EVALUATION OF THE STABILITY OF MICROENCAPSULATED LYCOPENE ISOMERS

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ABSTRACT OF THE THESIS

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Lycopene, a carotenoid primarily from tomatoes that is responsible for their red color, has been linked to several health benefits. In plant tissues, lycopene is found almost entirely as the all-trans isomer. However, in animal tissues, the lycopene isomer ratio is about 50% all-trans and 50% cis isomers. One reason for this is that cis isomers may be more bioavailable than the all-trans lycopene. Lycopene is labile to heat, light and oxygen. Cis isomers are less stable than all-trans lycopene, and can undergo retro isomerism. In an attempt to stabilize lycopene containing primarily 13-cis and 9-cis isomers, we microencapsulated lycopene via spray drying. We find that loss of lycopene during spray drying is significant and cis isomer content decreased to a larger degree than all-trans lycopene during spray drying, as determined by HPLC. Therefore optimization of spray drying parameters is especially important when spray drying cis isomers. We find no clear evidence that lycopene encapsulated with medium chain triglycerides (MCT) and gum acacia was more stable than lycopene in MCT only, and the isomer profile could not be maintained by either approach. Lycopene encapsulated with

maltodextrin and Tween 80 was found to be more stable than pure lycopene, but less stable than lycopene in MCT. All formulations experienced a change in the lycopene isomer profile. Further modifications to the encapsulation formula and procedures are needed to produce a spray dried microencapsulate that has good lycopene stability in terms of isomerism and degradation.

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1 Introduction

Lycopene belongs to a family of pigments called carotenoids. Carotenoids consist of 40 carbons and contain conjugated double bonds. Lycopene is highly unsaturated and contains 11 conjugated double bonds. The double bonds are responsible for the red color and beneficial chemical properties of lycopene.

Lycopene has been linked to reducing the risk of certain cancers (Wertz et al., 2004; Giovannucci, 1999). Lycopene acts as an antioxidant *in vivo* protecting proteins, lipids and DNA from oxidative damage (Rao and Agarwal, 1999; Wertz et al., 2004). Lycopene has the highest antioxidant activity compared to other carotenoids (Bohm et al., 2002). Lycopene has been shown to impact gene expression in a positive manner in cancer cell lines (Wertz et al., 2004).

Lycopene has poor bioavailability. Only 10-30% of ingested lycopene gets absorbed (Gartner et al., 1997). To be absorbed into the body, lycopene is released from the food matrix and is incorporated into lipid micelles. Lycopene moves from the micelles into mucosal cells of the duodenum by passive diffusion. Chylomicrons then transport lycopene to the blood stream (Parker, 1996).

A large number of geometrical isomers of lycopene are theoretically possible because of the double bonds. Only a small number of the possible total cis isomers are found because there is steric hindrance between side groups in most of the configurations. The favorable isomers are all-trans lycopene, where none of the double bonds are in the cis conformation, as well as 13-cis, 9-cis and 5-cis. Chasse et al. (2001) studied lycopene energies using an *ab initio* molecular modeling procedure. They found that relative stability is as follows: 5-cis > all-trans > 9-cis > 13-cis > 7-cis > 11-cis. Lambelet et al.

(2009) found that 13-cis was less stable in terms of isomerism than 5-cis, all-trans and 9-cis when isomers were isolated and stored in hexane.

Lycopene in dietary sources like tomato products is primarily in the all-trans isomer form. In contrast, lycopene in human tissues contains 50% all-trans and 50% cis isomers (Scherlie et al., 1997). The higher level of cis isomers in humans is thought to be because of higher bioavailablity of cis isomers (Boileau et al., 2002). It is theorized that cis isomers are more soluble in lipid micelles and have a lower tendency to crystallize, therefore are more bioavailable. Lycopene high in cis isomers would be beneficial to the food industry because of higher bioavailability.

Lycopene in tomatoes is all-trans lycopene as a result of the biosynthetic pathway (Cunningham and Grant, 1998). Lycopene produced during ripening of tomatoes is deposited in crystalline form into chloroplasts. Proteins and fiber in the tomato tissue stabilize lycopene (Britton, 1995; Parker, 1996; Moraru, 2004). In order to increase the amount of cis isomers, lycopene must first be released from the tomato matrix. Food processing partially achieves this. Cooking and the addition of oil increases the cis isomer content and the absorption of lycopene (Stahl and Sies, 1992). Heat partially breaks down the cellular structure to release lycopene and also induces isomerism.

Extraction of lycopene is a necessary step to significantly increase the cis isomer content. Once lycopene is removed from the tomato matrix, isomerism can take place. An inherent problem of extracted lycopene is that it is more susceptible to degradation than lycopene in tomato products (Moraru, 2004). Cis isomers are even less stable than all-trans lycopene because they are more susceptible to oxidation and can undergo retro

isomerism. A method of stabilizing lycopene is needed so that lycopene high in cis isomers can be stored in ambient conditions.

Microencapsulation by spray drying is a potential technique for stabilizing cis isomer. Spray drying is commonly used in the food industry due to high production capacity and minimal operational costs (Gharsallaoui et al., 2007). Nutracuticals benefit from encapsulation not only because stability can be enhanced but also because the encapsulated materials can be flowing powders and/or cold water dispersible.

The goal of this research is to encapsulate lycopene in order to maintain an isomer profile high in cis isomers and prevent loss of total lycopene. Encapsulation of lycopene in medium chain triglycerides (MCT) using spray drying and octenyl succinate (OSA) modified starch and gum acacia as the wall material has been evaluated. Lycopene in MCT simulates tomato oleoresin with other constituents removed. Pure lycopene was also tested with maltodextrin as the wall material and Tween 80 as an emulsifier. Close attention has been paid to the effects of spray drying on lycopene during the process. Stability of encapsulated lycopene high in cis isomers has been investigated at various storage conditions. Both total lycopene content and the isomer profile are of interest.

Our hypothesis is that encapsulation by spray drying is an effective method to slow degradation of lycopene and prevent cis to trans isomerism.

2 Literature Review

2.1 Lycopene

Carotenoids are essential components in photosynthetic organisms. Carotenoids quench reactive species during photosynthesis, and are also involved with other energy related functions (Cunningham and Grant, 1998). Carotenoids also provide color to flowers and fruits. For example, lycopene provides a red color to tomatoes and other fruits. Carotenoid structure is based on a carbon polyene chain of 30 carbons with side groups for a total of 40 carbons. Carotenoids are subdivided into carotenes (e.g., lycopene) and xanthophylls (e.g., lutein) depending on whether they contain oxygen in their molecular structure.

Cunningham and Grant (1998) reviewed the biosynthesis of carotenoids. The following information regarding lycopene synthesis has been summarized from their article. Isopentenyl pyrophosphate (IPP, $C_5H_{12}O_7P_2$) is the compound that serves as the building block for lycopene. The biosynthetic pathway is governed by gene expression of enzymatic activity. The enzymes facilitate the reactions that lead to the formation of carotenoids. IPP units condense to form longer carbon chains of 10, 15, and finally 20 carbons. Two C_{20} molecules of geranylgeranyl pyrophosphate react to form a C_{40} chain that then becomes phytoene. Phytoene undergoes desaturation steps to form lycopene, which can then be formed into α- or β-carotene by a cyclization reaction. Hydroxylation of α- or β-carotene creates xanthophylls. Tomatoes have low amounts of β-cyclase enzyme. Therefore, lycopene content remains relatively high. Lycopene does not contain a β-ionone ring, and therefore does not have vitamin A activity. When lycopene is formed in ripening tomatoes, lycopene is stored as crystals in intracellular chloroplasts.

Mammals cannot create carotenoids so they must be consumed in the diet. Carotenoids play a number of roles in the human body. For example, β-carotene is a precursor to vitamin A.

Lycopene contains thirteen double bonds. Eleven of the double bonds are conjugated and result in the red color in tomatoes. The double bonds in lycopene can theoretically be in cis or trans at each location. Cis configuration has the larger constituents on the same side of the double bond; cis is also written as Z-. The trans configuration has the larger constituents on opposite sides of the double bond; trans is also written as E-. Only small amounts of the total number of theoretical cis isomers are present in lycopene because there is steric hindrance resulting in high energy, unfavorable conformations. The favorable isomers are all-trans lycopene, where none of the double bonds are cis as well as 13-cis, 9-cis, and 5-cis, where the cis double bond starts at the 13^{th}, 9^{th} and 5^{th} carbon of the back bone respectively. Chasse et al. (2001) studied lycopene stability using an *ab initio* molecular modeling procedure. They stated that the relative stability is as follows: 5-cis > all-trans > 9-cis > 13-cis > 7-cis > 11-cis. Lambelet et al. (2009) found that 13-cis was less stable in terms of isomerism than 5-cis, all-trans and 9-cis when isomers were isolated from tomato extract and stored in hexane at room temperature.

2.2 Bioavailability and Role in Human Health

Upon consumption of tomatoes and other foodstuffs, lycopene is released from the food matrix and is absorbed into lipid micelles in the small intestine. Lycopene moves from the micelles into mucosal cells of the duodenum by passive diffusion. Chylomicrons then transport lycopene to the blood stream (Parker, 1996). There are a

number of factors that influence absorption, including the food matrix, amount and type of fat, fiber content, isomer profile and other carotenoids (van het Hof et al., 2000; Shi and Maguer, 2000). Lycopene has poor bioavailability. Only 10-30% of ingested lycopene becomes absorbed (Gartner et al., 1997). Even so, lycopene has the highest plasma levels of all carotenoids in humans.

Epidemiological studies have shown an inverse relationship between the intake of tomato products or blood lycopene levels, on the one hand, and risk of certain cancers. Giovannucci (1999) reviewed 72 articles on the subject and found that evidence was strongest for prostate, lung and stomach cancers. Lycopene is the predominant carotenoid present in the studies but lycopene has not been proven to be solely responsible for the health benefits. Lycopene in the epidemiological studies was from tomato products and not pure lycopene. Other phytochemicals may have a synergistic effect with lycopene.

Lycopene's antioxidant activity is thought to be the main mechanism of action *in vivo*. Lycopene has a high antioxidant activity compared to other carotenoids. Lycopene can quench singlet oxygen and other reactive species. Lycopene can absorb energy from singlet oxygen converting lycopene to its triplet state. The energy is then dispersed into the surrounding media through rotational and vibrational movements (Stahl and Sies, 1996). Lycopene can also quench reactive oxygen species by oxidizing. The reaction products may also have biological functions (Stahl and Sies, 1996). Researchers hypothesize that oxidation and cleavage products may be responsible for the health benefits (Lindshield et al., 2007; Caris-Veyrat et al., 2003). Lycopene reduces oxidative

stress by protecting lipids, proteins and DNA from reactive oxygen species (Rao and Agarwal, 1999; Wertz et al., 2004).

Wertz et al. (2004) discuss other modes of action that promote prostate health. Most of the literature reviewed by Wertz et al. (2004) used cell cultures to carry out the studies. To summarize, lycopene has an effect on the regulation of genes that are related to cancer risk. Some examples are that lycopene decreases cyclin D1, increases connexin 43 (involved in gap junction communication) and upregulates IGF-binding protein-3.

Lycopene levels vary greatly between people and vary between parts of the body (Bramley, 2000). Lycopene is more bioavailable in cooked tomato products than uncooked. This is because the food matrix is partially broken down and this helps in the release of lycopene from the food. Heating during processing not only breaks down the cellular structure but also increases the amount of cis isomers via heat-induced isomerism. Extracted lycopene such as tomato oleoresin is more bioavailable than from tomato products because lycopene has been removed from the food matrix (Shi and Maguer, 2000). Lycopene is also more bioavailable in food preparations that contain dietary lipids (van het Hof et al., 2000). Tomato juice that was heated with corn oil resulted in an increase in lycopene serum concentration compared to not heating with oil (Stahl and Sies, 1992). Decreasing the lycopene crystal particle size also increase absorption (Bohm et al., 2002).

