Extraction of Lycopene from Tomato Paste and its Immobilization for Controlled Release

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Abstract

Lycopene is one of the 600 carotenoids found in nature and can be easily identified in tomatoes. Several epidemiological studies report that lycopene rich diets have beneficial effects on human health, showing strong correlations between the intake of carotenoids and a reduced risk of cancer, coronary and cardiovascular diseases. The purpose of this study was to develop a simple and effective method for solvent extraction of lycopene from tomato paste, and to stabilise and encapsulate the lycopene in the form of sodium-alginate beads.

The Soxhlet method was used for the extraction of lycopene from commercially available tomato paste. Several solvents were tested, and ethyl acetate was found to be the best solvent for extraction, resulting in the separation of more lycopene than other solvents.

Due to the presence of unsaturated bonds in its molecular structure, lycopene is susceptible to oxidation and degrades easily when exposed to light and heat. In the food processing field, microencapsulation techniques have been widely used to protect food ingredients against deterioration, volatile losses, or interaction with other ingredients and factors.

Lycopene was encapsulated in 4% alginate (4g/100 mL), 1% agar-agar and chitosan. The stability of the resulting beads was tested under conditions to simulate those in the human intestine. The lycopene beads showed good survivability when exposed to the acidic conditions such as those in the gastric environment (pH 2.0–3.0). The lycopene release rate was best at higher pH levels (pH 6.6) such as in the intestine, which is where nutrient absorption occurs. The release rate of chitosan-coated alginate-lycopene was found to be much faster at body temperature (37°C) than at 24°C.

The experimental results show that Ca-alginate chitosan coated lycopene beads have a potential application as pH/temperature-controlled drug release carriers in the biomedical field.

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Chapter 1

Introduction

1.1 Lycopene

Tomatoes contain a wide variety of antioxidants including vitamin E, ascorbic acid, carotenoids, flavonoids and phenolic compounds. The scientific name for the tomato is *Lycopersicon esculentum*, so its major antioxidant has been named lycopene. It is also one of the most abundant non-vitamin analogues present in human blood from food consumption (Sathish *et al.*, 2009).

Lycopene is one of about 600 naturally occurring carotenoids and is responsible for the red colour in fruits (Cámara *et al.*, 2013; Roh *et al.*, 2013). Carotenoids are natural antioxidants which protect the cells of the body from the harmful effects of oxidation due to free radicals (Antioxidant, 2011). The lycopene molecule is a 40 carbon acyclic carotenoid, with a molecular mass of 536 Daltons. It consists of only hydrogen and carbon atoms and is one of the carotenoids synthesized by plants and photosynthetic microorganisms (Ishida *et al.*, 2001; Periago *et al.*, 2004). Lycopene has 13 double bonds, of which 11 are conjugated, resulting in excellent antioxidant properties. However, the unsaturated bonds in its molecular structure make lycopene susceptible to oxidants, sensitive to light and heat.

The biochemistry of lycopene is unique as it has no pro-vitamin A activity, in contrast to other carotenoids such as alpha-carotene and beta-carotene. Because of its non-polarity, lycopene is lipophilic, insoluble in water, and can be dissolved only in organic solvents and oils (Shi & Maguer, 2000).

The main source of lycopene is tomatoes because tomato products have high concentrations of lycopene, but it is also present in gac, watermelon, apricots, red grapes, pink grapefruit, pink guava, papaya, peaches, goji berry and cranberries. It is the most abundant carotenoid in tomatoes (0.9 to 4.2 mg per 100 g), followed by beta-carotene, gamma-carotene, phytoene and other minor carotenoids. The human body cannot produce lycopene so it must be obtained from food sources (Shi & Le Maguer, 2000).

Tomato processing waste is an excellent source of lycopene, as most of the lycopene is in the seeds and skin residues. Normally these are discarded as peel or pomace (solid remains after extracting juice), or used for animal feed. As well as being rich in lycopene, tomato seeds can also be used for producing edible oil or as a protein source.

1.2   Lycopene and human health

Free radicals are by-products of the metabolic processes of the body. Free radicals in the body react with other substances and can be potentially harmful if they initiate, promote or progress a chronic disease. The most effective way of combating free radicals is by including more antioxidant-rich foods in the diet. The antioxidants will find and quench (neutralize) these damaging molecules, thus preventing further or on-going harm. (Periago *et al.*, 2004; Cámara *et al.*, 2013; Lycocard, 2013).

Several epidemiological studies report that lycopene-rich diets have beneficial effects on human health (Borguini & Torres, 2009). The antioxidant activity of lycopene can help protect against degenerative diseases by neutralizing free radicals in the body, thus preventing DNA damage in the cells and improving cell function. High levels of lycopene in the blood and fatty tissues are associated with reduced risk of cancer, heart disease and macular degeneration (Wang & Chen, 2006). Lycopene can also improve the skin's ability to protect against harmful UV rays. A study at Manchester and Newcastle universities showed that tomatoes can protect against sunburn and keep the skin looking youthful (Food-info, 2013).

