presented for initial and final data sampling times. Emulsion stability refers to amount of liquid that separated from sample after centrifugation. Averages and standard deviations are shown for three replications.

Age (days old)	Time (w=weeks m=months)	pH	A_w	Emulsion Stability/Separation (%)
118	Initial	5.88 ± 0.01	0.950 ± 0.003	No separation
	6w-52	5.86 ± 0.04	0.950 ± 0.007	No separation
	6m-4	5.84 ± 0.14	0.962 ± 0.004	~11
	6m-38	5.84 ± 0.02	0.956 ± 0.009	No separation
162	Initial	5.82 ± 0.04	0.959 ± 0.004	No separation
	6w-52	5.90 ± 0.03	0.955 ± 0.004	No separation
	6m-4	6.02 ± 0.04	0.942 ± 0.003	Minimal
	6m-38	5.83 ± 0.08	0.950 ± 0.002	No separation
207	Initial	5.93 ± 0.05	0.947 ± 0.003	1.20 ± 0.01
	6w-52	5.80 ± 0.01	0.947 ± 0.004	0.90 ± 0.03
	6m-4	6.17 ± 0.12	0.949 ± 0.002	0.83 ± 0.18
	6m-38	5.93 ± 0.02	0.953 ± 0.10	Minimal

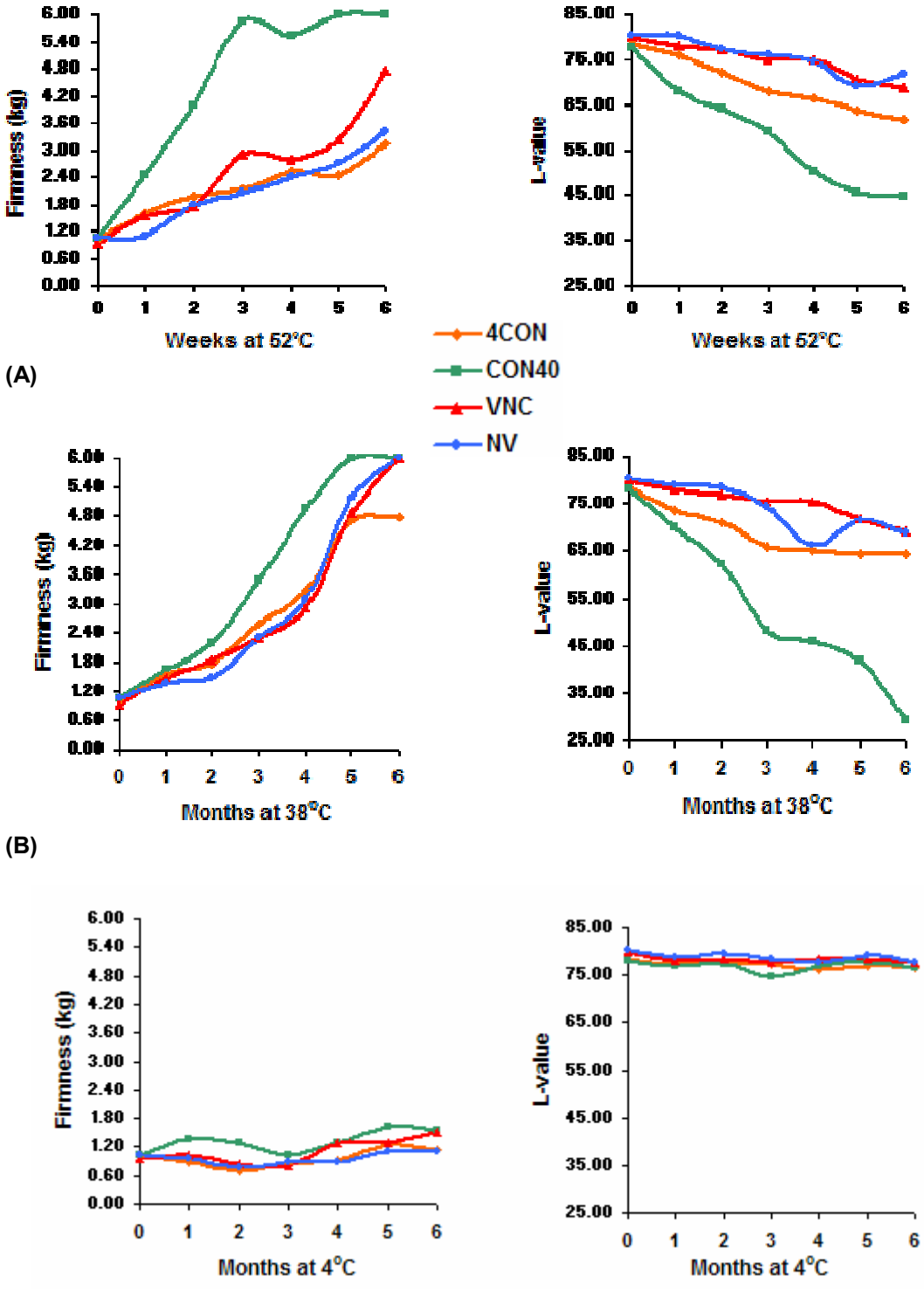
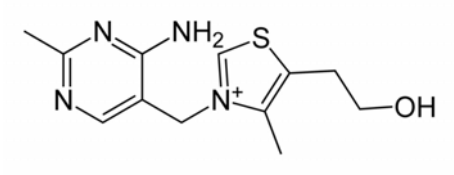


Figure C.3 Texture and L-value data for in-house produced cheese spread (A) 52°C for six weeks, and (B) 38°C and (C) 4°C for six months of batches used in vitamin comparisons. (4CON=control; CON40=control with 10x vitamin C added; VNC= vitamins other than C added; NV=no vitamins added)

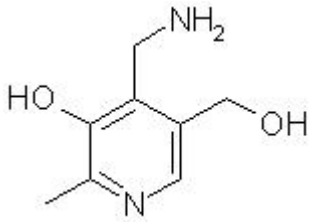
Table C.5 Least square means based on overall temperature and ingredient interactions at all three storage temperatures.

		Least Squares Means	
Temperature (°C)	**Ingredient variation**	**L-value**	**Firmness (kg)**
4	4CON	77.19[a]	0.968[a]
	CON40	76.80[a]	1.309[a]
	NV	78.78[a]	0.959[a]
	VNC	78.41[a]	1.108[a]
38	4CON	70.41[b]	2.808[b]
	CON40	53.62[c]	3.619[c]
	NV	74.08[a]	2.920[b]
	VNC	75.23[a]	2.890[b]
52	4CON	69.61[b]	2.114[c]
	CON40	58.49[d]	4.407[d]
	NV	75.59[a]	2.063[c]
	VNC	74.98[a]	2.554[b]

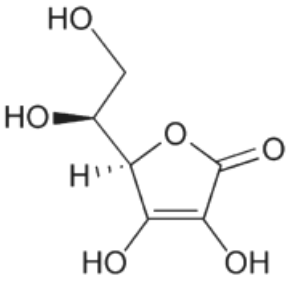
Data in columns followed by the same letter do not differ significantly at $p<0.05$. (4CON=control; CON40=control with 10x vitamin C added; NV=no vitamins added; VNC= vitamins other than C added)



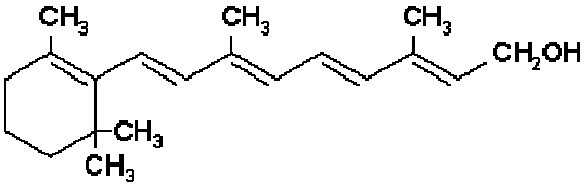
Thiamine (vitamin B1)



Pyridoxine (vitamin B6)

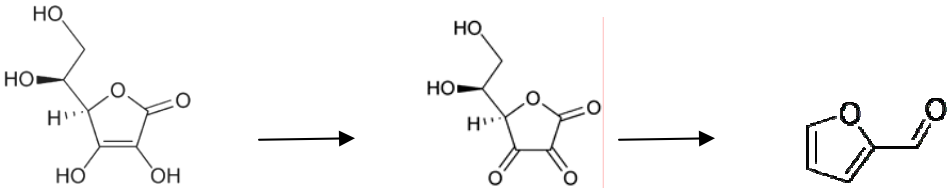


Ascorbic Acid (vitamin C)



Retinol (vitamin A)