A method to increase bioavailability, that may be applicable to lycopene, is the use of nanoemulsions. Delivery of nutracuticals by nanoemulsions has been shown to increase their absorption. Oral administration of curcumin nanoemulsion had a larger effect than a coarser emulsion on inhibition of TPA induced edema on mouse ears (Wang

et al., 2008). As mentioned before, decreasing the particle size of lycopene increased the bioavailablitly. Decreasing particle size is also a strategy used in the pharmaceutical industry. The mechanism at which nanoparticles are absorbed is through direct particle uptake. Small particle size also increases the surface area of the particle and can increase the dissolution of the active ingredient (Acosta, 2008). The surfactants used to stabilize the nanoemulsion can increase the stability of lycopene. Yuan et al. (2008) created β-carotene nanoeumulsions. The nanoemulsion did not effectively stabilize β-carotene as the content decreased significantly during storage. Tomato oleoresin can be homogenized into a nanoemulsion to increase bioavailablity.

2.3 Stability and Sources of Lycopene

Lycopene in tomato tissues is more stable than extracted lycopene. Long-term stability in tomato products is probably due to tomato tissue-lycopene interactions. Carotenoids interact with proteins, rendering them more stable in plant tissue (Britton, 1995; Parker, 1996; Moraru, 2004). Common dietary sources of lycopene include tomato products, watermelon and pink grapefruit. Lycopene is also present in leafy greens and other vegetables but in lower quantities. Specific molds synthesize lycopene and have been investigated as a source of carotenoids (Choudhari et al., 2008). Lycopene can also be produced synthetically. Lycopene supplements include pills that contain tomato oleoresin. Tomato oleoresin is a tomato extract that has had all of the solvent removed. Food items with tomato paste have high levels of lycopene. Lycopene in plant tissues is found primarily in the all-trans form. Even though dietary lycopene is primarily all-trans, human blood plasma contains above 50% cis isomers (Scherlie et al., 1997). Upon processing of tomatoes, the cis lycopene content increases. The increase in cis isomers

during processing can only partially explain why the cis isomer percentage is high in animals. Isomerism also occurs after ingestion in the stomach (Re et al., 2001; Moraru and Lee, 2005). Extent of isomerism varies under different gastric conditions. It is theorized that cis isomers are more soluble in lipid micelles and have a lower tendency to crystallize, and are therefore more bioavailable (Boileau et al., 1999). Boileau et al. (1999) tested bioavailability *in vitro* and *in vivo*. *In vitro*, cis isomers were preferentially incorporated into bile acid micelles. *In vivo*, cis isomers were found in higher proportions in lymph secretions compared to the isomer profile of the ingested lycopene. Tyssandier et al. (2001) concluded that absorption of carotenoids were inversely proportional to their hydrophobicity.

The most convenient way to increase the cis isomer content is by heating. The total effects of heating a tomato product are the release of lycopene from the tomato and then heat-induced isomerism. Processed tomato products only experience moderate increases in cis isomer content because lycopene is only partially released from the matrix. Incubation of lycopene in hexane increases the percentage of cis isomers to a certain isomeric distribution (Moraru, 2004; Boileau et al., 1999). At high temperatures, however, breakdown of pure lycopene occurs at a faster rate than isomerism (Lee and Chen, 2002). A source of cis lycopene isomers is the tangerine tomato. It contains a tetra-cis lycopene isomer, also known as prolycopene. Unlu et al. (2007) studied the bioavailability of lycopene in the tangerine tomato and concluded that tetra-cis lycopene is more bioavailable than all-trans. An oleoresin produced from the tangerine tomato was less stable than oleresins that contained mainly all-trans lycopene (Hackett et al., 2004). There are also a number of other ways such as melting crystals, light, iodine catalysis

with light, acid catalysis, isomerism by active surface, via boron trifluoride complexes and bio-stereoisomerization (Zechmeister, 1962).

A recent patent, (WO/2008/017401) STABLE AND BIOAVAILABLE COMPOSITIONS OF ISOMERS OF LYCOPENE FOR SKIN AND HAIR, explains a method of producing lycopene high in cis isomers that has a stable isomer profile. The method is essentially the same as in Lamebelet et al. (2008) which involves extented heating resulting in low amounts of 13-cis and high amounts of 9-cis and 5-cis. The patent claims that tomato oleoresin high in 5-cis lycopene is more bioavailable than lycopene high in 13-cis, 9-cis or all-trans lycopene. This bioavailability data has not been published in a peer reviewed journal to date. The patent also reviews other patents related to the stability and production of lycopene isomers.

Lycopene degrades via oxidation pathways. Oxygen reacts with the double bonds resulting in cleavage of the lycopene molecule. Ukai et al. (1994) determined by HPLC that an oxidation product is apo-6-lycopenal. Caris-Veyrat et al. (2003) analyzed lycopene, that has been oxidized, by HPLC-diode array detection and mass spectrometry and found that apo-lycopenals, apo-lycopenones and apo-carotendials were products. Kanasawud and Crouzet (1990) analyzed oxidation products of lycopene in an aqueous media by gas chromatography-mass spectrometry. They tentatively identified volatile compounds as 5-hexen-2-one, 2-methyl-2-hepten-6-one, hexane-2,5-dione, 6-methyl-3,5-heptadien-2-one, neral, geranial, geranyl acetate and psudoionone. Five non-volatile fractions were seperated by thin layer chromatography in the study but only the lycopene fractions could be identified and are probably the products that were found in Caris-Veyrat's study.

2.4 Analysis of Lycopene

2.4.1 Overview

Lycopene is most accurately measured by extraction and then analysis of the extract by spectrophotometry or HPLC. Other methods and variations are available. A method that does not destroy the tomato samples is by correlating L*a*b* to lycopene values determined by extraction (Arias et al., 2000). However, the correlation is not sufficient at higher concentrations. Davis et al. (2003) developed a puree absorbance method (PAM) that does not use organic solvents. This method entails pureeing tomatoes and then measuring absorbance with a Hunter Ultrascan XE. Chen and Tang (1998) used L*a*b* values to analyze the color stability of β-carotene powders during storage and found a correlation between b* values and carotene content. These methods are relatively fast but the cis isomer content cannot be estimated from them. A combination of extraction and spectrophotometry is better for quantifying total lycopene content, but can only provide an estimation of isomer content as indicated by a peak at about 360 nm and/or a hypsochromic shift. Extraction followed by HPLC analysis provides the most information because the isomers can be separated.

2.4.2 Extraction

There are many variations of extraction mixtures that are used to extract lycopene. The goal of extraction is to separate lycopene from water soluble components of a lycopene containing food item. Hexane, acetone, ethanol, dichloromethane, ethyl acetate, chloroform, benzene, petroleum ether and others have been used as solvents for extraction (Roldan-Gutierrez et al., 2007). The AOAC official methods of analysis (1990) use hexane and acetone for removal of carotenoids from plant materials.

Extraction methods often use high volumes of organic solvents, which is undesirable. Supercritical carbon dioxide is also used for extraction (Roldan-Gutierrez et al., 2007).

2.4.3 Lycopene Measurement

HPLC analysis of an extract is needed for isomer characterization. Determination of eluants of a sample in an HPLC method is critical to coming to accurate conclusions. Most commonly, the absorbance spectra are used for identification and quantification of carotenoids but mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) also can provide valuable information. Analyzing the absorption spectrum of an elution peak can identify lycopene, which has three absorption peaks close to 444, 470 and 502 nm. Lycopene's conjugated double bonds are a chromophore and absorb light. Cis isomers with centrally located cis bonds also have the 'cis peak' in the 360 nm region. Cis isomers also experience a hypsochromic shift. Confirmation of the molecular weight can be performed using HPLC-MS. MS ionizes the eluant and measures the mass/charge (m/z) ratio. Lycopene has a molecular weight of 536; protonated lycopene will have an m/z of 537. NMR spectroscopy, which measures magnetic resonance in nuclei with an odd number of protons or neutrons, can determine chemical structure of a pure compound. Shifts in the frequency at which protons resonate can be interpreted to determine the exact structural conformation of lycopene.

Iodine is often used as a catalyst for lycopene isomerization. The isomerized extract is commonly used to provide a reference for lycopene isomers in a researcher's HPLC method (Qui et al., 2006; Schierle et al., 2002).

Selected papers that include chromatograms of HPLC methods designed to separate isomers were reviewed. C30 columns are commonly used for analysis of

lycopene isomers. The mixtures of methyl tert-butyl ether (MTBE) and methanol are typically used as mobile phases. The concentrations used vary between method and sometime modifier solvents are added. Elution occurs in the following order in most of the methods: β-carotene, 13-cis lycopene, 9-cis lycopene, and then all-trans lycopene. Two exceptions were found where the order was reversed; they use a C18 column (Stahl and Sies, 1992) and a Suplex PKB 100 (Stahl et al., 1993). These papers were published relatively early compared to other papers.

The all-trans and 5-cis peak does get completely resolved in most methods that use a C30 column. These methods have been used by Boileau et al. (1999), Clinton et al. (1996), Ishida et al. (2000), Nguyen and Swartz (1998), Calvo and Santa-Maria (2008), Qiu et al (2006), and Yeum et al. (1996). Moraru and Lee's (2005) method does not separate 5-cis from all-trans, but the method is very quick. Lee and Chen (2002) identified a peak between 9-cis and all-trans as 5-cis lycopene because the spectral characteristics are the same as all-trans lycopene. The mobile phase of the method consists mostly of acetonitrile which is not typical. Fang et al. (2003) did achieve good peak separation of all-trans and 5-cis in a YMC C30 column (3um, 4.6 x 250 mm). The method employed a linear gradient over 40 minutes of 50:50 to 40:60 methonal/MTBE. Baseline is almost reached between all- trans and 5-cis peaks using this method. The methods used by Hengartner et al. (1992), Schierle et al. (1997) and Lambelet et al. (2009) are exceptions compared to typical methods but produce excellent peak resolution. They use 3 consecutive "Nucleosil 300-5" columns with n-hexane containing 0.15 % N-ethyl di-isopropylamine as the mobile phase. Nucleosil columns, which are made by Macherey-Nagel, consist of spherical silica beads. Five stands for 5 µm bead particle

size, and 300 refers to a bead pore size of 300 Å. The silica beads can be modified to contain functional groups like C18 chain, but the authors did not indicate that the media had been modified. The method achieved excellent resolution of all-trans and 5-cis in about 26 minutes.

An issue that should be addressed in future work is that the extinction coefficients for cis isomers are not well established. Cis carotenoid isomer absorption spectra exhibit a hypsochromic shift as well as a hypochromic effect (Britton, 1995). The spectral peaks shift to a shorter wavelength and decrease in absorbance. The quantities of cis lycopene isomers are underestimated, meaning that there is more cis lycopene than what is calculated when the all-trans extinction coefficient is used. Hengartner et al. (1992) reported the molar absorption coefficient (ε) of nine different lycopene isomers but did not include 13-cis or 9-cis. 5-cis lycopene did have the same absorption as all-trans lycopene. Muller et al. (1997) reports molar extinction coefficients of 13-cis and 9-cis. 13-cis has a ε of 136000 at 463 nm; 9-cis has a ε of 174000 at 464 nm. The values are significantly less than all-trans ε of 184900. The error in estimated cis isomer content is compounded because the absorption peaks are shifted. Data taken using one wave length for the absorption peak of all-trans lycopene will need to factor in another correlation. However, most researchers use the all-trans extinction coefficient even though the data is available. An explantion why all-trans absorption coefficient is used is because Muller et al. (1997) presented the data in a non-typical format with little discusion of the experimental procedures. The paper does not follow the introduction, materials and methods, results and discusion format.