In nature, most lycopene exists in the all-trans form. However, both isomerization and oxidation can occur when processing tomato products. The cis-isomers of lycopene have higher biological activity, probably because cis-isomers are more soluble in bile acid micelles. Several papers have demonstrated that the cis-isomers of lycopene could be adsorbed into the body more easily and played a more important role in biological function than all trans-lycopene. Lycopene in human plasma is an isomeric mixture where 50% of the total lycopene is the cis-isomers (Wang & Chen, 2006; Sathish *et al.*, 2009; Food-info, 2013).

1.3 Extraction methods

Several different methods have been used to extract lycopene such as supercritical fluid extraction (SCFE) with CO_2 and solvent extraction. Solvents for extracting carotenoids, include ethyl acetate (100%) or different mixtures of solvents such as ethanol/hexane (1:1), acetone/ethanol/hexane (1:1:2), ethyl acetate/hexane (1:1) or acetone/hexane (1:1) (Barba *et al.*, 2006b).

The solvents of choice, ethyl acetate and ethyl lactate, are non-toxic, approved as additives by the U.S. Food and Drug Administration, biodegradable and have a relatively high flash point (Strati & Oreopoulou, 2011). Recent studies describe a lycopene extraction process based on supercritical CO_2, which avoids using harmful solvents. Over 60% of the lycopene in tomato waste was extracted with this process (Periago *et al.*, 2004).

Methods for the analysis and identification of carotenoids include UV-spectrophotometry, colorimetric, thin layer chromatography (TLC) and HPLC (high-pressure liquid chromatography). The HPLC analysis of carotenoids is usually done with C18 or C30 reverse phase columns. A wide variety of organic solvents can be used as the mobile phase, and the column can be operated under isocratic or gradient elution conditions. The various compounds eluted can be detected using UV–vis (wavelength 480 nm) or a photodiode array (Barba *et al.*, 2006b; Roh *et al.*, 2013).

1.4 Microencapsulation

Microencapsulation techniques have been widely used to protect many labile compounds including food ingredients against deterioration, volatile losses, or interaction with other ingredients (Goula & Adamopoulos, 2012). In the food industry, encapsulation has been applied for a variety of reasons. It is a useful tool to improve delivery of bioactive molecules (e.g. antioxidants, minerals, vitamins, lutein, fatty acids, lycopene) and living cells (e.g. probiotics) into foods.

In most cases, encapsulation refers to a technology where the bioactive components are completely enveloped, covered and protected by a physical barrier, without any protrusion of the bioactive components. It is also the

technology used for packaging solid or liquid materials in small capsules that release their contents at controlled rates over prolonged periods and under specific conditions. Particles released from these beads usually have diameters ranging from a nanometre to a few millimetres.

A gel bead system based on natural polysaccharides, which has been widely accepted as a safe and inert oral drug dosage form, is an effective matrix. Such a polysaccharide gel bead system would both reduce the limitations associated with using lycopene such as its instability and also allow the entrapped lycopene to be released into the digestive tract in a controlled manner so lycopene can be more efficiently absorbed by the human body (Kim *et al.*, 2008).

Alginates, which are derived from seaweeds, have been extensively used for micro-encapsulation because they are easy to prepare on a small-scale. The encapsulation process using these materials is simple, mild and non-toxic. Because alginate is biocompatible and biodegradable under normal physiological conditions is pH-sensitive and relatively inexpensive, it is often selected as a matrix for entrapping and delivering lycopene, drugs, and cells (Gouin, 2004; Deng *et al.*, 2010).

1.5 Aim of this research

The aim of this research is to investigate the extraction, identification, purification, stability and applications of lycopene and its functional components from tomatoes and tomato paste. The main focus will be on solvent extraction, followed by methods of identification and purification. The analysis of pure lycopene will be achieved by chromatographic methods such as HPLC, TLC silica plates and column chromatography. The effects of temperature, pH and time on the release of encapsulated lycopene will be investigated.

Sodium alginate, agar agar and calcium chloride will be used to produce lycopene-immobilized alginate beads. A chitosan solution will then be added to coat the lycopene-alginate beads, reducing lycopene release and increasing bead stability. Stabilization of all-trans-lycopene from tomato by encapsulating it using α-, β-, and γ-cyclodextrins (CDs) has been evaluated (Blanch *et al.*, 2007).

Encapsulating lycopene extracted from tomato pulp waste has also been achieved using gelatin and γ-poly-glutamic acid (γ-PGA, a biodegradable and nontoxic polymer) as the carrier (Chiu *et al.*, 2007).

The selected encapsulation process should not require complicated equipment and able to done at ambient temperatures and pressures. Moreover, it should not use toxic materials or produce any harmful by-products. The easiest way to encapsulate lycopene within an alginate matrix is by using the dripping-gelation method. Alginate-agar liquid droplets containing lycopene are extruded from an orifice and allowed to fall into a gelling bath. The surface of the droplets will solidify almost instantly when they contact the gelling bath e.g. calcium chloride ($CaCl_2$).

In this research, a novel pH temperature sensitive hydrogel bead composed of calcium alginate (Ca-alginate) and poly-(D) glucosamine (chitosan) will be prepared for use as the delivery system. The release of lycopene at different pH values (using phosphate buffer solution) and temperatures will be determined.