Figure C.4 Structures of the vitamins used in cheese spread fortification



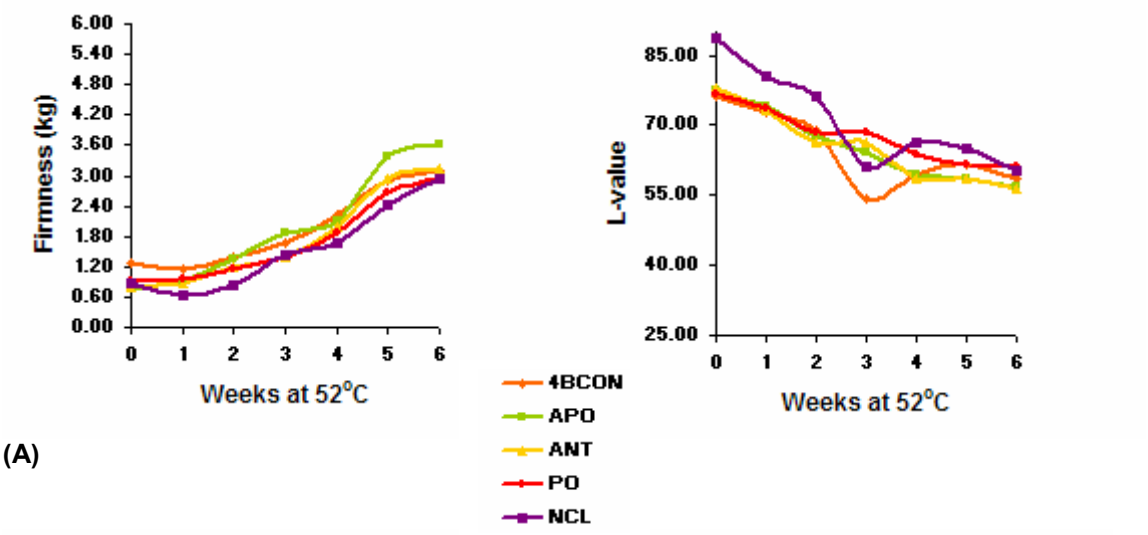
L-Ascorbic acid **L- Dehydroascorbic acid** **Furfural**

Furfural
(or other reaction intermediates) → Amino Acids → Brown polymers

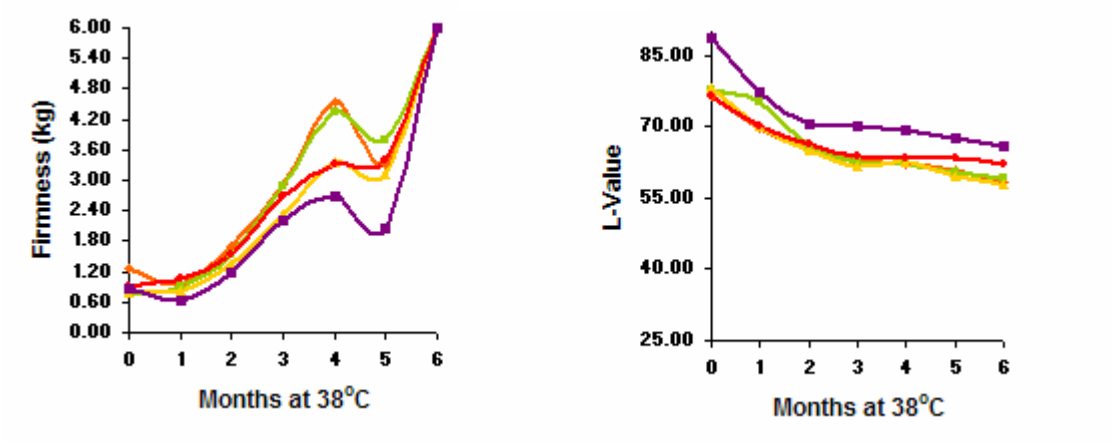
Figure C.5 Basic ascorbic acid degradation pathway resulting in formation of brown polymers

Table C.6 pH, water activity, and emulsion stability of cheese spreads of different vitamin inclusion. Averages and standard deviation are shown for three replications.

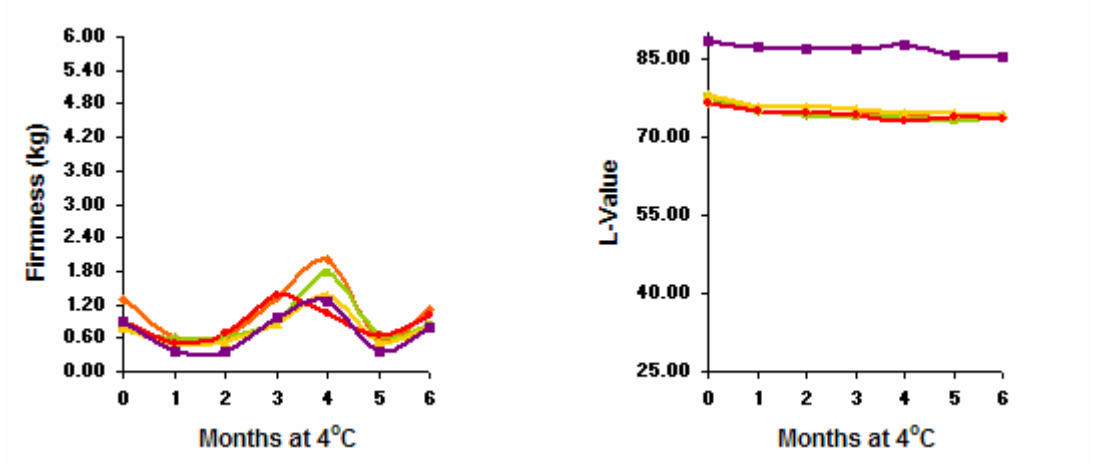
Batch	Time	pH	A_w	Emulsion Stability/ Separation
4CON	Initial	5.49 ± 0.03	0.965 ± 0.004	No separation
	6w-52	5.52 ± 0.03	0.936 ± 0.001	No separation
	6m-4	5.63 ± 0.01	0.951 ± 0.004	No separation
	6m-38	5.63 ± 0.02	0.925 ± 0.004	No separation
CON40	Initial	5.53 ± 0.02	0.952 ± 0.003	No separation
	6w-52	5.36 ± 0.01	0.928 ± 0.004	No separation
	6m-4	5.66 ± 0.07	0.955 ± 0.003	No separation
	6m-38	5.63 ± 0.06	0.909 ± 0.002	No separation
VNC	Initial	5.77 ± 0.03	0.960 ± 0.001	No separation
	6w-52	5.56 ± 0.02	0.918 ± 0.008	No separation
	6m-4	5.98 ± 0.09	0.955 ± 0.005	No separation
	6m-38	5.78 ± 0.03	0.918 ± 0.009	No separation
NV	Initial	5.82 ± 0.12	0.963 ± 0.001	No separation
	6w-52	5.59 ± 0.02	0.919 ± 0.007	No separation
	6m-4	6.05 ± 0.05	0.949 ± 0.003	No separation
	6m-38	5.76 ± 0.01	0.896 ± 0.003	No separation



(A)



(B)



(C)

Figure 4.6 Texture and L-value data for in-house produced cheese spread (A) 52°C for six weeks, and (B) 38°C and (C) 4°C for six months of batches used in colorant comparisons. (4BCON=control; APO=apo-carotenal; ANT=annatto; PO=paprika oleoresin; NCL=no color)

Table C.7 Least square means based on overall temperature and ingredient interactions at all three storage temperatures.