Bohm et al. (2002) compared the antioxidant activity of isomers, but did not take into account the different molar absorptivities of the isomers. Higher antioxidant activities reported can be due to the underestimation of actual cis lycopene concentrations. Most research is not affected when the same extinction coefficient is used for all isomers because the quantification of changes in the isomer profile is comparative.

One could determine the molar absorptivity of 13-cis and 9-cis by using the semi-preparative HPLC method, thoroughly drying isomers to remove all solvent, accurately weighing sample and completely dissolving isomer in hexane, then measuring absorbance using a spectrophotometer. This technique is inherently difficult because all of the solvent must be evaporated for accurate weighing, incomplete dissolution may occur. As an alternative, isomers can be separated by HPLC then analyzed by photodiode array detection in line with mass spectrometry. The MS signal strength can be correlated to PDA signal for accurate correlation between absorbance of cis isomers to all-trans lycopene.

The extinction coefficient for all-trans lycopene should also be re-measured. A mass extinction coefficient of 3450, which corresponds to a molar extinction coefficient of 184900 from Britton (1995), is commonly used. Hengartner et al. (1992) reported a molar extinction coefficient of 187000, which is about 1% different from Britton's value. Most researchers use Britton's value.

HPLC methodology and quantification of lycopene isomers should be standardized across research groups. At a minimum, journal articles should include a

typical chromatogram that the method produces so that readers may interpret and make judgments about the method on their own.

2.5 Microencapsulation Overview

Lycopene that has been extracted from tomatoes has a significantly diminished stability compared to a shelf stable tomato product like pasta sauce. A method that may be useful to increase the stability of extracted lycopene is microencapsulation. There are many reasons to microencapsulate an ingredient, but the main reason to encapsulate lycopene in this research is to provide a barrier to oxygen and light, and to prevent isomerism. Other reasons to microencapsulate lycopene would be to produce lycopene as a flowable powder and/or to have lycopene dispersible in water.

Microencapsulation by spray drying is commonly used in the food industry due to high production capacity and minimal operation costs. A typical encapsulation is composed of a wall material and a core that contains the active ingredient. The sizes of microcapsules are 5-300 microns (Gibbs et al., 1999). Common wall materials include carbohydrates, gums and proteins (Gharsallaoui et al., 2007). The core materials are often hydrophobic in nature. The first step of the encapsulation process by spray drying is mixing the wall material with water. Then the core material is dispersed in the polymer solution to form a coarse emulsion. The emulsion is then homogenized and fed into a spray dryer. The feed is atomized into droplets on the scale of micrometers. As the solvent evaporates, the hydrocolloid becomes concentrated and creates a shell around the core particles. There are a number of encapsulation parameters that can be adjusted to reach the desired attributes of the encapsulation ranging from the ingredient formula to the spray drying parameters.

Microencapsualtion has been used as a successful method to extend the shelf life of lycopene. Shu et al. (2006) encapsulated purified lycopene in a gelatin/sucrose matrix. The study investigated the effect of different processing parameters to determine the best encapsulation procedure. Stability of the best encapsulate was compared to tomato oleoresin ("raw material of lycopene" could be referring to pure lycopene) to show that encapsulation is effective in enhancing the stability.

On the other hand, Wang and Chen (2006) attempted to encapsulate lycopene by spray drying but found that it was not the best method. They were interested in producing a source of lycopene high in cis isomers. They cited significant losses of total lycopene during spray drying as the reason why they investigated freeze drying as an alternative. They also found that encapsulating lycopene by dissolving purified lycopene in ethyl acetate, adding carriers including maltodextrin and sodium alginate, then evaporating the solvent by a stream of nitrogen produced an encapsulation high in cis isomers. The investigation did not test the stability of the encapsulations.

A homogenized emulsion can be freeze-dried, dried under vacuum, or oven dried as alternatives. Spray drying is an efficient one step process to create capsules but has its drawbacks. Spray drying can expose the ingredients to high temperatures. The temperature of the drying air must be above 100˚C to evaporate water efficiently. In theory, however, when spray dry conditions are optimal, the encapsulate only experiences a limited increase in temperature due to evaporative cooling of the solvent. The encapsulate is also exposed to oxygen if air is used as the drying gas; nitrogen can be used for oxygen sensitive materials. Other drying methods also have disadvantages.

Freeze-drying for example has a significantly longer drying time. Freeze dried material must then be ground to make a powder.

Other microencapsulation techniques include spray chilling, extrusion, fluidized bed coating, liposome entrapment, coacervation, and inclusion complexation (Gibbs et al., 1999). Spray chilling may be a successful process that will prevent lycopene isomerism. Lycopene would be dispersed in a lipid carrier above its melting temperature then atomized at a temperature below the lipid melting point. Lycopene surrounded by a solid lipid matrix may prevent movement of lycopene and therefore isomerism.

Chiu et al. (2007) encapsulated lycopene in gelatin and poly(γ-glutamic acid) by freeze drying. They report that lycopene loss during freeze drying was 23.5%. Stability of encapsulated lycopene is determined but non-encapsulated lycopene was not measured to provide a reference. Their degradation rate constants were similar to Sharma and Maguer (1996) who measured lycopene stability in dried tomato pulp waste.

Lambelet et al. (2009) discovered a process that maintains a lycopene isomer profile high in cis without encapsulation. The research shows that 13-cis lycopene is significantly less stable than the other isomers. They were able to isomerise tomato oleoresin by refluxing the oleoresin in ethyl acetate for one week to produce a product high in total cis isomers but low in 13-cis. The isomerized extract remained stable in the absence of light, moisture and oxygen for 1 year at room temperature in terms of the isomer profile and total lycopene content. The study does not provide stability information of the isomerized extract when it is stored in low barrier packages such as a plastic bottle. Stability in other packaging formats needs to be investigated to determine its applicability for use in food products.

Researchers have grafted dextran to casein in order to encapsulate β-carotene. Casein and β-carotene hydrophobically interact to form a nano sized core while dextran forms a shell rendering the particle water soluble under a range of conditions. The nano-particles in solution showed high resistance to oxidation by $FeCl_3$ (Pan et al., 2007).

In inclusion complexation, a core molecule or guest molecule would be confined in a host molecule. Encapsulation is on a molecular level whereas in microencapsulation groups of molecules such as an oil droplet are surrounded by any number of wall material molecules. Blanch (2007) encapsulated lycopene in cyclodextrins. Lycopene hydrophobically interacts with the core of the cyclodextrin. The encapsulates were stable for 6 months according to Raman spectroscopy data.

3 Materials and Methods

3.1 Isolation of Lycopene

Lycopene was purified from three different sources: tomato paste, tomato oleoresin (Lycored, Lyc-O-Mato 6%) and synthetic lycopene (Roche, 10% lycopene in corn oil). Purification of lycopene from the tomato sources used an extraction procedure whereas isolation of lycopene from the synthetic source only used a re-crystallization method.

The procedure for extraction of lycopene from tomato paste and oleoresin, which was adapted from Moraru (2004), is as follows. Extraction was carried out using a solvent mixture of hexane, acetone, and ethanol in a 2:1:1 ratio as well as enough water to disperse large clumps of tomato paste. This slurry was stirred for 15 – 30 minutes and then filtered through a Buchner funnel with Watman filter paper. The filtrate was then transferred into a separatory funnel and successively washed with water until the polar portion was colorless. Lycopene in the upper hexane layer was then rotoevaporated and re-suspended in hexane. The extract was then passed through a 0.45 μm syringe filter. The lycopene that was trapped in the filter was then washed through with more hexane. At this stage, the extract contains a significant amount of β-carotene; therefore, re-crystallization is recommended.

Re-crystallization was performed by a modified method from Shu et al. (2006). The extract or synthetic lycopene was diluted with hexane, then heated to dissolve all of the lycopene. The solution was then placed at -20˚C to cause crystallization of lycopene. Lycopene crystals precipitated and were removed with a pipette.

Re-crystallization is recommended when using tomato sources because β-carotene and cis lycopene isomers do not completely separate when isolating cis isomers using the semi-preparative high performance liquid chromatography (HPLC) method. Open column chromatography is an alternative method for removal of β-carotene but is more labor intensive. We recommend purifying lycopene from a synthetic source mainly because the crystallization process is easier.

Lycopene high in cis isomers was prepared by semi-preparative HPLC of the purified tomato extracts or crystallized synthetic lycopene. Lycopene is primarily in the all-trans form in tomato products and synthetic lycopene. To increase the cis isomer content, the purified extracts were heated at 80˚C for anywhere from 10 minutes to 2 hours. After heat-induced isomerism, the cis isomer content was about 25-35%. The fraction containing primarily 13-cis was collected. Table 1 shows the chromatographic condition, and Figure 1 is a typical chromatogram of collection. Rotoevaporation or a stream of nitrogen gas was then used to remove the elution solvent. The resulting lycopene fraction contained between 65-90% cis isomers with 13-cis and 9-cis comprising a majority of the cis content.

The method used in this study to isolate lycopene high in cis isomers is extremely time consuming, and uses solvents that pose food safety issues. Commercial lycopene oleoresins use ethyl acetate or ethanol. There are various methods to increase the cis isomer percentage such as heat, melting crystals, light, iodine catalysis with light, acid catalysis, isomerism by active surface, via boron trifluoride complexes and bio-stereoisomerization (Zechmeister, 1962). Use of heat or refluxing lycopene solutions is the most convenient method. However, the maximum cis isomer percentage achieved is

close to 60% cis isomers by Lambelet et al. (2009); Wang and Chen(2006) achieved 47% cis isomers. Nguyen and Swartz (1998) observed that lycopene will isomerize in an organic solvent at room temperature, resulting in an isomeric ratio of about 50% cis. The tangerine tomato is a potential source of cis isomers where tetra-cis lycopene makes up 98% of the total lycopene (Zechmeister, 1962).

The current study used lycopene high in the 13-cis isomer because its levels were the highest in the heat-isomerized extract used for collection. Using limited amounts of 13-cis and higher amounts of 5-cis and 9-cis to stabilize lycopene may be a better approach. One experiment used lycopene enriched in 9-cis because β-carotene and 13-cis were overlapping during semi-preparative HPLC separation.

3.2 HPLC Analysis of Lycopene

Lycopene was quantitatively analyzed by HPLC. Equipment includes a Waters 600E multi-solvent delivery system equipped with a Waters 484 tunable absorbance detector connected to a Galaxie chromatography software system.

The HPLC method was modified from Moraru and Lee (2005) who based the method on Yeum et al. (1996). Refer to Table 2 for the HPLC parameters used. Figure 2 is a typical chromatogram of isomerized lycopene sample using the Develisol RP-Aqueous column. The injection solvent causes an absorbance peak at 1-3 minutes due to a change in the refractive index of the solvents. A modification made was lowering the temperature of the column. Using the same solvents, peak resolution was increased by holding the temperature of the column at 0˚C (submerged in ice water). The rate of the gradient change was also tuned to find a balance between peak resolution and method time. Presence or absence of 2% water was also investigated because water is only

slightly miscible in MTBE. Water can settle to the bottom of the solvent reservoir; this can be prevented by vigorous mixing. Presence or absence of water did not have an effect on retention times or resolution by visual comparison of the chromatograms. The HPLC column used in most of the experiments was a Develosil RP-aqueous column. However, a YMC column was used toward the end of experimentation because of better peak resolution. Polymeric reverse phase columns have better shape selectivity than monomeric columns (Sanders et al., 1999). The Develosil RP-aqueous column is made with monomeric C30 molecules, whereas the YMC columns are made with a polymeric methodology. Develosil RP-Aqueous-AR should be used if one wants to use the Develosil brand because it is made with trifunctional C30 molecules. Figure 2 and Figure 3 are chromatograms of the same sample under the same operating conditions, but Figure 2 used the Develosil column, whereas Figure 3 used a YMC column.