1.6   Thesis structure

The first chapter introduce general information and background about lycopene. The second chapter is a literature review with details about sources of lycopene, structure and characterization, role of lycopene in the human body, health aspects, bioavailability and functional uses. This chapter also describes the different methods used for extracting and immobilizing lycopene for controlled release.

The third chapter describes the materials and methods used in this research, including extraction and analysis methods and the methods for immobilization and controlled release of lycopene using chitosan-coated alginate beads. In the fourth chapter the results of the research are presented and discussed. The fifth chapter presents the conclusions and outlines recommendations for future work.

Chapter 2

Literature Review

Lycopene is an important biological compound and has received great interest in the past decade because of its important role in preventing chronic diseases such as atherosclerosis, skin cancer and prostate cancer (Helmenstine, 2014). It is an antioxidant that displays higher efficiency than vitamin E and other kinds of carotenoids. A well-balanced diet usually contains an adequate lycopene intake. The recommended daily intake of lycopene is 70 to 75 milligrams, and consuming some lycopene every day is beneficial to health (Basu & Imrhan, 2007).

Lycopene is a bright red carotenoid pigment, and gives the red coloration to fruits such as tomatoes, red carrots, red bell peppers, watermelons, papayas and gac fruit. It is naturally synthesized by plants. It is one of the pigments widely used by the food industry as a food additive due to its strong colour and non-toxicity. Lycopene is registered as E160d (Perkins-Veazie *et al.*, 2001) and approved for food use. It has many health benefits and is in increasing demand as a red colorant and antioxidant agent. The importance of natural food additives has gained more attention due to the increased use of natural rather than synthetic compounds in food, cosmetics and pharmaceuticals (Ishida *et al.*, 2001; Kong *et al.*, 2010).

This chapter summarizes the literature on the sources, properties and uses of lycopene in the food industry. It then goes on to examine the methods used to extract lycopene from natural sources, and finishes with immobilization and encapsulation techniques.

2.1 Sources of lycopene

Lycopene can be obtained from natural sources or synthesized chemically. It can be obtained in supplement form, usually as tablets or capsules or in soft gel. Sometimes other nutrients are added to the formulation for additional antioxidant benefits.

2.1.1 Chemical synthesis

Lycopene can be extracted from natural raw materials or chemically synthesized by a series of reactions using synthetic reagents and chemical solvents (Figure 2-1). The final product may often contain traces of chemical solvents, impurities and reaction by-products, which could be toxic and therefore these products cannot be included in food. This industrial process of synthetic lycopene has a deleterious environmental impact due to the large amount of chemical solvents used (EFSA, 2006; Lycopene, 2013).

LYCOPENE CAS : 502-65-8

SYNTHETIC — **NATURAL**

Made by chemical reactions — Made by biosynthesis in vegetables

Synthetic raw materials — Natural raw materials (Tomatoes)

Free of Restrictions (GE[1] varieties, pesticides) — Organic Certified (No GE[1] var. ; No pestic.)

Chemical Solvents — Extraction by Chemical solvents — Extraction by Supercritical CO_2

SYNTHETIC LYCOPENE (With chemical solvents) — **NATURAL LYCOPENE** (With chemical solvents) — **ORGANIC LYCOPENE** (Free of chemical solvents)

Figure 2-1: Flow diagram for synthesising of lycopene (U.S. Food & Drug Administration, 2003).

Synthetic lycopene is highly concentrated with a purity of 90-95%. These concentrations are used for soaps, creams and cosmetics but not for human consumption. It has a low bioavailability and is very labile to oxygen (from the atmosphere) and light (Lycopene, 2013).

2.1.2 Biological sources

Lycopene occurs naturally in plant (vegetable) sources such as tomatoes, and in microbial sources and in cells that have been genetically engineered to accumulate lycopene.

2.1.2.1 Microbial sources

Microbial sources of lycopene offer an alternative natural product considering that until very recently, the only known source of lycopene was tomatoes.

Genetic engineering has made it possible to enhance lycopene content in bacteria, fungi and mammalian cells. Several patents disclose DNA modification and methods for producing carotenoids in biological sources (Olempska-Beer, 2006). It is desirable to obtain edible (suitable for consumption) lycopene from these biological sources while eliminating the need to ingest the biological source.

Blakeslea trispora, a fungal plant pathogen, produces lycopene and is also the microbe used for producing commercial β-carotene for dietary supplements and food additives (*Wikipedia: Blakeslea trispora*, 2013). Lycopene from *B. trispora* contains at least 90% all-trans-lycopene and minor quantities of 13-cis-lycopene and β- and γ-carotene (Olempska-Beer, 2006). Lycopene-producing *B. trispora* cells are produced by mating and co-fermentation of two non-pathogenic and non-toxigenic (plus and minus) strains. Lycopene is solvent extracted with isopropanol and isobutyl acetate from the biomass and purified by filtration and crystallization. Pure lycopene crystals are unstable when exposed to oxygen and light so the lycopene is stored under nitrogen or other inert gases in light-proof containers.