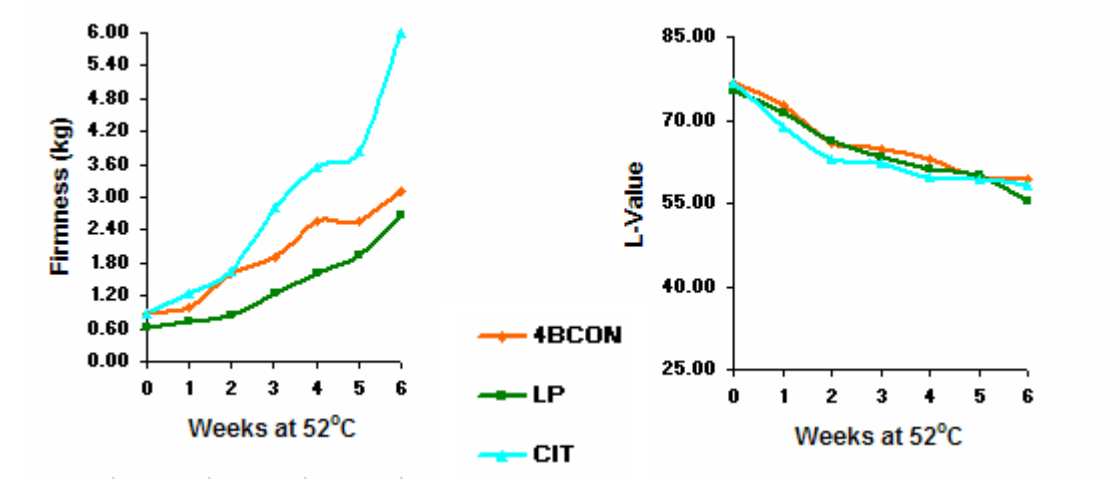
		Least Squares Means	
Temperature (°C)	**Ingredient variation**	**L-value**	**Firmness (kg)**
4	4BCON	74.53[a]	1.077[a]
	ANT	75.41[a]	0.768[b]
	APO	74.49[a]	0.870[b]
	NCL	86.95[b]	0.711[b]
	PO	74.39[a]	0.883[b]
38	4BCON	65.20[c]	2.964[c]
	ANT	64.99[c]	2.541[d]
	APO	65.39[c]	2.896[c,e]
	NCL	72.72[a,d]	2.240[f]
	PO	66.53[c,e]	2.700[d,e]
52	4BCON	64.58[c]	1.962[g]
	ANT	65.29[c]	1.763[g,h]
	APO	65.53[c]	1.998[g]
	NCL	71.24[d]	1.548[i]
	PO	67.76[e]	1.698[h,i]

Data in columns followed by the same letter do not differ significantly at $p<0.05$. (4BCON=control; APO=apo-carotenal; ANT=annatto; PO=paprika oleoresin; NCL=no color)

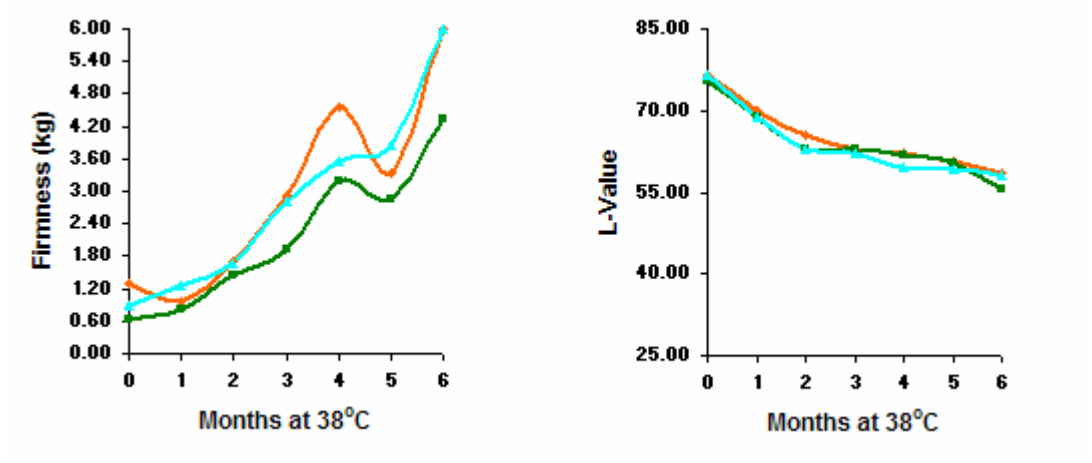
Table C.8 pH, water activity, and emulsion stability of cheese spreads of different colorant inclusion. Averages and standard deviations are shown for three replications.

Batch (ingredient variations)	Time (w=weeks m=months)	pH	A_w	Emulsion Stability/ Separation (%)
4BCON	Initial	5.77 ± 0.01	0.958 ± 0.001	No Separation
	6w-52	5.76 ± 0.05	0.919 ± 0.005	No Separation
	6m-4	5.83 ± 0.07	0.951 ± 0.004	No Separation
	6m-38	5.70 ± 0.02	0.891 ± 0.010	No Separation
APO	Initial	5.76 ± 0.02	0.955 ± 0.002	No Separation
	6w-52	5.76 ± 0.11	0.914 ± 0.007	No Separation
	6m-4	5.81 ± 0.12	0.947 ± 0.003	1.55 ± 0.51
	6m-38	5.70 ± 0.02	0.901 ± 0.010	No Separation
ANT	Initial	5.83 ± 0.01	0.957 ± 0.003	No Separation
	6w-52	5.88 ± 0.09	0.917 ± 0.005	No Separation
	6m-4	5.87 ± 0.06	0.950 ± 0.001	3.44 ± 0.97
	6m-38	5.68 ± 0.01	0.893 ± 0.009	No Separation
PO	Initial	5.80 ± 0.01	0.954 ± 0.003	No Separation
	6w-52	5.76 ± 0.07	0.920 ± 0.006	No Separation
	6m-4	5.78 ± 0.01	0.946 ± 0.004	0.82 ± 0.19
	6m-38	5.67 ± 0.03	0.917 ± 0.008	No Separation
NCL	Initial	5.98 ± 0.31	0.963 ± 0.003	No Separation
	6w-52	5.82 ± 0.07	0.925 ± 0.005	No Separation
	6m-4	5.75 ± 0.04	0.953 ± 0.005	2.52 ± 0.40
	6m-38	5.70 ± 0.02	0.901 ± 0.011	No Separation

(4BCON=control; APO=apo-carotenal; ANT=annatto; PO=paprika oleoresin; NCL=no color)



(A)



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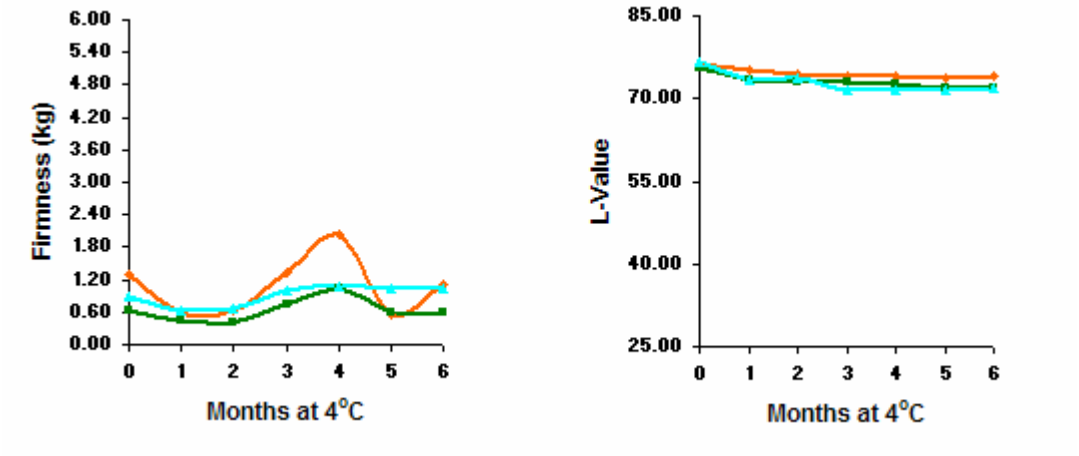


(C)

Figure C.7 Texture and L-value color data for (A) 52°C for six weeks, and (B) 38°C and (C) 4°C for six months of batches used in emulsifying salt comparisons. (4BCON=control; CIT=citrate; LP=adjusted phosphate mix)

Table C.9 Least square means based on overall temperature and ingredient interactions at all three storage temperatures.

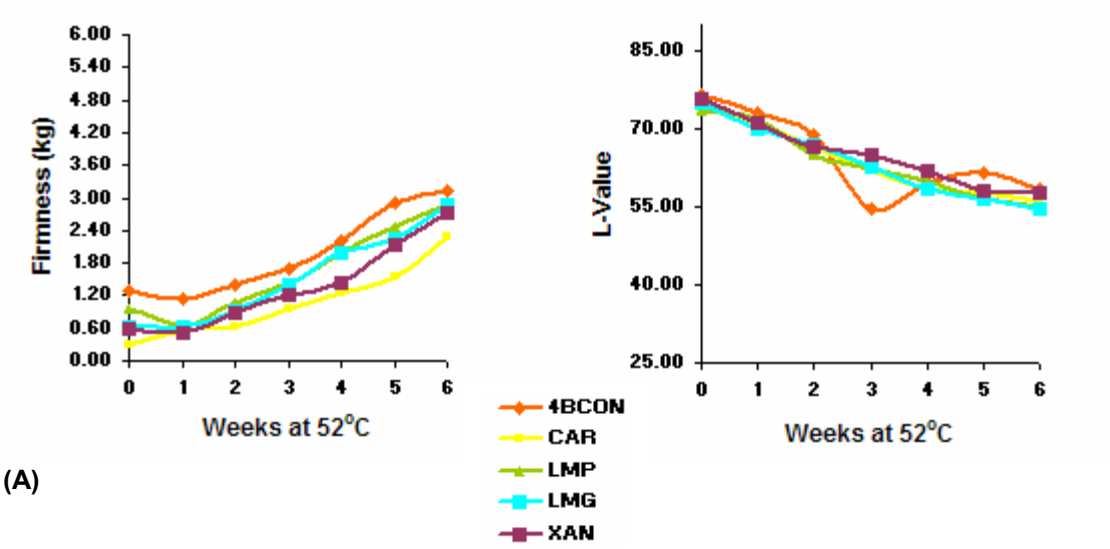
		Least Squares Means	
Temperature (°C)	**Ingredient variation**	**L-value**	**Firmness (kg)**
4	4BCON	74.53[a]	1.077[a]
	CIT	72.97[a]	0.906[a,b]
	LP	73.03[a]	0.642[b]
38	4BCON	65.20[b]	2.964[c]
	CIT	63.98[b]	2.860[c]
	LP	63.99[b]	2.165[c]
52	4BCON	64.58[b]	1.962[d]
	CIT	66.03[b]	1.942[d]
	LP	64.66[b]	1.378[e]