Identification of HPLC peaks was done by photodiode array detection and comparison with chromatograms present in literature. Six elution peaks are present in heated tomato paste extract. The elution peak at 16-18 minutes has ultraviolet-visible (UV-VIS) spectral peaks at 380, 402, 425 and 450 nm. This elution peak has been tentatively identified as the co-elution of β-carotene and ζ-carotene. β-carotene has spectral peaks at 425, 450 and 477nm while ζ-carotene has peaks at 377, 399 and 425 nm (Britton, 1995). No other testing regarding the identification of the carotenoids was done because the focus of the experiment is on lycopene. The peak eluting at 16-18 minutes can also be eliminated by crystallization of the tomato extract. An eluant at 21 minutes with a smaller peak immediately before it, at 23 minutes, and two poorly separated peaks at 26 minutes have been identified as lycopene. The YMC column resolves another small

peak immediately after the peak at 21 minutes. The peaks at 21 and 23 minutes are cis isomers because they have an absorption peak in the region of 360 nm. They have been assigned as 13-cis and 9-cis respectively by comparison to the literature. The first peak at 26 minutes is identified as all-trans lycopene. It is the only peak present at high level in fresh tomato extract or from freshly diluted lycopene crystals. The peak after trans is identified as 5-cis based on comparison of this method and chromatogram to references. Studies that include similar methods and chromatograms were reported by Clinton et al. (1996), Yeum et al. (1996) and Schierle et al. (1997).

Clinton et al. (1996) identified the peaks by 1) confirming that the peaks are lycopene by mass spectrometry; 2) identifying cis-isomers by the presence of a peak in the 360 nm region of their absorption spectra; and 3) comparing HPLC chromatograms of α-carotene to that of lycopene concluding that the peak after all-trans is 5-cis. Schierle et al. (1997) identified peaks by comparing the chromatogram of iodine isomerized lycopene with that reported by Hengartner et al. (1992), where NMR was used to identify isomers.

The 5-cis isomer peak was not present in most of the chromatograms in the study when a Develosil RP-aqueous column was used. The 5-cis peak was easily identifiable in lycopene samples that were high in 13-cis and 9-cis that were stored at room temperature for a few days. A 5-cis peak was not present in a sample of heat isomerized (80˚C for 10 minutess) 98% all-trans lycopene, even though significant 13-cis and 9-cis peaks were present. 5-cis may not be resolved in all samples. High levels of all-trans may overlap with a small 5-cis peak. Calvo and Santa-Maria (2007) also experienced this phenomenon. Samples with high levels of an isomer have a sharp peak. If low levels

of an isomer are present, the peak appears broad compared to its height. Some lycopene samples contain a tailing shoulder in the all-trans peak that is 5-cis isomer. Because the shoulder is indistinguishable from the main peak, the entire peak area was assigned to all-trans lycopene. 5-cis and all-trans lycopene have the same absorption spectra and cannot be distinguished by UV-VIS detection. Implementing a YMC brand column significantly increased peak resolution. Figures 2 and 3 are chromatograms using the same method and same sample but different column. The chromatogram using the YMC column clearly separates all-trans from 5-cis lycopene. During the stability test using the YMC column, 5-cis and all-trans lycopene were accounted for separately.

Calibration of lycopene measurement on HPLC was performed using high purity all-trans lycopene. A standard of lycopene crystals dissolved in hexane was used. Concentration of the standard was determined by measuring absorbance at 471 nm using an absorption coefficient ($A_{1cm}^{1\%}$) of 3450 (Britton, 1995) (Figure 4). The standard was then analyzed by HPLC (Figure 5). A HPLC peak area (PA) of 78602023 corresponds to 1 mg of lycopene. The concentration of lycopene in an extract is calculated by equation 1.

Equation 1: Concentration (mg/ml) =

PA (observed)/ [Injection volume(ml) x 78602023(PA/mg)]

The same correlation factor was used for all isomers. The cis isomer content is underestimated because 13-cis and 9-cis have a lower extinction coefficient but judgments based on the changes in the isomer profile are still valid (Hengartner et al., 1992). Cis isomer content was determined by totaling the sum of 13-cis including the

shoulder, 9-cis and 5-cis. Cis isomer percentage is equal to total cis lycopene / total lycopene x 100.

Precision of the analytical HPLC method was analyzed by measuring lycopene content of one sample six times. The percent relative standard deviation (coefficient of variation) was calculated using the peak area of all-trans lycopene (Table 3). The percent relative standard deviation is 1.9%.

3.3 Encapsulation Materials and Methods

3.3.1 Encapsulation Formula and Procedures

Three different encapsulation systems were evaluated. The first system tested used octenyl succinate (OSA) modified starch (HiCap100, National Starch, Bridgewater, NJ) as the wall material with lycopene in medium chain triglycerides (MCT) (Neobee 1053, Stepan, Northfield , IL) as the core material. The ingredients were homogenized using a rotor-stator type homogenizer then spray dried. The second formulation was very similar but substituted gum acacia, also known as gum Arabic, (Encapsia, Colloides Naturels Inc,. Bridgwater, NJ) for OSA modified starch. The third formulation used maltodextrin (Cargill Dry MD 01956, Cargill Inc., Cedar Rapids, LA) as the wall material and no oil. Lycopene was dissolved into ethyl acetate, then added drop wise to form dispersion. Tween 80 (Fisher Scientific, Pittsburg, PA) was used as an emulsifier. A high-pressure homogenizer was used before spray drying.

The procedure for preparing emulsions with HiCap 100 or gum acacia is as follows. 20 g hydrocolloid was mixed with 60 ml water until it was completely hydrated. 1 g of MCT containing 0.1-1 mg lycopene per gram was then added to the slurry to form a coarse emulsion. The remaining lycopene in oil was placed into vials for stability

controls. The slurry was then homogenized using a rotor-stator homogenizer. The rotor is about 1 cm in diameter. Homogenization was performed at 9,000 rpm for about 2 minutes, then 18,000 rpm for 5 minutes. Multiple trials were performed; lycopene concentration ranged from 5 – 50 μg/g dry weight.

The lycopene and maltodextrin dispersion was prepared by dispersing 0.35 g Tween 80 into 35 g water that was then homogenized with a rotor-stator type homogenizer until Tween 80 was completely dispersed. 1.5 ml lycopene in ethyl acetate solution was added drop wise to the water/Tween with the homogenizer on. A new drop was added after the previous drop was completely dispersed. 15 g maltodextrin was added to the dispersion and mixed until the maltodextrin was completely dissolved. The dispersion was then passed through the high-pressure homogenizer 5 times at 1500 bar (EmulsiFlex-C3, Avestin Inc., Ottawa Canada). Lycopene concentration was about 43 μg/g dry weight.

The spray drier used is a Yamato Pulvis Mini-Spray Model GA-32 (Yamato Scientific America, Inc., South San Francisco, CA). The inlet air temperature is 197ºC for OSA modified starch and gum acacia; 185ºC for the maltodextrin system. Drying air speed is adjusted to 0.45 m^3/min with the atomizing air pressure equal to 0.15 MPa. The feed rate is 3 ml/min at ambient temperature. The liquid nozzle orifice (part of atomizer) used is part number 64 with an orifice size of 711 μm; the air nozzle part number is 2850.

3.3.2 Evaluation of Encapsulates

The change in lycopene because of spray drying was determined by comparing the lycopene content immediately after spray drying to before. Total lycopene as well as the isomer profile was measured. A method of high extraction efficiency is needed in

order to evaluate the effect that the procedures have on lycopene. A high efficiency extraction technique was developed because low concentrations of lycopene were being encapsulated. Other extraction techniques use large quantities of solvent but cannot be used because lycopene concentration would be too low to measure without rotoevaporation, which would change the isomer profile (Desobry et al., 1997).

To extract lycopene from OSA starch and gum acacia encapsulations, 0.120 – 0.130 g encapsulated lycopene is placed in a microfuge tube with 0.75 ml water and 0.75 ml extraction solvent (2 hexane: 1 acetone: 1 ethanol). The mixture is shaken until no clumps are visible, then the vial is centrifuged for 7 minutes at maximum speed. The upper hexane layer and interface layer (layer between polar layer and hexane layer) are transferred into separate microfuge tubes. The interface layer is re-extracted with 0.75 ml water and 0.75 ml extraction solvent (2 hexane: 1 acetone: 1 ethanol). The mixture is shaken and centrifuged, and the hexane layers are pooled together. The pooled hexane layers were filtered through a 0.45 micron filter, then lycopene content is measured by HPLC. The lycopene concentration was increased by removing solvent by a stream of nitrogen gas if the concentration was near the limit of detection.

To extract lycopene from the formulation with maltodextrin and Tween 80, 0.120-0.130 g encapsulated lycopene is placed in a microfuge tube with 0.75 ml water and 0.75 ml extraction solvent (2 hexane: 1 acetone: 1 ethanol). The mixture is shaken until no clumps are visible and centrifuged for 1 minute at maximum speed. The upper phase and lower phase are separated. 0.4 ml ethanol is added to the upper phase containing hexane and maltodextrin. The maltodextrin precipitates and becomes white while the upper phase contains lycopene. 0.4 ml dichloromethane is added to the lower phase and then

mixed vigorously. After centrifugation for 2 minutes, the lower dichloromethane phase is red while the upper phase has no pigment. The pooled lycopene layers were filtered through a 0.45 micron filter then lycopene content is measured by HPLC. The lycopene concentration was increased by removing solvent by a stream of nitrogen gas if the concentration was near the limit of detection.

Precision of extraction method used for gum acacia was evaluated by extracting lycopene from a batch of encapsulated lycopene. The extraction procedure was completed six times, and lycopene content of each extract was measured by absorbance at 471 nm in a spectrophotometer (Table 4). The relative standard deviation (coefficient of variation) of the extraction method is 3.5%. The precision of the method used for the maltodextrin/Tween 80 formulation was not tested.

3.4 Precision of Measurements

Reproducability of measurements has been a problem when measuring the stability of lycopene during this study. The precision test of HPLC analysis and extraction was performed to determine the most efficient way to collect data and to determine the source of variation. The variability of extraction was greater than HPLC determination, 3.5% to 1.9% respectively. To increase the precision of the measurements, replicate samples should be measured. To most efficiently collect data, a replicate measurement should consist of one extraction and one HPLC measurement. Variation in the extraction procedure is because it involves multiple steps. Limiting the number of steps during extraction will increase precision. A test that concentrated lycopene after extraction using a stream of nitrogen gas had high variability. This was probably because lycopene was carried onto the sides of the vial by hexane vapors and

did not get transferred from the walls back into solvent for HPLC analysis. Another source of variation that was remedied occurred when measuring pure lycopene and lycopene in oil. 100 µl of hexane was used to disperse lycopene for injection into HPLC, the total volume immediately before analysis varies because hexane is highly volatile. To improve the method, the total volume before injection was measured. An effort to increase precision was continually made throughout the study.

A strategy that can be used to increase precision while limiting time requirements would be to measure total lycopene content spectrophotometricaly and only perform one HPLC analysis to obtain the isomer profile. Another way to increase precision is to use cyclohexene instead of hexane to reduce evaporation of the solvent. An internal standard could also be used as a reference.

3.5 Stability of Lycopene During Storage

The stability of encapsulated lycopene was compared to lycopene in MCT formulation and, in some tests, pure lycopene. The same starting material of lycopene in hexane was used for each formulation to normalize for variations between batches of lycopene. A different batch of lycopene was used in each encapsulation trial. Stability tests were performed in the absence of light. No attempt at removing oxygen from the vials was made and relative humidity was not controlled. Storage temperatures ranged from –20 to 40˚C.

Lycopene in oil was prepared by adding lycopene in hexane to MCT. A stream of nitrogen gas then removed the solvent. Aliquots would then be placed into vials for stability storage. A portion of the lycopene in oil would also be encapsulated.