The high concentration of lycopene in certain microorganisms indicates that microbial sources are attractive alternatives for the industry (Table 2-1). The *Blakeslea* group, particularly *B. trispora*, is of primary industrial interest as a source of β-carotene. This organism is preferred in carotenoid production processes because of the increased yield in cells due to the high amount of biomass and because intracellular neutral lipids in the form of tri-acylglycerols increase lycopene solubility (Mantzouridou & Tsimidou, 2008).

Table 2-1: Maximum lycopene production form various microorganisms (Mantzouridou & Tsimidou, 2008).

Microorganism	Lycopene (µg/g biomass dry wt)	Reference
Blakeslea trispora	15,000	Swarthout, 1963; Ninet *et al.*, 1968
Escherichia coli	500	Sandmann *et. al.*, 1999
Flavobacterium R1519	1000	McDermott *et al,* 1974; Britton *et al.* 1977
Mycobacterium aurum	7000	Kerr *et al.*, 2004
Mycobacterium marinum	80	Howes & Batra, 1970
Rhodotorula spp.	90	Squina & Mercadante, 2005
Streptomyces chrestomyceticus	580	Bianchi *et al.*, 1969

Commercial lycopene preparations for food usage are formulated as suspensions in edible oils or as water-dispersible powders and are stabilized with antioxidants. The concentration of lycopene from *B. trispora* when used in certain foods and beverages as a colorant ranges from 10 mg/kg to 50 mg/kg (Olempska-Beer, 2006).

Production of lycopene in the fungal cells is due either to chemical inhibition of cyclization reactions leading to β-carotene production or to genetic regulation. Special compounds commonly referred to as "bio-regulators", "stimulants", "inducers", or "inhibitors" are added to regulate lycopene formation. Low concentrations of these chemicals enhance lycopene yield at the expense of β-carotene (Mantzouridou & Tsimidou, 2008).

There are published reports on microbial production strategies using *Escherichia coli* as a host strain and some studies using yeasts. Early studies (Sandmann *et al.*, 1999) with *E. coli* as a host strain report lycopene levels of up to about 0.5 mg/g dry weight of cells. The yield from *Candida utilis*, after extensive re-engineering of the ergosterol pathway, was up to 7.8 mg (dry wt.) of lycopene/g (dry wt.) (Shimada *et al.*, 1998).

More recently, a combination of rational systems, biological design and random screening approaches gave lycopene levels of up to 18 mg/g (dry wt.) in *E. coli* cultures (Wang *et al.*, 2012).

2.1.2.2 Plant sources

Tomatoes have one of the highest known concentrations of natural lycopene and most people get much of the lycopene in their diet from tomato products. Other plants known to contain high concentrations of lycopene can also be utilized. They include fruits such as Ruby Red or Red-blush grapefruit, watermelon, and persimmons (Ausich & Sanders, 1999).

Research shows that lycopene is absorbed more efficiently by the body after it has been processed into juice, sauce, paste, or ketchup (Helmenstine, 2014). Processed tomato products have higher levels of lycopene than raw tomatoes, where lycopene is enclosed in the cells and tissues of the fruit. One ripe tomato contains 3.7 milligrams of lycopene whereas one cup of tomato soup contains almost 25 milligrams of lycopene. The higher content is because the cooking process breaks down the cell walls of the tomato, which makes the lycopene more available due to the increase in surface area of molecules. This also makes it more easily absorbed by the body.

Because lycopene is fat-soluble (as are vitamins, A, D, E, and β-carotene), absorption into tissues is improved when oil is added to the diet (Helmenstine, 2014). Therefore, eating tomato products with oil (e.g. adding olive oil to tomato salad) dramatically improves absorption and utilization of lycopene by the body (Ausich & Sanders, 1999).

The lycopene content in tomatoes typically ranges from 70 to 130 mg/kg and depends on the variety, geographic location, cultivation technique, climatic conditions and ripeness of the tomato. The lycopene content increases as the fruit ripens. Tomato sauce and ketchup contain lycopene at concentrations of 33 to 68 mg per 100 g, while raw tomatoes contain lycopene at concentrations of 3.1 mg per 100 g (Rath, 2009).

Table 2-2 shows the amount of lycopene in different tomato products (Rao & Agarwal, 1999). The enhancement during processing can be shown by lycopene

content in fresh tomatoes being less than 50 µg/g dry matter whereas tomato powder contains over 1000 µg/g dry matter.

Table 2-2: Lycopene content of common tomato-based foods (Rao & Agarwal, 1999).

Tomato products	Lycopene (µg/g dry matter)
Fresh tomatoes	8.8- 42.0
Cooked tomatoes	37.0
Tomato sauce	62.0
Tomato paste	54 – 1500
Tomato soup (condensed)	80
Tomato powder	1126.3-1264.9
Tomato juice	50 -116
Pizza sauce	127.1
Ketchup.	99 -134.4

Lycopene extracted from tomato is a dark-red viscous liquid, which is freely soluble in ethyl acetate and n-hexane, partially soluble in ethanol and acetone, and insoluble in water. A solution of lycopene in n-hexane has a maximum absorption at approximately 472 nm (Rath, 2009). The all-trans configuration is the predominant lycopene isomer in raw tomatoes but it changes to the cis isomer during cooking, food processing and storage.