Data in columns followed by the same letter do not differ significantly at $p<0.05$. (4BCON=control; CIT=citrate; LP=adjusted phosphate mix)

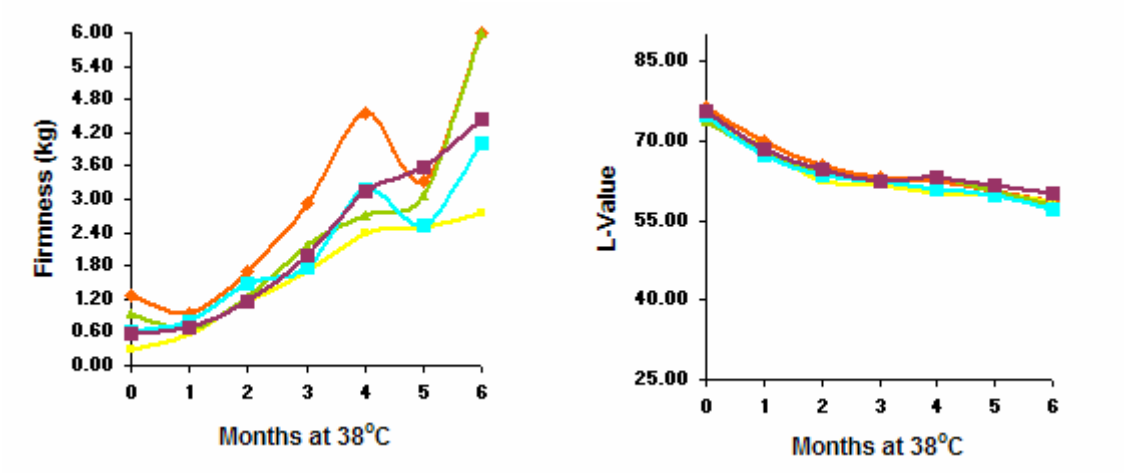
Table C.10 pH , water activity, and emulsion stability of cheese spreads with different emulsifying salt inclusion. Averages and standard deviations are shown for three replications.

Batch (ingredient Variations)	Time (w=weeks m=months)	pH	A_w	Emulsion Stability/ Separation (%)
4BCON	Initial	5.77 ± 0.01	0.958 ± 0.001	No Separation
	6w-52	5.76 ± 0.05	0.919 ± 0.005	No Separation
	6m-4	5.83 ± 0.07	0.951 ± 0.004	No Separation
	6m-38	5.70 ± 0.02	0.891 ± 0.010	No Separation
LP	Initial	5.96 ± 0.01	0.963 ± 0.003	No Separation
	6w-52	5.81 ± 0.15	0.932 ± 0.005	No Separation
	6m-4	5.70 ± 0.10	0.950 ± 0.006	3.94 ± 0.60
	6m-38	5.50 ± 0.12	0.916 ± 0.002	No Separation
CIT	Initial	5.92 ± 0.00	0.959 ± 0.001	No Separation
	6w-52	5.74 ± 0.02	0.934 ± 0.004	No Separation
	6m-4	5.72 ± 0.04	0.954 ± 0.006	1.08 ± 0.28
	6m-38	5.69 ± 0.02	0.923 ± 0.004	No Separation

(4BCON=control; CIT=citrate; LP=adjusted phosphate mix)



(A)



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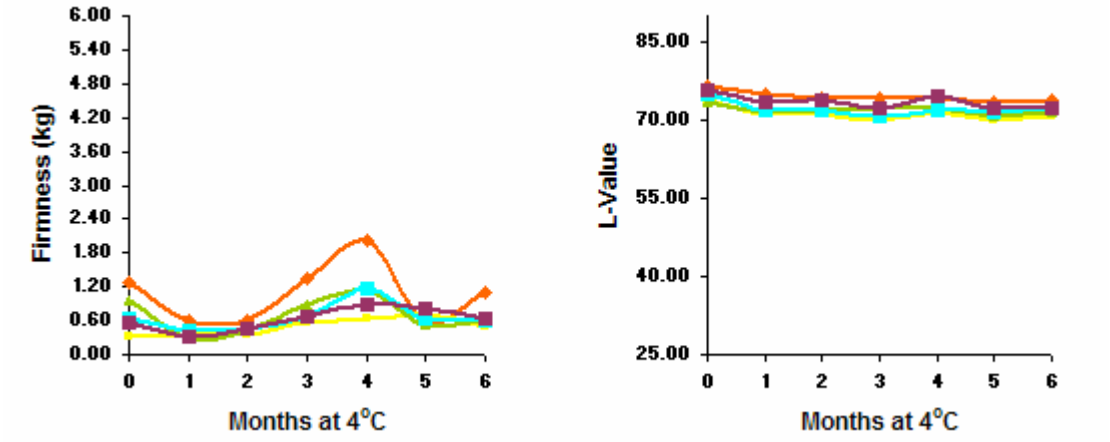

(C)

Figure C.8 Texture and L-value color data for (A) 52°C for six weeks, and (B) 38°C and (C) 4°C for six months of batches used in stabilizer comparisons. (4BCON=control; CAR=carrageenan; LMG=lower mixed-gums; LMP= low-methoxy pectin; XAN=xanthan)

Table C.11 Least square means based on overall temperature and ingredient interactions at all three storage temperatures.

		Least Squares Means	
Temperature (°C)	**Ingredient variation**	**L-value**	**Firmness (kg)**
4	4BCON	74.53[a]	1.077[a]
	CAR	71.20[b]	0.490[b]
	LMG	72.15[b,c]	0.662[b]
	LMP	71.98[b]	0.687[b]
	XAN	73.46[a,c]	0.624[b]
38	4BCON	65.19[d]	2.964[c]
	CAR	63.54[e]	1.627[d]
	LMG	63.72[e]	2.056[e]
	LMP	64.22[d,e]	2.401[f]
	XAN	65.19[d]	2.220[e,f]
52	4BCON	64.58[d,e]	1.962[e]
	CAR	63.62[e]	1.079[a]
	LMG	63.48[e]	1.525[d]
	LMP	63.45[e]	1.631[d]
	XAN	65.22[d]	1.350[a]

Data in columns followed by the same letter do not differ significantly at $p<0.05$. (4BCON=control; CAR=carrageenan; LMG=lower mixed-gums; LMP= low-methoxy pectin; XAN=xanthan)

Table C.12 pH, water activity, and emulsion stability of cheese spreads with different stabilizer inclusion. Averages and standard deviations are shown for three replications.