Measurement of lycopene was executed by adding hexane to suspend lycopene, then injection to HPLC for total lycopene measurement and the isomer profile.

The pure lycopene formulation was prepared by pipetting an aliquot of lycopene in hexane into vials. A stream of nitrogen gas then removed the solvent. Measurement would be the same as for lycopene in oil.

4 Results and Discussion

4.1 Lycopene Stability During the Encapsulation Process

4.1.1 Retention of Total Lycopene Content

There is a pattern of a loss of total lycopene content during spray drying. The percentage lost during spray drying was determined by comparing the lycopene content measured in the emulsion to lycopene content measured in the encapsulate. Lycopene content was normalized to the dry weight of the sample. Lycopene content decreased 20, 33, 36, and 43% during spray drying where lycopene was measured to a reasonable degree of accuracy. Variability in the loss of lycopene may be due to the isomer profile. The 20% decrease was observed when lycopene high in all-trans was spray dried, whereas the highest loss of 43% was measured when lycopene primarily containing 13-cis was spray dried. Goula and Adamopolous (2005) found that lycopene loss ranged from 8 to 21% during spray drying of tomato pulp. Lycopene experienced a decrease of 24% during the spray drying of watermelon pulp in the example given by Quek et al. (2007). Wang and Chen (2006) encapsulated lycopene by spray drying and experienced significant losses.

Degradation of lycopene is most likely due to oxidation. Lee and Chen (2002) heated lycopene at 150˚C and only observed a decrease of 24% in total lycopene content after 2 minutes. During spray drying, the residence time at high temperatures is less than 1 second. Loss of lycopene cannot be attributed to high temperatures alone. Lycopene exposed to light did not decrease significantly after 1 hour (Lee and Chen, 2002). The high flux of air during spray drying is probably a major contributing factor in the degradation of lycopene. A spray dryer that uses nitrogen as the drying gas would limit

degradation of lycopene during drying. A spray dryer that can recirculate the drying gas is needed to use nitrogen as the drying gas. Nitrogen as the drying gas has not been tested in this lab nor been reported in literature.

4.1.2 Stability of the Isomer Profile

The isomer profile is susceptible to change during spray drying. The percentage of cis isomers (of the total lycopene) before and after spray drying of all encapsulations is reported in Table 5. When starting with lycopene high in cis isomers, the isomer profile shifts to a higher percentage of all-trans. Lycopene containing primarily the all-trans isomer did not change dramatically. Table 6 is a more detailed look at lycopene during spray drying which provides the mass of each isomer and percentage (of the total lycopene) of each isomer. In the cis enriched encapsulation, the mass of 13-cis decreases to a greater extent than all-trans lycopene. In the all-trans lycopene trial, each isomer decreases to a similar degree. This can be explained by the fact that cis isomers are less stable than all-trans lycopene and that cis can isomerize into all-trans (Moraru 2004; Lambelet et al., 2009). Note that 13-cis was in the highest proportion in all high cis trials except for the trial starting as 79.6% cis which was primarily 9-cis lycopene. The sample containing primarily 9-cis did not change significantly. Lambelet et al. (2009) found that 9-cis was less susceptible to isomerism than 13-cis.

Shu et al. (2006) observed an increase in cis isomer percentage during spray drying. However, the homogenized feed was held at 55˚C prior to spray drying. Also, lycopene was dispersed using hot acetone. The impact of each step on isomerism was not measured. The increase in cis isomers could have been from other processing steps besides spray drying. Wang and Chen (2006) spray dried lycopene to obtain

microcapsules containing 28.4-34.5% cis isomers. The isomer profile of the starting lycopene material was not specified and significant losses in total lycopene content were noted because of spray drying.

4.1.3 Encapsulation Yield

Another important factor is the recovery of solid materials after spray drying. Encapsulation yield is defined as the ratio of the mass of microcapsule obtained to the mass of starting materials including oil and wall material but not water (Shu et al., 2006). Spray drying was initially attempted at an inlet temperature of 150˚C in our experiment. But because encapsulation yields were below 50%, the temperature was increased to 197˚C. Yield was low because of poor drying of the particles; moist hydrocolloid particles can adhere to the walls of the drying chamber. The spray drying conditions affect the physical structure of the capsules as well as attributes including moisture content, water activity, retention of lycopene, particle size and polydispersity. The moisture content (MC) and water activity (a_w) of an encapsulation trial of gum acacia were 0.13 and 3% respectively. These values fall below the glass transition temperature for gum acacia (Collares et al., 2004). After collecting data that showed lycopene is degrading during spray drying of lycopene with gum, the inlet temperature was lowered to 185˚C for the maltodextrin/Tween formulation. In this case, encapsulation yield was 40%. Incomplete recovery of the dispersion after high-pressure homogenization may have contributed to this loss.

Lycopene yield is dependent on the spray drying conditions. Optimizing spray-drying conditions is challenging because there are many factors that can be adjusted. Experience and knowledge are very helpful when developing a procedure because of its

complexity (Gharsallaoui et al., 2007). The parameters that can be adjusted with our spray dryer are the following: 1) drying air temperature at the inlet, 2) drying air speed, 3) atomizing air pressure, 4) sample feed speed, and 5) spray nozzle orifice diameter. The two parameters that have a high impact on encapsulation yield are drying air temperature and atomizing air pressure. Temperature affects the drying rate and atomizing air pressure affects the particle size.

Shu et al. (2006) provides a template for the optimization of spray drying conditions. They tested the effect of a number of different processing conditions on encapsulation efficiency and encapsulation yield. One parameter, such as drying air temperature, was adjusted while other variables were kept constant. A similar study can be performed with the formulations used in this study and the Yamato Pulvis Mini-Spray Model GA-32 spray dryer. A test design using response surface methodology can also be useful.

Spray drying of lycopene requires significant process development or experience to achieve good retention and yield of lycopene. Lycopene high in cis isomers possesses a technical challenge that is greater than in all-trans lycopene because isomerism can occur and cis isomers are more susceptible to degradation.

4.2 Stability of Lycopene During Storage

4.2.1 Storage of Lycopene at -20˚C

Cis lycopene in MCT did not degrade and the isomer profile remained the same over a 27-day test period. Cis lycopene in hexane did not degrade or isomerize after 5 months of storage. There is a strong correlation between temperature and degradation. Increasing the storage temperature increases the rate of degradation of lycopene (Hackett

et al., 2004; Ax et al., 2003; Lee and Chen, 2002). This study shows that storage at -20˚C prevents isomerism for an extended period of time and agrees with other studies (Lambelet et al., 2009).

4.2.2 Stability of Lycopene Encapsulated with Octenyl Succinate Modified Starch

During preliminary testing, emulsions prepared with 25% octenyl succinate (OSA) modified starch with 1.25% oil, were not forming a stable emulsion even with high sheer homogenization. This was either because the starch level was too low or the emulsion particle size was too large. The level of OSA starch used did not increase the viscosity enough to stabilize the emulsion. At the time, 25% starch by weight was being used in the emulsion. National Starch recommended using 30% starch to supply sufficient viscosity. Using an emulsifier in the formulation would also assist in forming a stable emulsion. The emulsion was kept under constant agitation during spray drying to evenly distribute lycopene. Quantitative data regarding the stability of lycopene in OSA modified starch during storage is not available because poor measurements were taken. Even so, the capsules were completely bleached after 2 weeks storage at room temperature in aluminum lined pouches and no lycopene was extractable from the encapsulates using a low recovery extraction method. Lycopene was detected immediately after spray drying. This was regarded as a non-successful encapsulation method because the emulsion was not stable and lycopene was not sufficiently stable to have a reasonable shelf life for a shelf stable dry food product. Comparisons to lycopene in oil or pure lycopene were not made at this time.

4.2.3 Stability of Lycopene Encapsulated with Gum Acacia

Three encapsulation batches of cis enriched lycopene were made by dispersing lycopene in oil then forming an emulsion with 25% gum acacia and 1.25% oil. The emulsions were then spray dried using the parameters specified in the methods section. The lycopene content and isomer profile are different in each encapsulation and the storage conditions vary. The concentration of lycopene in MCT is approximately 20-30 times greater than capsules on a weight basis because oil was used at a 5% usage rate and lycopene degraded during spray drying. The quality of data varies because of difficulties with data collection.

Trial A: The encapsulate, which contained 5 μg lycopene per gram powder which was 72% cis isomers, was not stable at room temperature in vacuum sealed aluminum lined pouches over a two week period. No lycopene was detected after two weeks using the developed extraction procedure. Lycopene content in oil decreased by 99% after 2 weeks storage at room temperature. Figure 6 is a chromatogram of lycopene that was initially high in cis isomers after storage at room temperature. The sample of lycopene in MCT experienced an increase in all-trans lycopene content. The sample also produced degradation products eluting between 3 and 11 minutes. Ukai et al. (1994) produced a similar chromatogram of an oxidized lycopene sample using a C18 column. They identified one of the peaks as apo-6-lycopenal, an oxidation product. Caris-Veyrat (2003) also found apo-lycopenals as degradation products when analyzing oxidized samples of lycopene.

Trial B: Lycopene high in 13-cis isomer was encapsulated. The resultant powder contained 3.3 μg lycopene per gram with 79.5% as cis isomers. Replicate samples were

placed into 3 ml glass vials. Replicates of cis lycopene in oil and pure cis lycopene (HPLC purified with all solvent removed) were also placed in 3 ml glass vials. All samples were placed in an incubator at 35˚C in the dark. Duplicate samples were removed and assayed immediately before storage and at 12-hour intervals (Figure 7). Pure lycopene was not detectable after 12 hours, which would be less than 10% of its original content. Pure lycopene (total content) decreased much faster than total lycopene in oil or the encapsulated lycopene. Retention of total lycopene in oil and the encapsulate is not significantly different ($P < 0.05$) during the first 36 hours. A difference is present beyond 36 hours with encapsulated lycopene content being more stable than lycopene in oil. The slight increase in stability does not compensate for the loss of lycopene during spray drying (43% loss).

The isomer profile is not stable during storage in both the encapsulate and oil (Figure 8). The percentages of lycopene as cis in both formulations initially decrease and then stop decreasing after 24 hours. The isomer profile of lycopene in oil and encapsulated lycopene change in an identical pattern leading to the conclusion that the encapsulation of lycopene in oil does not influence isomerization during storage.

Trial C: The third encapsulation contained 17 μg lycopene per gram powder with 79% cis isomer; 9-cis was 43% of the total lycopene. Storage conditions were 35˚C in the absence of light. The data does not show a significant difference between lycopene in oil and lycopene that has been encapsulated (Figure 9). There is high deviation in the data which indicates there was poor precision during the measurements. Pure lycopene showed only minor degradation during storage, most likely because not all of the solvent was removed from the vials. In this trial, lycopene was relatively more stable than

comparable trials. The isomer profile also does not change significantly. This may be because 9-cis was present at higher concentrations.

4.2.4 Stability of Lycopene Encapsulated with Maltodextrin/Tween 80

One stability test was performed with maltodextrin and 2.3% Tween 80 and no oil in the formulation. This method was not successful at forming a stable dispersion; lycopene would flocculate and float if not stirred. A modification of the homogenization process or formulation is needed in future work. Lycopene was kept well dispersed during spray drying by a stir bar at a high speed. Samples were placed into 2 ml microfuge tubes and placed in a 40˚C incubator in the dark. Measurements were taken in triplicate. The encapsulate produced contained 19 μg lycopene per gram powder with 52% cis isomers. Lycopene in MCT contained 354 μg lycopene per gram oil. In terms of total lycopene content, lycopene in MCT was most stable followed by encapsulated then pure lycopene (Figure 10). A shift toward a higher percentage of all-trans lycopene in the isomer profile was prevalent in all formulations. Figure 11 displays the amount of each isomer during storage in oil. All-trans and 5-cis lycopene content increased while 13-cis decreased. Figure 12 displays cis isomers and all-trans in the encapsulate. Even though the all-trans lycopene content does not increase, cis to trans isomerism probably is taking place because there is not a decrease in all-trans. One could explain the higher rate of cis degradation and slower rate of all-trans degradation because 13-cis is less stable in terms of degradation and not because of isomerism. Either way, the total lycopene content decreased more rapidly than in oil.