2.1.3   Tomatoes as a source of lycopene

The tomato fruit consists of skin, pericarp, and locular contents (Figure 2-2). The epidermal layer has a heavily cutinised outer surface and the skin consists of four or five layers of cells under a thin cuticle. The locular cavities are filled with jelly-like parenchyma cells that surround the seeds (Science, n.d.). The chemical composition of the tomato fruit depends on its genetics, ripeness and the conditions under which it was cultivated.
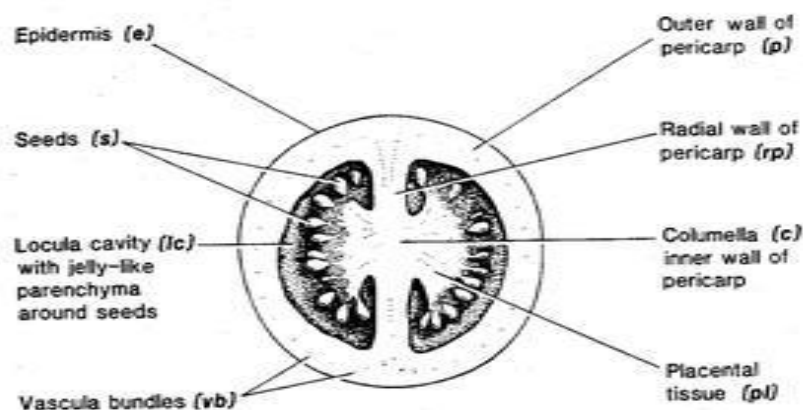
Figure 2-2: Main anatomical features of mature tomato (Science, n.d.).

Tomatoes and tomato products are rich in antioxidant compounds and are considered an important source of carotenoids, in particular lycopene, ascorbic acid and phenolic compounds (Table 2-3) (Borguini & Torres, 2009).

Table 2-3: Tomato composition given in USDA* Food Composition Data, value per 100 g (Borguini & Torres, 2009).

Nutrient	Value	Nutrient	Value
Water (%)	95	Potassium (mg)	237
Protein (g)	0.88	Sodium (mg)	51
Total lipid (fat)(g)	0.20	Zn, Cu, Mg (mg)	0.10 - 100
Carbohydrate (g)	3.92	Vitamin C, total ascorbic acid (mg)	12.7
Fibre total dietary (g)	1.20	Thiamine (mg)	0.037
Calcium (mg)	10	Niacin (mg)	0.594
Iron (mg)	0.27	β-carotene (μg)	449
Magnesium (mg)	11	α-carotene (μg)	101
Phosphorus (mg)	24	Lycopene (μg)	2573

*USDA: United States Department of Agriculture

Because tomato skins were found to give the highest yield of lycopene, it has been reported that tomato processing residues, including skins, are ideal for extracting large amounts of lycopene. Tomato peels, usually eliminated during tomato processing, are a valuable source of carotenoids. Tomato paste enriched in tomato peels (ETP) contained 48 mg lycopene (58% more than classically made tomato paste CTP) and 1.75 mg β-carotene (99% more than CTP) per 100 g of paste.

Enrichment of tomato paste with tomato peel is an interesting option for increasing lycopene and β-carotene intakes (Reboul *et al.*, 2005).

Discarded tomato skins from the production of tomato juice have been found to be the best source for lycopene extraction. The lycopene concentration in the non-blanched tomato peels was 62.92 mg/100 g, whereas it was 134 mg/100 g in the blanched tomato peels (Figure 2-3) (Urbonaviciene *et al.*, 2012).
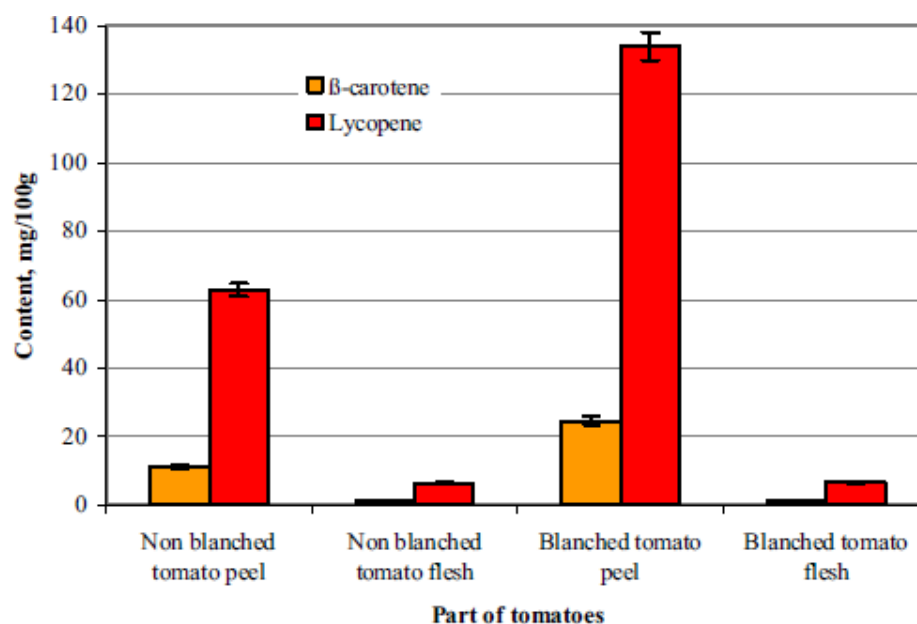
Figure 2-3: Content of β-carotene and lycopene in the blanched and non- blanched peels and flesh (Urbonaviciene *et al.*, 2012).