Batch (ingredient variation)	Time (w=weeks m=months)	pH	A_w	Emulsion Stability/ Separation (%)
4BCON	Initial	5.77 ± 0.01	0.958 ± 0.001	No Separation
	6w-52	5.76 ± 0.05	0.919 ± 0.005	No Separation
	6m-4	5.83 ± 0.07	0.951 ± 0.004	No Separation
	6m-38	5.70 ± 0.02	0.891 ± 0.010	No Separation
LMG	Initial	5.89 ± 0.01	0.961 ± 0.002	No Separation
	6w-52	5.69 ± 0.01	0.925 ± 0.003	No Separation
	6m-4	5.67 ± 0.05	0.941 ± 0.007	3.09 ± 0.68
	6m-38	5.50 ± 0.06	0.913 ± 0.009	No Separation
XAN	Initial	5.81 ± 0.01	0.959 ± 0.002	No Separation
	6w-52	5.72 ± 0.07	0.931 ± 0.002	No Separation
	6m-4	5.76 ± 0.07	0.954 ± 0.004	4.40 ± 0.36
	6m-38	5.60 ± 0.02	0.913 ± 0.004	No Separation
CAR	Initial	5.92 ± 0.07	0.959 ± 0.001	No Separation
	6w-52	5.79 ± 0.02	0.940 ± 0.001	No Separation
	6m-4	5.99 ± 0.06	0.953 ± 0.006	5.06 ± 0.48
	6m-38	5.63 ± 0.08	0.923 ± 0.002	0.68 ± 0.42
LMP	Initial	5.85 ± 0.03	0.954 ± 0.001	No Separation
	6w-52	5.71 ± 0.05	0.914 ± 0.002	No Separation
	6m-4	5.92 ± 0.11	0.948 ± 0.003	4.88 ± 0.59
	6m-38	5.63 ± 0.01	0.906 ± 0.004	No Separation

(4BCON=control; CAR=carrageenan; LMG=lower mixed-gums; LMP= low-methoxy pectin; XAN=xanthan)

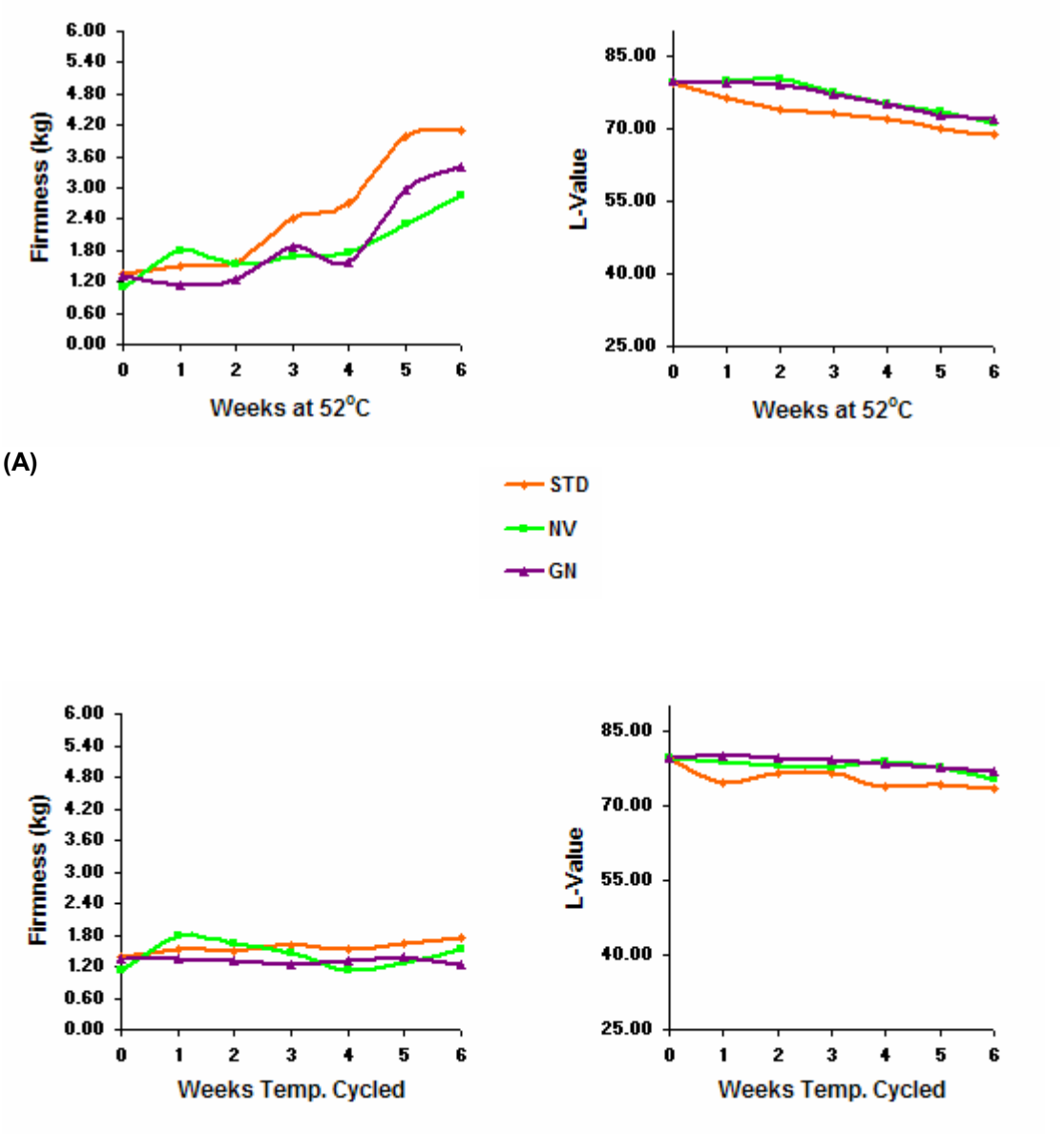

Figure C.9 Texture and L-value color data for (A) 52°C and (B) temperature cycled for six weeks of batches made with suggested formulation changes. (STD=standard/control; NV=no vitamins added; GN=partial gum substitution and no vitamins added)

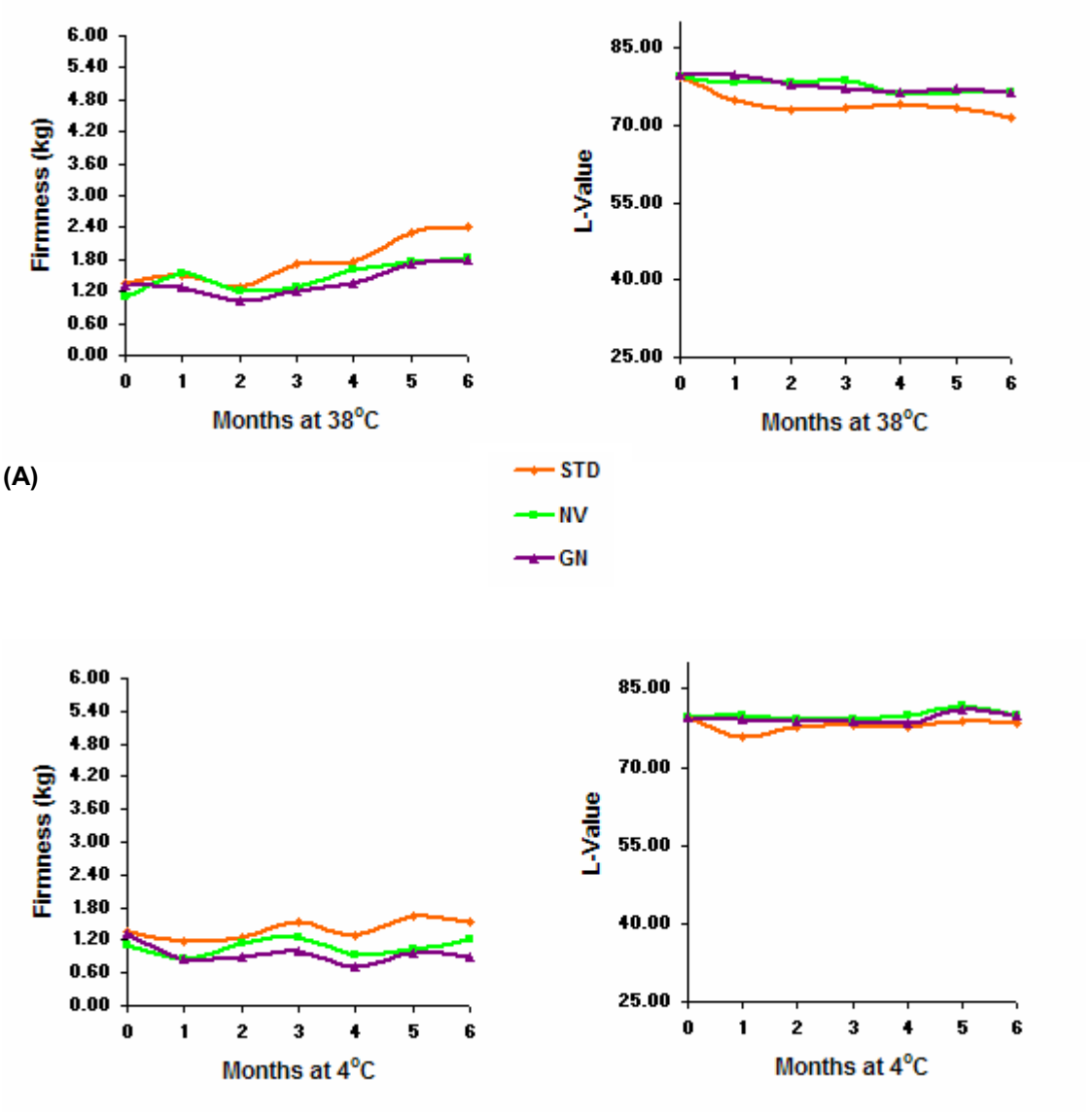

Figure C.10 Texture and L-value color data for (A) 38°C and (B) 4°C for six months of batches made with suggested formulation changes. (STD=standard/control; NV=no vitamins added; GN=partial gum substitution and no vitamins added)

Table C.13 Least square means based on overall temperature and ingredient interactions at all three storage temperatures.