4.2.5 Summary of Total Lycopene Stability During Storage

In terms of total lycopene content, lycopene in MCT encapsulated with gum acacia provided the most stable product. But because the increase in stability is small and lycopene degradation occurs during spray drying, lycopene dispersed in oil is a better formulation so far. Encapsulation of lycopene in maltodextrin with Tween 80 improved the stability of total lycopene content over pure lycopene, but was not as good as lycopene in MCT. The stability of lycopene encapsulated with gum acacia is primarily due to the protective effect of oil, not the wall material. Medium chain triglycerides have a protective effect on lycopene probably because oil provides an oxygen barrier. MCT are highly resistant to oxidation.

Lycopene stability is influenced by many factors including light, oxygen content, temperature, moisture content, solids (fiber, pectin, sugars, proteins, etc.), antioxidants (vitamin E, etc.), isomeric confirmation, physical form (crystalline, dissolved or hydrophobic complex) and pH. The stability of lycopene in this study is low compared to data in the literature (Hackett et al., 2004; Chiu et al., 2007; Ribeiro and Schubert 2003; Lambelet et al., 2009). The stability of lycopene in this test is very reasonable because it is not that different from most of the literature which contains a wide rage of stabilities. The differences can be explained by the fact that lycopene in this study is highly purified and in an unfavorable formulation that does not contain other antioxidants as in tomato oleoresins, it is not bound in the tomato structure, and is stored with exposure to air.

4.2.6 Summary of Lycopene Isomerism During Storage

Lycopene in MCT and encapsulated lycopene in MCT experienced a change in the isomer profile during storage at 35˚C (Figure 8) and 40˚C (Figure 11). The data

indicates that the isomer profile of 13-cis enriched tomato oleoresin would not be stabilized by microencapsulation with gum acacia. Oil will be occupying hydrophobic regions of the wall material and limit the amount of lycopene-wall interactions. Lycopene hydrophobically bound with the wall material may inhibit lycopene from isomerizing by preventing lycopene isomer intermediates from freely rotating to different conformations. Lycopene as crystals are less likely to isomerise because the crystal lattice prevents bond rotation. The isomer profile in tomato products remains stable, most likely because it is in crystalline form and interacts with tomato solids. Encapsulation with maltodextrin and Tween 80 was an attempt to recreate those conditions. We were unable to determine if isomerism was taking place because both 13-cis and all-trans content decreased. 13-cis content declined faster than all-trans but this could just be because 13-cis is degrading faster and not because 13-cis is isomerizing. Stability of encapsulated all-trans lycopene would provide baseline degradation rates for comparison.

4.3 Application of Cis Lycopene Isomers in Food Products

Even though lycopene high in 13-cis is not stable at room temperature, it still does have potential applications in food products. Cis isomers are sufficiently stable at low temperatures. Ice cream is a suitable vehicle for lycopene. Lycopene content and the isomer profile would remain stable in ice cream because it would be in the freezer. Ice cream is high in fat and low in fiber and therefore will increase the absorption of lycopene. Lycopene would also provide natural color. One drawback of using lycopene high in cis instead of high in all-trans is that cis isomers do not provide as much color per unit mass of lycopene.

5 Conclusions and Future Work

5.1 Conclusions

Significant losses of lycopene occurred during spray drying ranging from 20 – 43%. The lycopene isomer profile changed significantly when 13-cis lycopene was encapsulated. 13-cis content reduced dramatically where all-trans decreased to a lesser extent. Lycopene high in all-trans lycopene experienced little isomerism during spray drying. Processing conditions need to be optimized to maximize lycopene retention and yield during encapsulation by spray drying. This is especially important when spray drying cis isomers because they are even more labile than all-trans lycopene.

A spray dried encapsulation of lycopene using MCT and gum aciaca did not prove to be successful enough at retaining an isomer profile high in cis isomers and preventing loss of total lycopene. Lycopene did not show a meaningful increase in shelf life after encapsulation as compared to lycopene in medium chain triglycerides. The stability of lycopene in the gum acacia encapsulate is probably due to the protective effect of the oil. A formulation of maltodextrin and Tween 80 was also used to encapsulate lycopene. The encapsulate was more stable than pure lycopene but did not stabilize the isomer profile and was much less stable than lycopene in oil. Besides spray drying parameters, there are numerous variables that can be adjusted, such as the wall material and emulsifiers, to meet the research objectives and exhaustively test the hypothesis.

5.2 Recommendations For Future Work

More research is needed in the area of lycopene isomer stability. We recommend continuing the research above by testing other encapsulation methods. Encapsulation via

spray drying may yet prove to be an effective method to encapsulate lycopene isomers. The spray drying conditions are important and should be optimized. Nitrogen should be compared to air as the drying gas because we hypothesize that the presence of oxygen in air is a major contributing factor in the degradation of lycopene during spray drying.

Other stabilization methods should be studied. For example, a preliminary test was performed to determine if a solid lipid matrix could prevent isomerism. Lycopene, primarily 13-cis, in palm kernel oil experienced a much faster degradation rate than in MCT at room temperature. The isomer profile remained high in cis-isomers in the palm kernel oil formulation, even after significant losses. More investigation is needed.

Degradation and isomerism occur simultaneously. Determining the degradation rates of lycopene isomers in conditions where isomerism will not take place will determine the relative stabilities of the isomers, and will help elucidate if encapsulated cis lycopene is isomerizing or degrading. A test could be performed where lycopene would be stored at -20˚C to prevent isomerism and an oxidation catalyst could be added. Measuring the stability of encapsulated all-trans lycopene would also provide a reference degradation rate that could be compared to encapsulated cis isomers. One could conclude that isomerism is not occurring if the degradation rate of all-trans lycopene is the same.

We identify a knowledge gap regarding the extinction coeffiecents of cis isomers. More published data is needed so that researchers can take into account that isomers have different molar absorption coefficients. Two laboratory approaches are outlined in the literature review section on the analysis of lycopene that can provide valuable data. The 13-cis and 9-cis extinction coefficients should be accurately determined and published.

An issue that was not discussed thoroughly in this research but needs more attention in future works is that the cis isomers have different properties. Isomers are typically grouped as cis isomers or all-trans, but which cis isomers are present is important. Cis isomers vary in stability and isomerism rates and should be accounted for individually when discussing data. The stability of each isomer needs to be studied.

Degradation products of lycopene may be biologically active (Lindsheild et al., 2007). It would be interesting to determine if the degradation products of each isomer are different.

The pharmacokinetics of lycopene depends on multiple factors and requires more research. We hypothesize that nano-sized lycopene crystals will be more bioavailable than microcrystalline lycopene. The use of nanoemulsions may also yield a highly bioavailable source of lycopene.

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Table 1. Semi-preparative lycopene isomer collection parameters. Changes in solvent follow a linear gradient. Detection is by absorbance at 471 nm [modified from Moraru (2004)].

Time (min)	Flow (ml/min)	%A	%B
Start	4	90	10
0.5	4	90	10
4	4	55	45
8	4	5	95
20	4	5	95
21	4	90	10

Solvent A: 83% Methanol, 15% MTBE, 2% Water
Solvent B: 10% Methanol, 90% MTBE, 2% Water

Column: Develosil RP-Aqueous C30 (5 um, 250 mm x 10 mm)

Column Temperature = 0 ºC

Table 2. Analytical HPLC method parameters for lycopene quantification. Change in solvent follows a linear gradient. Detection is by absorbance at 471 nm [modified from Moraru (2004)].

Time (min)	Flow (ml/min)	%A	%B
0	1	90	10
4	1	90	10
15	1	5	95
26	1	5	95
28	1	90	10

Solvent A: 83% Methanol, 15% MTBE, 2% Water
Solvent B: 10% Methanol, 90% MTBE, 2% Water

Column: Develosil RP-Aqueous or YMC carotenoid (3 um, 150 mm x 4.6 mm)

Column Temperature = 0 ºC

Table 3. Precision of HPLC method. A sample of lycopene in hexane was measured 6 times by HPLC. Average, standard deviation and % relative standard deviation were calculated on the peak area of all-trans lycopene.

Inj #	Inj V (ml)	Peak Area all-trans
1	0.02	53058
2	0.02	54252
3	0.02	51850
4	0.02	53850
5	0.02	51884
6	0.02	53141
	Average:	53005
	Standard Deviation:	988.02
	% Relative Standard Deviation:	1.8639

Table 4. Precision of extraction method on gum acacia encapsulates. Identical samples were extracted using the developed procedure. Lycopene content was measured spectrophotometrically. Average, standard deviation and % relative standard deviation were calculated.

Dry mass (g)	Volume (ml)	Dilution factor	AU (471)	mg/ml	mg/g
0.100	1.14	5	0.4036	0.005849	0.066682
0.102	1.21	5	0.389	0.005638	0.066878
0.105	1.17	5	0.4064	0.005890	0.065630
0.106	1.35	5	0.387	0.005609	0.071432
0.107	1.34	5	0.384	0.005565	0.069695
0.119	1.31	5	0.4235	0.006138	0.067566
				Average:	0.068063
				Standard Deviation:	0.002411
				% Relative Standard Deviation:	3.5416

Table 5. Percentage of cis isomer before and after spray drying of various encapsulation trials.

%Cis Before	%Cis After
90.4	79.5
87.3	72.1
79.6	79.1
78.5	44.1
62.2	55.6
57.5	41.3
23.5	23.4
11.8	15.7
1.6	3.2

Table 6. Lycopene isomer content and isomer percentage of the gum acacia emulsion and in the encapsulate after spray drying for lycopene high in cis isomers and lycopene containing mostly all-trans lycopene.

Lycopene High in Cis Isomers:

	13-cis	9-cis	all-trans	5-cis		
Mass	*mg*	*mg*	*mg*	*mg*	*total mg*	*% recovered*
Emulsion	60.0	38.4	15.9	3.8	118.1	100.0%
Encapsulated	27.8	20.2	13.7	5.2	66.9	56.7%
Percentage	*%*	*%*	*%*	*%*	*% Cis*	
Emulsion	50.9	32.5	13.4	3.2	86.6	
Encapsulated	41.5	30.3	20.5	7.7	79.5	

Lycopene High in All-trans Isomer:

	13-cis	9-cis	all-trans	5-cis		
Mass	*mg*	*mg*	*mg*	*mg*	*total mg*	*% recovered*
Emulsion	65.8	7.3	242.2	-	315.2	100.0%
Encapsulated	51.9	5.9	189.4	-	247.3	78.4%
Percentage	*%*	*%*	*%*	*%*	*% Cis*	
Emulsion	20.9	2.3	76.8	-	23.2	
Encapsulated	21.0	2.4	76.6	-	23.4	

Figure 1. A typical chromatogram of isomer collection from heated crude tomato paste extract.