Lycopene extract from tomatoes is used as a food/dietary supplement in products where the presence of lycopene provides specific properties (e.g. antioxidant or other claimed health benefits). Consumption of tomatoes is reported to have enormous health benefits including reducing cholesterol, improving vision, maintaining the gut, lowering hypertension, alleviating diabetes and preventing urinary tract infections and gallstones (Abano *et al.*, 2012). Regular consumption of tomatoes and tomato products has been correlated with reduced susceptibility to various types of cancers and cardiovascular diseases. This positive effect is attributed to antioxidants, in particular, carotenoids (lycopene and β-carotene) and phenolic compounds (Borguini & Torres, 2009).

2.2 Physical and chemical properties of lycopene

The physical properties of lycopene are summarised in Table 2-4

Table 2-4: Properties of lycopene (Shi & Maguer, 2000).

Molecular formula	$C_{40}H_{56}$
Molecular weight	536.89 Da
Melting point	172–175˚C
Crystal form	Long red needles from a mixture of carbon disulphide and ethanol
Powder form	Dark reddish-brown
Solubility	Soluble in chloroform, hexane, benzene, carbon disulphide, acetone, petroleum ether Insoluble in water, ethanol, methanol
Sensitivity	Light, oxygen, high temperature, acids

Chemistry of lycopene

Lycopene is an unsaturated acyclic hydrocarbon (Figure 2-4) with the chemical formula $C_{40}H_{56}$. Common names include Ψ,Ψ-carotene, all-trans-carotene, and (all-E)-lycopene. It has 13 double bonds, of which 11 are conjugated (EFSA, 2006). The chemical name for lycopene is 2,6,10,14,19,23,27,31-octamethyl-2,6,8,10, 12,14,16,18,20,22,24,26,30- dotriacontatridecaene (Olempska-Beer, 2006).
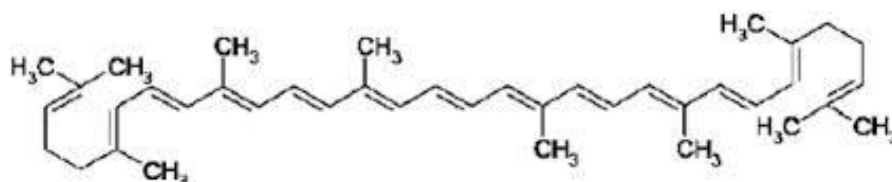
Figure 2-4: Molecular structure of lycopene (Organic Herb Inc, 2012).

Lycopene occurs in the all-trans and various cis configurations. In some literature sources, all-trans-lycopene is referred to as all-E-lycopene and cis isomers are referred to as Z-isomers. Lycopene found in human plasma is a mixture of approximately 50% Z-lycopene and 50% all-E-lycopene (Olempska-Beer, 2006). Naturally- occurring lycopene consists predominantly of all-trans-lycopene. The cis form of lycopene is more bioavailable and is less likely to precipitate and form crystals, which affect solubility (Kun *et al.*, 2006). For example, lycopene present in red tomato fruits typically contains 94-96 % of all-trans-lycopene (Schierle *et al.*, 1997).

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2.3 Measuring lycopene

Various analytical methods have been developed to measure and analysis of lycopene content in food and biological samples. These include ultraviolet-visible (UV-VIS) spectrophotometry (Davis et al. 2003), liquid chromatography (LC), thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Xu et al. 2006; Ishida and Chapman 2004; Lee and Chen 2002).

Many other methods high-speed counter current chromatography (Baldermann *et al.*, 2008), fibre optic visible reflectance spectroscopy (Choudhary *et al.*, 2009) and infrared spectroscopy (De Nardo *et al.* 2009) are also developed. However, they consume too much time (usually >20 min) and solvent in order to achieve separation of the all-E-lycopene with its Z isomers.

UV-VIS spectrophotometry is more convenient, faster and less expensive than HPLC analysis and large numbers of samples can be processed in a relatively short time. However, UV-VIS spectrophotometry cannot detect very small quantities of lycopene (less than 1 µg) whereas HPLC can detect quantities as small as 1 ng   (Hyman *et al.*, 2004). Although HPLC analysis allows accurate quantification of pigments and separation of isomers, it is laborious and a high level of skill is required to produce consistent results (Hyman *et al.*, 2004).