		Least Squares Means	
Temperature (°C)	**Ingredient variation**	**L-value**	**Firmness (kg)**
4	STD	77.99[a]	1.395[a]
	NV	79.88[c]	1.077[c]
	GN	79.39[c]	0.950[c]
38	STD	74.20[b]	1.761[b]
	NV	77.55[a]	1.475[a]
	GN	77.80[a]	1.388[a]
52	STD	73.31[e]	2.522[e]
	NV	76.68[d]	1.860[b,d]
	GN	76.44[d]	1.933[d]
TC	STD	75.62[d]	1.556[f]
	NV	78.02[a,f]	1.422[a]
	GN	78.77[c,f]	1.304[a]

Data in columns followed by the same letter do not differ significantly at $p<0.05$. (STD=standard/control; NV=no vitamins added; GN=partial gum substitution and no vitamins added)

Table C.14 pH, water activity, and emulsion stability of cheese spreads using the suggested formulary changes. Averages and standard deviations are shown for three replications.

Batch (formulation variation)	Time (w=weeks m=months)	pH	A_w	Emulsion Stability/Separation (%)
STD	Initial	5.75 ± 0.04	0.950 ± 0.002	No Separation
	6w-52	5.54 ± 0.07	0.950 ± 0.003	No Separation
	6w-TC	5.56 ± 0.01	0.950 ± 0.003	No Separation
	6m-4	5.72 ± 0.02	0.961 ± 0.003	No Separation
	6m-38	5.62 ± 0.02	0.959 ± 0.005	No Separation
NV	Initial	5.85 ± 0.03	0.953 ± 0.002	No Separation
	6w-52	5.64 ± 0.01	0.946 ± 0.003	No Separation
	6w-TC	5.64 ± 0.02	0.947 ± 0.004	No Separation
	6m-4	5.91 ± 0.02	0.963 ± 0.003	No Separation
	6m-38	5.73 ± 0.03	0.958 ± 0.007	No Separation
GN	Initial	5.80 ± 0.07	0.952 ± 0.001	No Separation
	6w-52	5.63 ± 0.03	0.946 ± 0.006	No Separation
	6w-TC	5.64 ± 0.04	0.954 ± 0.000	No Separation
	6m-4	5.87 ± 0.02	0.964 ± 0.001	No Separation
	6m-38	5.71 ± 0.02	0.960 ± 0.009	No Separation

(TC=temperature cycled) (STD=standard/control; NV=no vitamins added; GN=partial gum substitution and no vitamins added)

Table C.15 Results of sum ranking sensory test for preference of cheese spread

Samples	Sum Ranking
STD	215^{A}
NV	193^{AB}
GN	162^{B}

Values in column with like letters were not significantly different at (p=0.05)
(STD=standard/control; NV=no vitamins added; GN=partial gum substitution and no vitamins added)

Table C.16 Results of nine-point hedonic sensory test for three formulations of cheese spread

Attributes	Samples		
	STD	NV	GN
Appearance	6.40^{B}	7.08^{A}	7.02^{A}
Color	6.69^{B}	7.29^{A}	7.31^{A}
Flavor	5.54^{B}	5.77^{B}	6.35^{A}
Overall Liking	5.56^{B}	5.79^{B}	6.41^{A}

Values in rows with like letters were not significantly different at (p=0.05)
(STD=standard/control; NV=no vitamins added; GN=partial gum substitution and no vitamins added)

6. Rheological Differences in Process Cheese Spread Due to Ingredient Variation

Abstract

Processed cheese spread is a popular commercially available product. Manufacturing processed cheese spreads require the appropriate balance of ingredients in order to deliver a top quality product throughout the whole shelf-life. Common quality deterioration during storage under unfavorable conditions includes hardening and emulsion breakdown. Alteration of type and/or quantity of the emulsifier and stabilizer may improve the quality and extend the shelf-life. One important parameter to be considered in selection of such additives is their effect on the flow properties of the spreads. Therefore, the objective of this research was to determine rheological differences in processed cheese spread when formulation substitutions are used for emulsifying salts and stabilizers. A standard cheese spread was processed, packaged, and stored at room temperature for 150 days. Alterations to the standard formulation included gum substitution with xanthan gum, low-methoxy pectin, or carrageenan; and emulsifying salt alterations included a different mixture of phosphate salts or sodium citrate. Samples from each of the six formulations were taken and yield stress measurements were determined using a controlled stress rheometer equipped with a texturized plate and plate geometry. The yield stress of the standard formulation was 2.33 kPa. Replacement of standard stabilizers with xanthan gum, low-methoxy pectin, or carrageenan resulted in creamier texture and lower yield stress: 1.40, 1.12, and 1.12 kPa, respectively; while alteration of emulsifying agents resulted in either lower (phosphate mixture, 1.24 kPa) or higher (Na-citrate 2.55 kPa) yield stress. Yield stress of the varied formulations differed significantly from the standard ($p<0.05$). The differences in yield stress due to variations of emulsifying salts or gums are an important indication

that any change in formulation requires ample consideration of their affect on product flow properties.

Introduction

Pasteurized process cheese spread is a commercially available product. The production of cheese spread varies among manufacturers; however, the basics of product ingredients and processing remain the same. As defined by the Food and Drug Administration (FDA) 21CFR133.179, a pasteurized process cheese spread is prepared by the mixing of cheese with the aid of heat and the additions of emulsifying agents, stabilizers, acidulants, salt and colorants. Manufacturers are constantly working on formulary changes in an effort to increase product stability, functionality, and popularity. Common problems observed with shelf-stable cheese spreads are phase separation and hardening, thus necessitating a formulary change in emulsifier and/ or stabilizer to help minimize these changes over the lifetime of a product. One important parameter to be considered in selection of such additives is their effect on the flow properties of the spreads. Since the product is expected to be spreadable at room temperature, a small change formulation can affect overall texture of the finished product. Any change in texture, or apparent viscosity may have repercussions later that affect thermal processing conditions.

Process cheese is an oil-in-water emulsion in which the casein proteins act as natural emulsifiers. The emulsification capacity of the proteins can be enhanced by the addition of emulsifying salts such as sodium phosphates or citrates (Lee et al., 2004). The emulsifying salts interact with the calcium present in the casein by acting as a chelating agent. When the behavior of the calcium changes in the structure of the cheese, the integrity of the casein is jeopardized and solubility increases. The removal

of calcium from the protein system allows for further hydrating, swelling, and dispersion, ultimately forming a gel network structure upon cooling (Awed et al. 2002). The final structural composition of the process cheese is not only dependant on the type of emulsifying salt used that sequesters the calcium, but also on the type of cheese used, fat ratio, and other components present in the mixture (Scar et al. 2002). Polysaccharide-based stabilizers such as pectin, xanthan gum, and carrageenan can also be added to assist with emulsion stabilization, enhancing viscosity, improving texture, or increasing shelf-life (Lee et al., 2004; Cernikova et al., 2007).

Alterations in a formulation can have significant effects when performing a large production cheese spread operation. The rheological properties of cheese are important to study in order to identify textural or structural characteristics. One way rheology measurements are reported is in terms of 'yield stress'. Yield stress is defined as the point at which a product begins to flow (Steffe 1996). Changes in the flow properties can be greatly affected and significant differences in yield stress values are indicative to such changes. Recognizing yield stress as a physical reality has been under debate, however the utilization of this rheological parameter is highly regarded in the food processing industry (Gunasekaran et al. 2003).

Therefore, the objective of this research was to determine rheological differences in process cheese spread when formulation substitutions are used for emulsifying salts and stabilizers by measuring the yield stress. The emulsifying salts to be used include disodium and trisodium phosphate and sodium citrate. The stabilizers include low-methoxy pectin, carrageenan, and xanthan gums. Structures and dissociation constants (pKa) are shown in Figure D.1 (All figures and tables appear in Appendix D at the end of the chapter.)