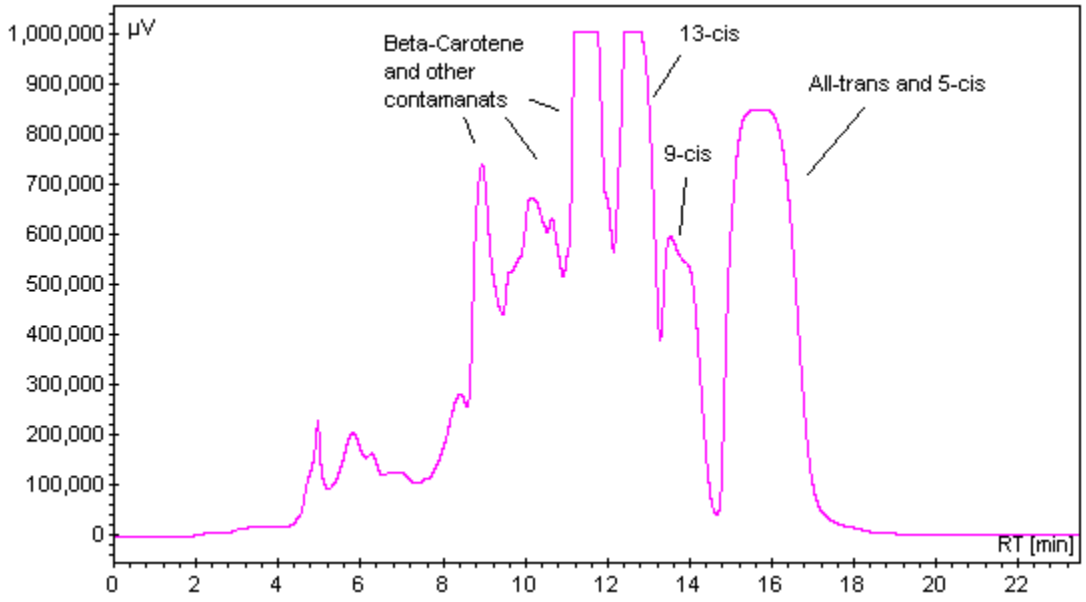

Figure 2. A typical chromatogram of isomerized lycopene using a Develosil RP-Aqueous column with the analytical HPLC method.

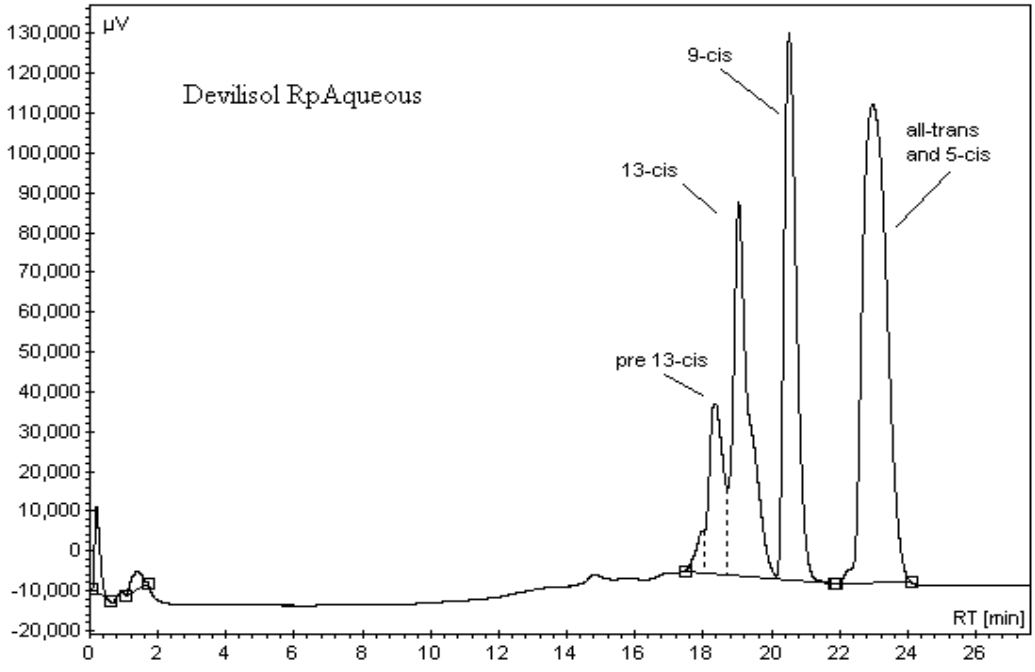

Figure 3. A typical chromatogram of isomerized lycopene using a YMC carotenoid column with the analytical HPLC method.

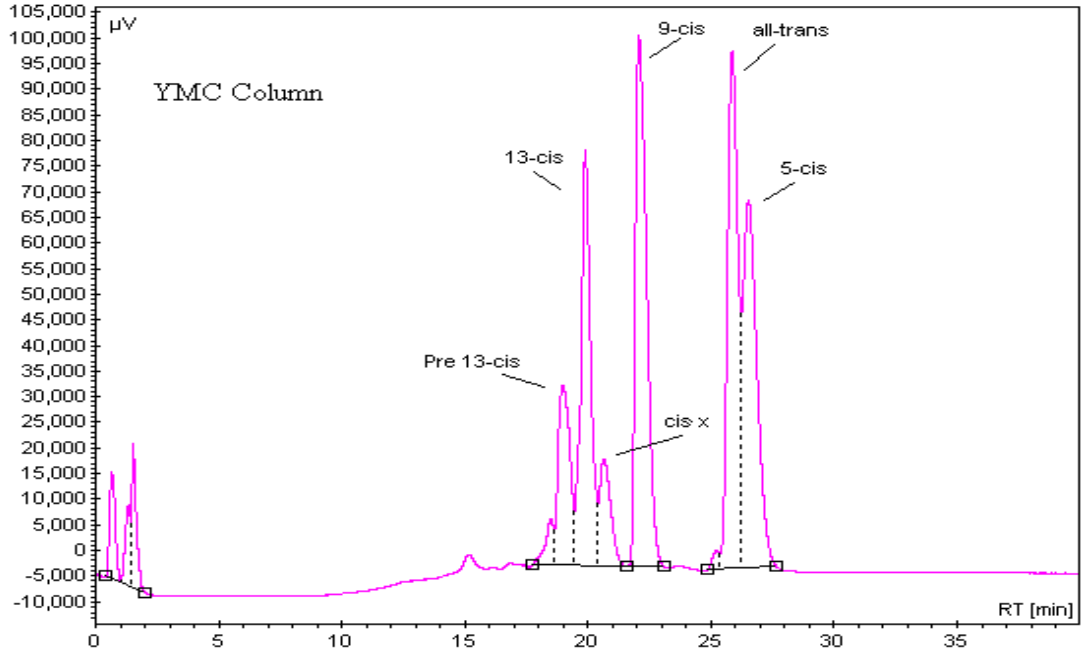

Figure 4. Spectrophotometric determination of lycopene concentration in hexane. Various volumes of lycopene in hexane were diluted in hexane to a total volume of 1 ml. Absorbance at 471 was measured. Concentration calculated using $A_{1cm}^{1\%}$ 3450. The concentration of the stock used to calibrate HPLC is 0.0348 mg/ml.

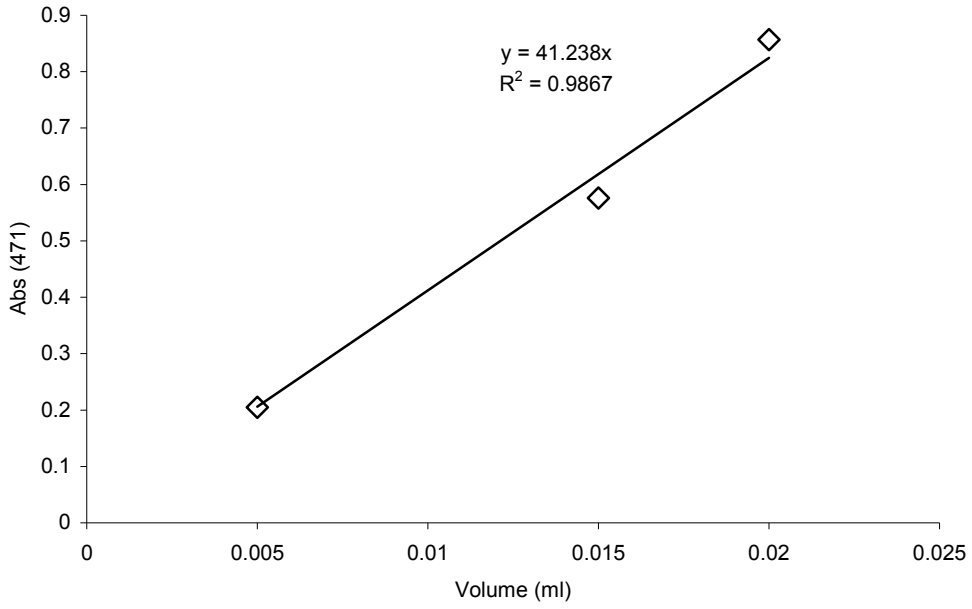

Figure 5. Correlation of peak area (PA) to lycopene mass on HPLC. Various volumes of lycopene in hexane were injected into HPLC. PA/mg lycopene = 78602023

Concentration (mg/ml) =

PA (observed)/ [Injection volume(ml) x 78602023(PA/mg)]

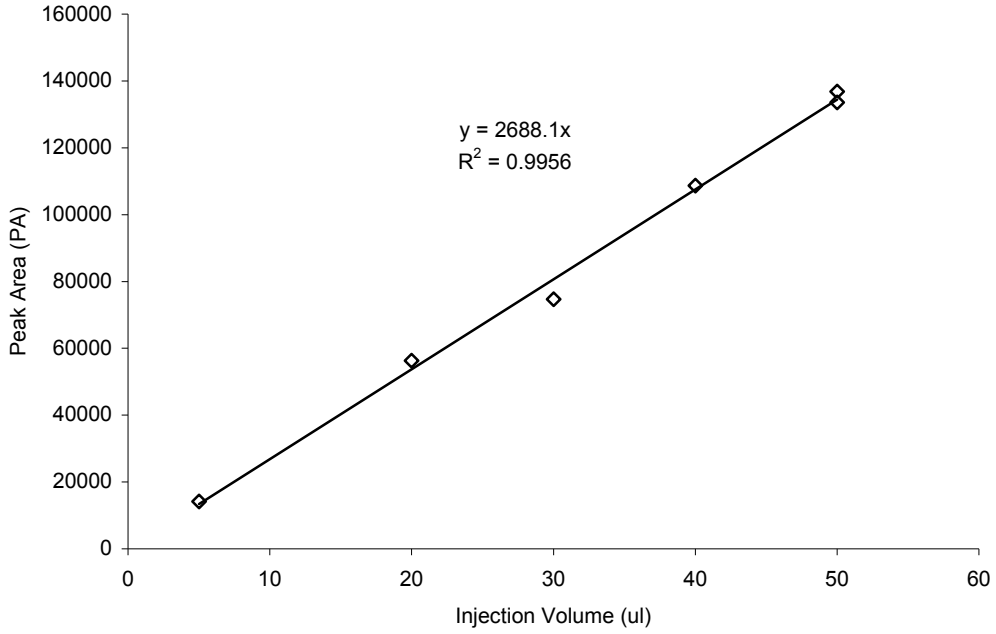

Figure 6. Chromatogram of lycopene high in 13-cis and 9-cis after storage at room temperature in MCT. Significant amounts of all-trans and 5-cis isomers are present as well as degradation products that are eluting between 3 and 11 minutes.