2.4 Biological activity of lycopene

The potential health benefits of lycopene in the diet (Figure 2-5) on various conditions are due to lycopene having the following attributes:

- antioxidant function
- enhancement of cellular gap junction communication
- induction of phase II enzymes by activation of the antioxidant response element (ARE) transcription system
- suppression of insulin-like growth factor-1-stimulated cell proliferation by induced insulin-like growth factor binding proteins
- anti-angiogenesis and inhibition of cell proliferation associated with malignant tumors
- induction of apoptosis (Mein *et al.*, 2008).
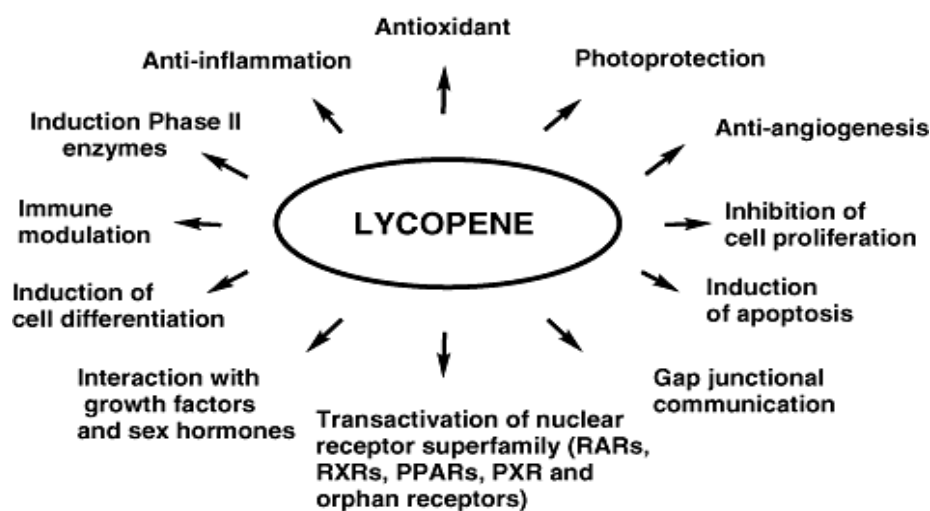
Figure 2-5: Biological functions of lycopene (Mein *et al.*, 2008).

2.5 Role of lycopene in the human body

Lycopene accounts for 50% of the carotenoids found in human serum, with particularly high concentrations in the prostate gland, adrenal glands, skin, liver and kidneys. Absorption of lycopene from processed tomato products such as tomato paste is higher than from raw tomatoes. Because lycopene is a hydrocarbon, it can be directly absorbed in the gut. Absorption is enhanced in the presence of oils and surfactant, which disperse the lycopene. Unlike other carotenoids, lycopene cannot be converted into vitamin A (Borguini & Torres, 2009).

Lycopene consumption has been associated with many health benefits (Figure 2-6), including decreased risk of cancer and neurodegenerative diseases. It is also beneficial in reducing urinary tract symptoms (BPH benign prostatic hyperplasia, enlargement of the prostate) and cardiovascular risk associated with type 2 diabetes (Ranveer *et al.*, 2013). An increased lycopene status in the body may regulate gene function, improve inter-cell communication, modulate hormone and immune response and regulate metabolism, thus lowering the risk of chronic disease (Agarwal & Rao, 2000).
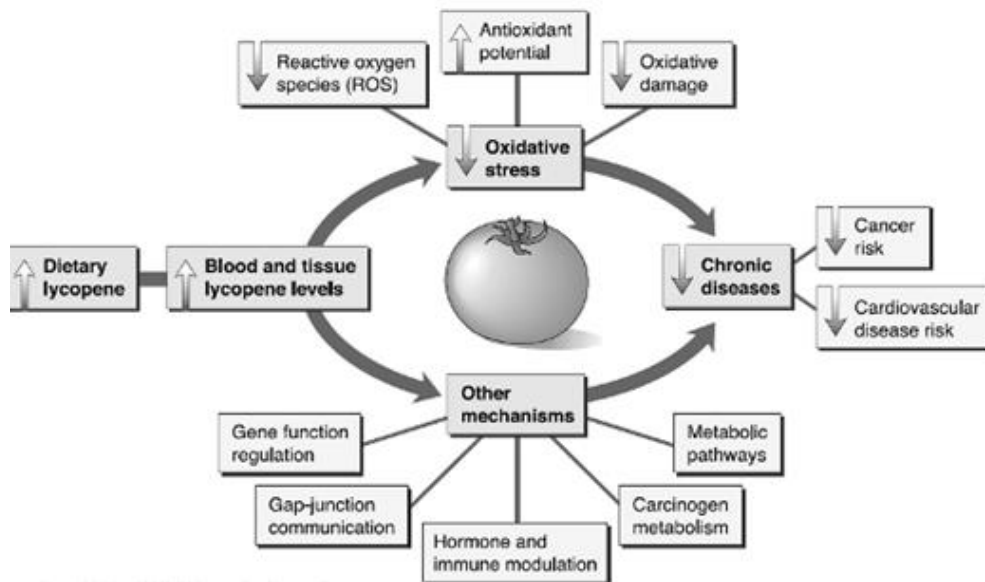
Figure 2-6: Role of lycopene in the body (Agarwal & Rao, 2000).