Materials and Methods

Formulation and Storage

A standardized cheese spread formulation was prepared following current military ingredient specifications (). The ingredients used include butter, water, cheddar cheese, colorant, emulsifiers, stabilizers, and vitamins. Variations to the emulsifying salts, or stabilizers were substituted to produce five alternate formulations. The standard formulation was prepared with disodium phosphate (DSP) and trisodium phosphate (TSP) emulsifying salts in a 3:1 ratio, respectively, in total concentration of 2% (w/w). The stabilizer used in the control was a pre-blended mixture of locust bean, guar, and xanthan gums, with a concentration of 0.22% (w/w). Two varied formulations focused on emulsifying agent substitution where an adjusted ratio of DSP and TSP (1:1) or total replacement with sodium citrate was used, still totaling 2% (w/w) of the added ingredients. The remaining three formulations focused on stabilizer substitution which included carrageenan, low-methoxy pectin, or xanthan gum (all gums were supplied courtesy of CP Kelco, Atlanta, GA, after consultation with representative; gums are part of the GENU or KELGUM series). All substitutions were added in the same percentage as the standard formulation dictated. The pH of all the produced batches was ~5.5-5.9 allowing for negatively charged moieties to be present (see pKa values listed in Figure D.1). Sample processing and packaging were performed in the University of Tennessee Food Science and Technology pilot plant (see previous section entitled "*Quality Improvement of Process Cheese*" for complete description). Upon completion of processing, the samples were then stored at room temperature for 150 days.

Equipment:

An AR-2000 Rheometer by TA Instruments (New Castle, DE) was used to measure rheological differences. A texturized plate and plate geometry with a diameter of 40 mm was employed in determination of the sample yield stress. A 5-g sample of cheese spread was placed directly onto the texturized plate. A continuous ramp speed from 1 to 500 Pa over a 3-minute time interval was used to collect a 500-point yield stress data plot. Between each sample and replication, the machine required a zero-gap adjustment. All samples were analyzed at 23 ± 2°C. Three replications for each cheese spread formulation were performed. The cubed root of the shear stress data was used to perform calculations that assisted in determining the yield stress of the samples. According to Baker et al. (2007), using a cube root displacement produces two straight-line intersecting segments that suggest a definite yield stress upon extrapolation.

Statistical Analysis

The average mean and standard deviation of the measured yield stress for the three replications (n=3) were calculated and Duncan's Multiple Range Test was used to determine if differences in mean separation were significant (v.9.1, SAS Institute, Cary, NC, USA). The data analysis was used to determine if differences exist in the flow properties; it is not meant to indicate if one additive is better than the other.

Results and Discussion

The collected data representing the Shear Stress (kPa) vs. a Cubed Root Shear Rate for the control formulation and emulsifying salt alterations is shown in Figure D.2. The data for the control formulation and stabilizer changes is shown in Figure D.3.

The yield stress data shows an increase in flow property at lower shear stresses, when compared with the control formulation. The exception was Na-citrate which exhibited a reduced flow potential and need for an increased shear stress, thus resulting in a more solid-like cheese spread. The mean separations are shown in Table D.1 for all the sample formulations.

The increased yield stress for the Na-citrate was expected based on previous work of citrate salt and cheese interaction performed by Pastorino et al. (2003). When Na-citrate is added to a cheese blend, the citrate acted as a chelating agent with calcium thus decreasing the amount of bound calcium in the cheese. At this point, protein-protein interactions are decreased and fat emulsification by the caseins is increased. The action of the Na-citrate is similar to the polyphosphates that were used in the control. However, when added in the same amounts (%w/w), the amount of bound calcium was likely greater in the citrate system than in the phosphate systems given the increased yield stress values obtained.

The reduced yield stress value obtained in the adjusted phosphate formula indicates that there is a significant difference in the amount of shear stress required to promote flow when compared to the control formulation. In the control formulation, the increased DSP content likely promoted calcium ionic interactions within the casein due to the increased negative charge expressed by the DSP. An increase in ionic interactions will result in decreased protein-protein interactions and emulsification capacity of the fat by the caseins is increased. In the adjusted formulation, there were equal parts of DSP and TSP, thus resulting in reduced ionic interaction opportunities between the emulsifying salts and the calcium, ultimately indicated by the lower yield stress value. In all the systems, the moisture content and pH were equalized to reduce the influence of water on the ionic environment such that ionization of the calcium

phosphate complexes and interaction with the different amino acid functional groups were minimized (Lee et al., 2004). New protein-protein interactions are ultimately responsible for the emulsification, stability and structure formation upon cooling of the cheese spread product (Dimiteli et al., 2005).

The data obtained for the changes in stabilizers indicated that there was a significantly lower yield stress value for the products where xanthan gum, carrageenan, and the low-methoxy pectin (LMP) were individually used. These are all polysaccharide-based gums requiring a specific environment for gelation and emulsion stabilization to occur. Since the focus of this research was to determine rheological differences based on formulary changes in a product held at room temperature, it can only be stated that the emulsion remained stable under these conditions with each of the stabilizers used. The differences were seen with the significantly reduced yield stress values of carrageenan, LMP, and xanthan gum which were 1.12, 1.12, and 1.40, respectively. It is not surprising that carrageenan and LMP produced similar results that were significantly less than that of the control (2.33 kPa). A similarity between these two gums is observed in their anionic structural ability to sequester cationic calcium present in the cheese spread matrix. The resulting gels are softer and more flexible by nature presumably due to increased hydrophilicity, production of fewer junction zones, or cross-linking and alignment between the polysaccharide chains (Whistler et al., 1997). The reduction in yield stress observed when xanthan was used independently is the result of increased hydrophilic interaction within the given environment. At low temperatures xanthan gums form stiff helical-structured gels, but at higher temperatures, such as those reached during cheese spread production, xanthan gums exist as a relaxed random-coil. Under certain conditions, the helical regions may associate themselves resulting in a weak gel (McClements 2005), ultimately displaying a creamier texture and

lower yield stress value. The higher yield stress observed in the control formulation, 2.33 kPa, is partly due to the mixture of gums being used. Different polysaccharide molecules can form mixed junction zones and synergistic interaction either increases viscosity through the increase in molecular size or sufficient junction zone formation results in a stiffer gel (Whistler et al., 1997).

Conclusion

Emulsifying salts and stabilizers are of major importance in process cheese production since they are primarily responsible for yielding a uniform structure, texture, and emulsion. Careful selection of such components is necessary when considering formulation changes. The work presented indicates that despite the many options available for product improvement, formulary changes should not be made unless certain physical parameters are measured. The yield stress data clearly indicates that the flow properties of process cheese spread can be significantly altered using different emulsifying salts or stabilizers. Again, this research was not designed to determine if one additive was better than the other, but simply if rheological differences could be detected using the method developed.

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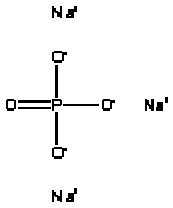
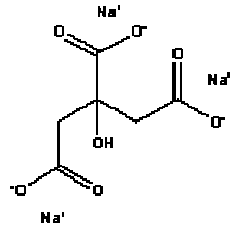
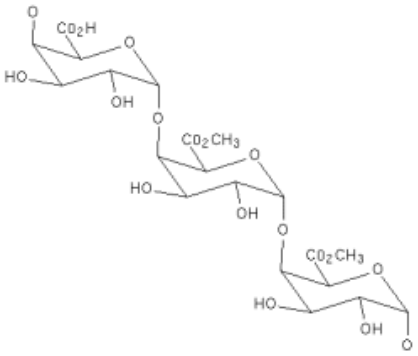
Appendix D



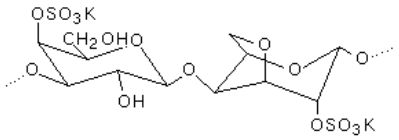
(A) Trisodium Phosphate
pKa =2.12, 7.21, 12.67



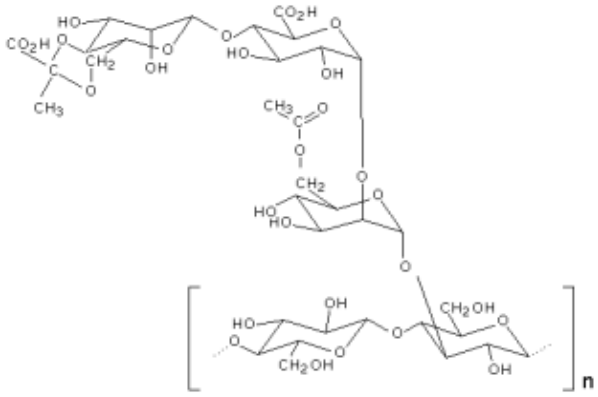
(B) Trisodium Citrate
pKa= 3.13, 4.76, 6.40