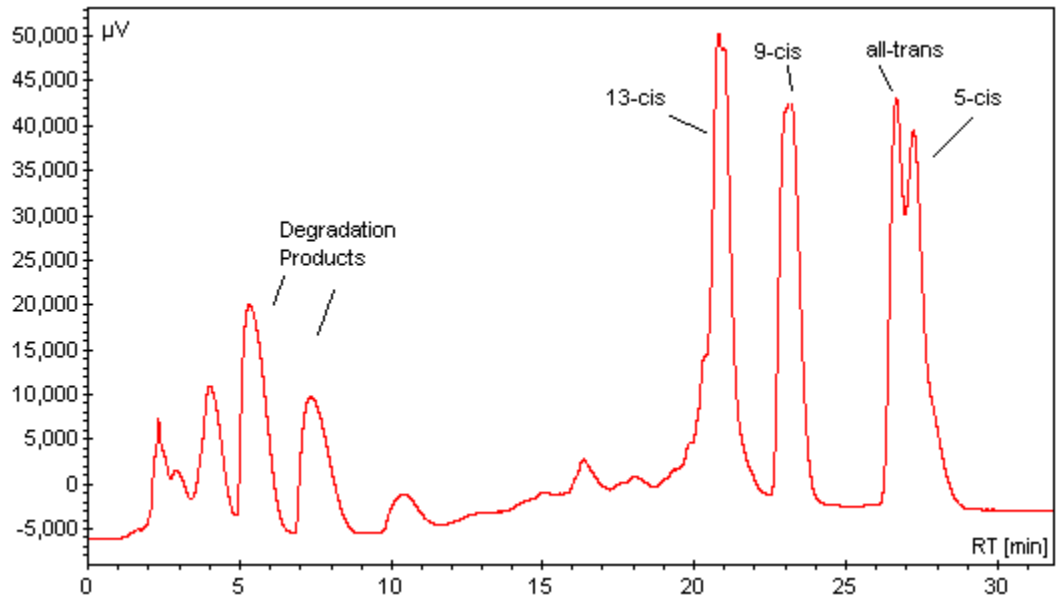

Figure 7. Retention of total lycopene (high in 13-cis) during storage at 35˚C; (——) cis lycopene in MCT, (----) cis lycopene in MCT encapsulated in gum acacia (1.25% MCT dry weight).

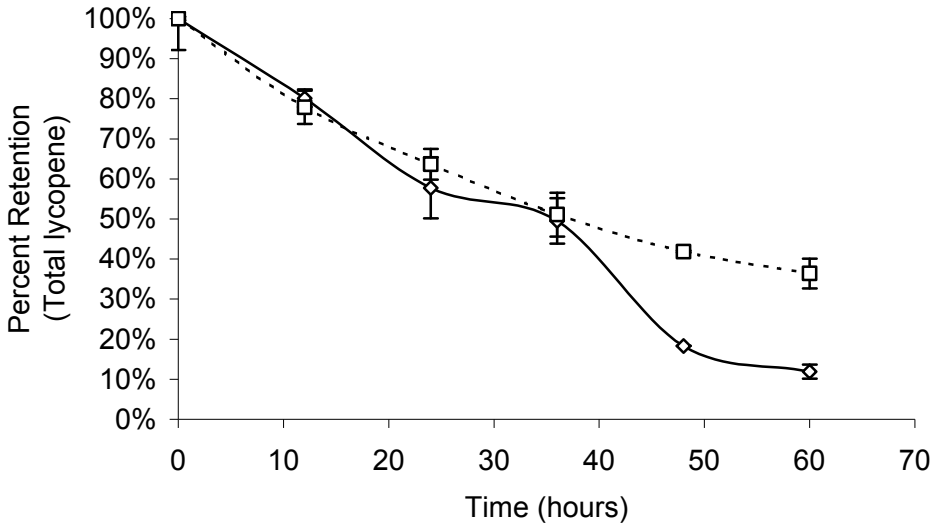

Figure 8. Percentage of cis isomers (of the total lycopene) during storage at 35˚C; (——) cis lycopene in MCT, (----) cis lycopene in MCT encapsulated in gum acacia (1.25% MCT dry weight).

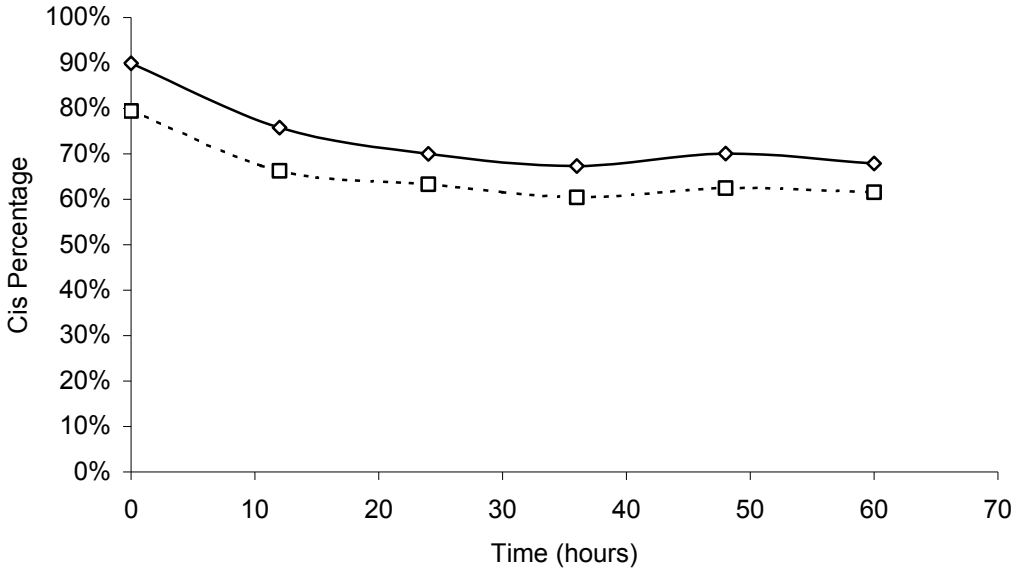

Figure 9. Retention of total lycopene (high in 9-cis) during storage at 35˚C; (——) cis lycopene in MCT, (----) cis lycopene in MCT encapsulated in gum acacia (1.25% MCT dry weight), (–·–·–) pure.

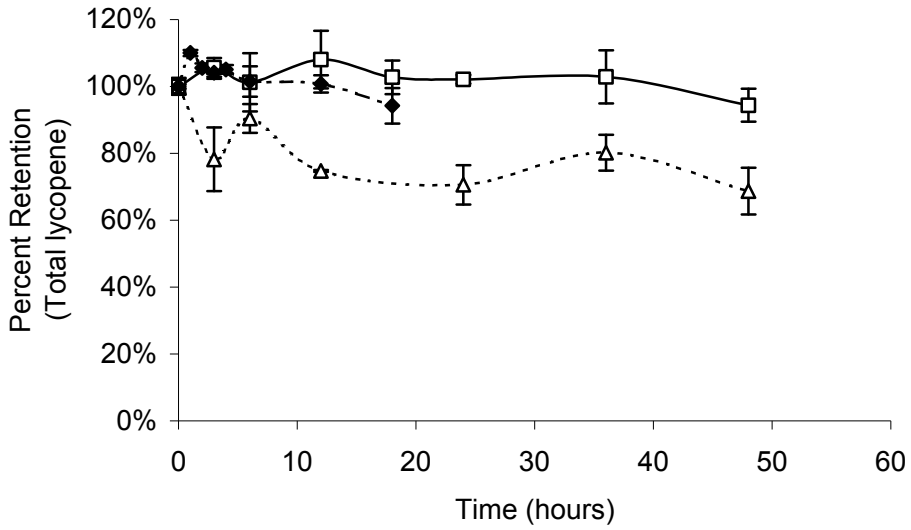

Figure 10. Retention of total lycopene (high in cis isomers) during storage at 40˚C; (——) cis lycopene in MCT, (----) pure cis lycopene encapsulated with maltodextrin and 2.3% Tween 80, (–·–·–) pure cis lycopene.

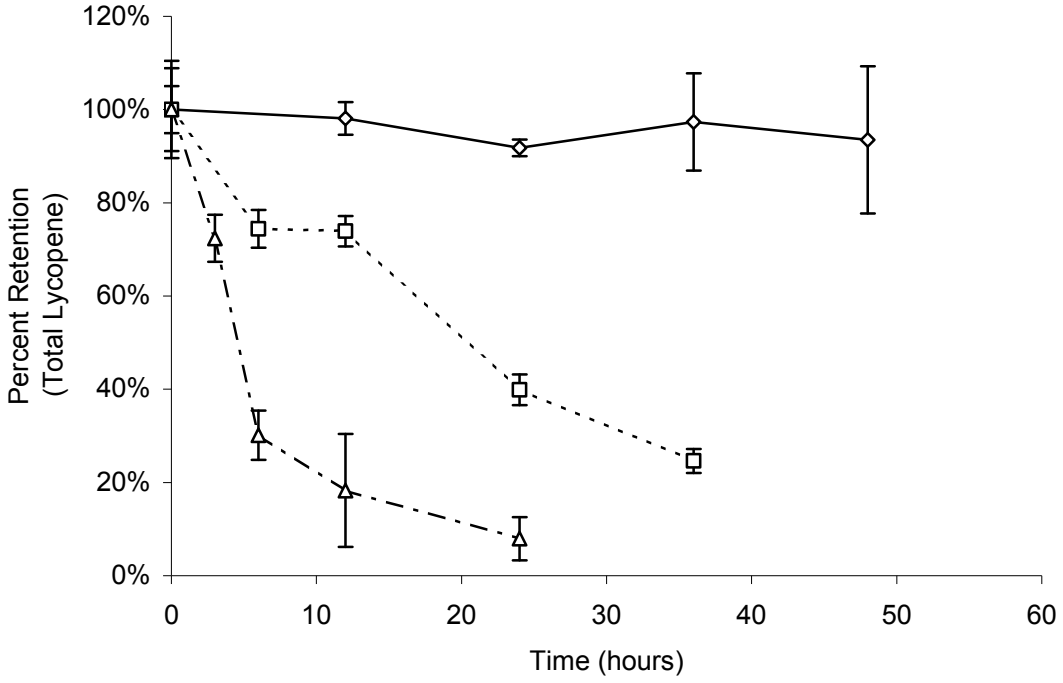

Figure 11. Stability of lycopene in oil during storage at 40˚C including all isomers.

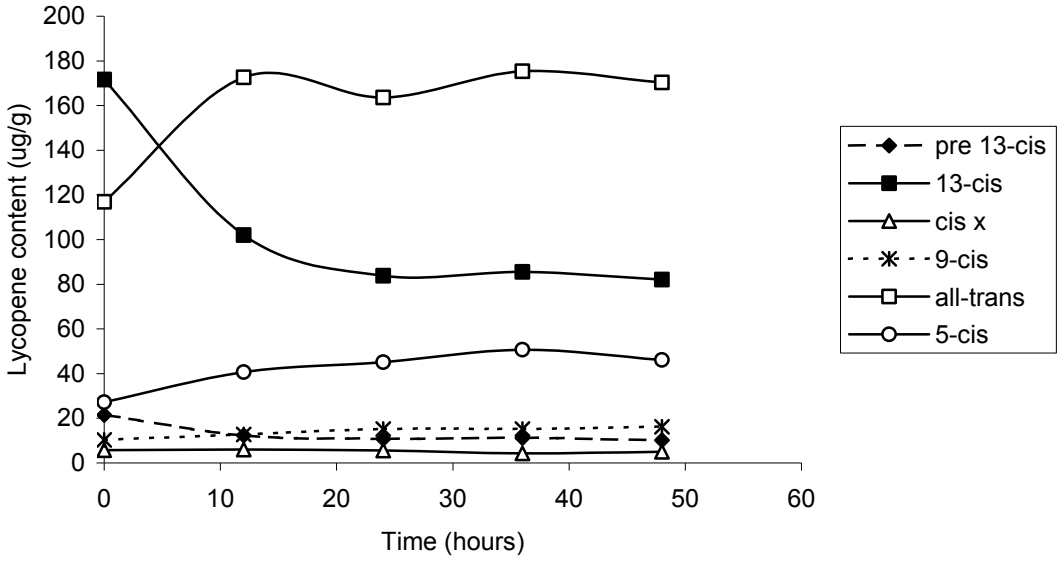

Figure 12. Stability of pure cis lycopene encapsulated with maltodextrin and 2.3% Tween 80 at 40˚C; (----) total of cis isomers, (–·–·–) all-trans isomer, (——) total lycopene.