(C) Pectin
pKa (COO-)= 4.3



(D) Carrageenan
pKa (SO_3^-)= 1.81



(E) Xanthan

Figure D.1 Structures and pKa values: Emulsifying salts (a) Trisodium Phosphate and (b) Sodium Citrate (courtesy of chemfinder.com); Stabilizers include (c) low-methoxy pectin (d) carrageenan, and (c) Xanthan gum (courtesy of cybercolloids.com)

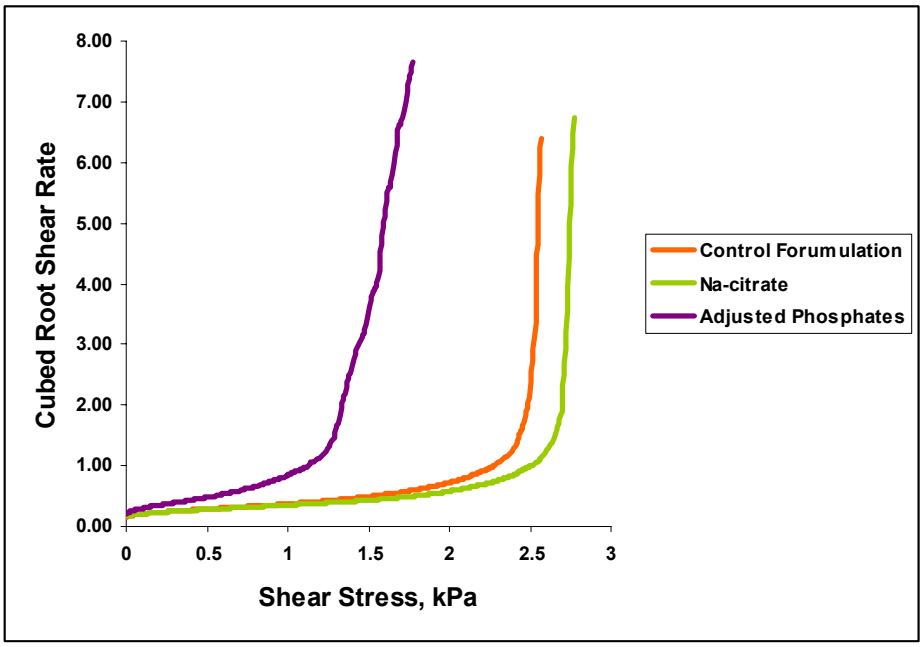


Figure D.2 Cube root displacement of the cheese spread control formulation and emulsifying salt variations when subjected to a continuous ramp speed from 1 to 500 Pa over a 3-minute time interval.

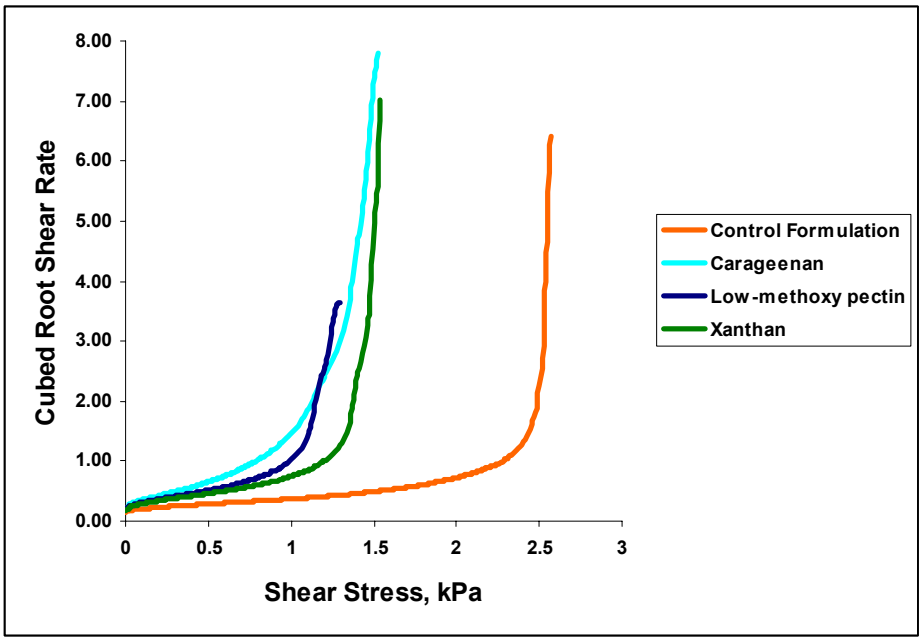


Figure D.3 Cube root displacement of the cheese spread control formulation and stabilizer variations when subjected to a continuous ramp speed from 1 to 500 Pa over a 3-minute time interval.

Table D.1 Yield stress (kPa) measurements of the control vs. emulsifiers and stabilizers at 25 ºC.

Sample	Yield Stress (kPa)
Control	2.33 ± 0.14^{b}
Sodium Citrate	2.55 ± 0.20^{a}
Adjusted Phosphates	1.24 ± 0.06^{cd}
Carageenan	1.12 ± 0.12^{d}
Low-methoxy pectin	1.12 ± 0.16^{d}
Xanthan gum	1.40 ± 0.07^{c}

Results with like letters indicate no significant difference ($p<0.05$), n=3 (except in Adjusted Phosphates, n=2)

7. Overall Summary

The work completed on these projects was diverse and challenging. The cheese spread project was based primarily on studying the physical parameters and the changes that occur when subjected to inappropriate environments. The carotenoid projects monitored chemical changes and evaluated the responses to the surrounding environment.

For the cheese spread, the removal of vitamins from the currently used formulation is likely the best approach to reduce the amount of darkening and hardening that has plagued this product for quite some time. Although the military currently requires fortification of the cheese spread, possible solutions would be to encapsulate the vitamins and keep them present in the cheese spread, or simply remove them and supply another means of subjection. One means of encapsulation would be by the used of cyclodextrins. The technology for this already exists and could easily be applied.

Textural properties are one of the key recognizable traits in any food. When formulary changes are needed, it is wise to determine the effects of the changes not only on shelf-life considerations, but rather on other physicochemical properties. Rheology is one way to determine these changes. When the cheese spread was produced using different emulsifiers and stabilizers, it was determined that there was a change in the flow, or viscoelastic, property of the cheese spread. Since all samples were spreadable at room temperature, a requirement of process cheese products, there was no overall significant effect detected if a new emulsifier or stabilizer was chosen. There were differences; however, between the emulsifiers and stabilizers themselves.

Color properties were evaluated in a variety of ways. The research focused on carotenoid stability when exposed to different environmental stresses. Structural degradation was observed when atmospheric or environmental changes were applied. The differences observed in the presence of oxygen or nitrogen, and light or dark

conditions serve as indication that controlling these parameters has a significant effect on the overall stability of the carotenoid. Five different carotenoid colorants were examined and each offered something different in their response to the surroundings. The most interesting of which is Annatto as it is a commonly used food colorant industry, but was actually one of the weakest in our model system. However, model systems are very controlled and food systems are much more complex, so responses to the system will vary.

The final colorant study involved the attempt at determining the antioxidant capacity of three selected carotenoids. The carotenoids were tested using lipophilic ORAC methods. The results highly varied and definitely more research is necessary for this topic.

Vita

Ann Marie Doneski Craig was born in rural Missouri in late 1973. She graduated from high school and began college at Drury University in 1992. At Drury, she obtained a Bachelor of Arts degree in both Chemistry and Biology in 1996. After graduating from college Ann Marie worked as a Research Assistant at Baylor College of Medicine in Houston, TX. Mrs. Craig decided to return to school and later earned a Master of Science degree in Chemistry from Missouri State University in 2000. Upon graduation she entered the graduate program in Chemistry at the University of Tennessee and began work in radionuclide separation in collaboration with the Oak Ridge National Laboratory. During her time in the Chemistry department, Ann Marie discovered her desire for teaching and was named Head Teaching Assistant for several laboratory sessions. She also coordinated numerous summer chemistry camps for middle-school students. A change came in 2005 where Ann Marie transferred graduate programs and joined the Department of Food Science and Technology working in food chemistry; this was likely one of the best decisions she has ever made.

Upon completion of her Doctorate, Ann Marie will be accepting a tenure-track faculty position at California State Polytechnic University in Pomona, CA as an Assistant Professor of Food Chemistry.