The major health benefits of lycopene include:

- **Antioxidant activity**

  Lycopene is one of the most potent antioxidants. Its singlet-oxygen-quenching ability is twice that of β-carotene and ten times higher than that of α-tocopherol (Agarwal & Rao, 2000). As an antioxidant, it traps reactive oxygen species, increasing the overall antioxidant potential and reducing the oxidative damage to lipids (lipoproteins, membrane lipids), proteins (important enzymes) and DNA (genetic material), thereby lowering oxidative stress. This reduced oxidative stress leads to reduced risk of cancer and cardiovascular heart disease. As lycopene levels in the blood increase, the levels of oxidized lipoproteins, proteins and DNA compounds decrease. A recently study showed lycopene antioxidant activity improved sperm quality (Rao, 2013).

- **Reducing prostate cancer**

  Studies show that taking high doses of lycopene can slow the progression of prostate cancer. The estimated intake of lycopene from various tomato products was inversely proportional to the risk of prostate cancer, a result not observed for any other carotenoid. Consuming ten or more servings of tomato products per week reduced the risk by almost 35%. The protective effects were highest for more advanced or aggressive prostate cancer (Agarwal & Rao, 2000).

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- **Inhibiting cancer cells**

  Lycopene has a protective effect against stomach, colon, lung and skin cancers. Free radicals in the body can damage DNA and proteins in the cells and tissues, resulting in inflammation which may lead to cancer. Hence, the antioxidant properties of lycopene in eliminating free radicals may reduce the risk of cancer (Hussain *et al.*, 2003). Research in breast, lung and endometrial cancers has shown that lycopene is even more effective than the other bright vegetable carotenoids α- and β-carotene in delaying the cell cycle progression from one growth phase to the next, thus inhibiting growth of tumour cells. Lycopene also plays a role in modulating intercellular communication by regulating irregular pathways that may be associated with cancer (Singh & Goyal, 2008).

  Multiple studies have investigated whether intake of tomatoes or tomato-based products helps prevent digestive tract cancers, including oral, pharyngeal, oesophageal, gastric, colon, and rectal cancer. People with a higher intake of lycopene have been shown to have a reduced risk of developing cervical and breast cancer (Lycopene, 2012).

- **Reducing blindness**

  Age-related macular degeneration (ARMD) is the most common form of blindness in elderly people in the Western world. Lycopene is the only micro-nutrient whose serum level is shown to be inversely related to the risk of ARMD. Lycopene also helps reduce the incidence of cancers and cardiovascular diseases which play a role in eye health (Lycopene, 2012).

- **Reducing atherosclerosis and heart disease**

  Lycopene may be helpful in people with high cholesterol, atherosclerosis or coronary heart disease, possibly due to its antioxidant properties. Lycopene prevents oxidation of low density lipoprotein (LDL) cholesterol and reduces the risk of arteries becoming thickened and blocked. Most published studies in this area used tomato juice as a treatment. Drinking two to three 8-oz (200 mL) glasses of a processed tomato juice would provide more than the 40 mg lycopene per day recommended for reducing LDL cholesterol (Lycopene, 2012).

- **Reducing osteoporosis**

  Epidemiological data indicates, lycopene prevents osteoporosis in post-menopausal women. This is a new and exciting finding and could stimulate serious dietary considerations for all people seeking to protect against this disease (Rao, 2013).

- **Preventing skin damage**

  Lycopene can reduce inflammation and help to protect the skin from damage resulting from UV sun exposure. It is a common ingredient in anti-aging creams and lotions but because it degrades easily, containers must be properly sealed between uses (Rao, 2013).

2.6 Functional uses of lycopene in food

Because of lycopene benefits, there is a growing interest in using lycopene as a value-added or functional ingredient in food products. Lycopene extract from tomatoes can be used as a nutritional supplement in several food categories such as baked goods, breakfast cereals, dairy products including frozen dairy desserts, dairy product analogues, spreads, bottled water, carbonated beverages, fruit and vegetable juices, soybean beverages, candy, soups, salad dressings, and other foods and beverages. Lycopene is a natural food colouring, thus eliminating the adverse effects of artificial food colorants. It provides colour shades ranging from yellow to red (Choksi & Joshi, 2007).

The levels of tomato extract expressed as lycopene levels, added to food depend on the intended function and may vary from 2 mg/L in bottled water to 130 mg/kg in ready to-eat cereals. Food and beverage products are formulated to provide about 2 mg lycopene per serving (Rath, 2009).

There are initiatives by food scientists to recycle lycopene-rich by-products as food ingredients. Fortifying dry fermented sausage with lycopene can be achieved by adding dried tomato peel to the meat mixture during sausage production (Altan *et al.*, 2008). Extrusion processing allows barley-tomato pomace blends to be formulated into snacks (Altan *et al.*, 2008).

Research shows that heat processing converts lycopene in tomatoes to a form the body can absorb more easily. One study showed that lycopene is absorbed 2.5 times better from tomato paste than from fresh tomatoes (Borguini & Torres, 2009).

The main concerns in using lycopene extracted from tomatoes and tomato products are solubility and stability. Lycopene is insoluble in water but soluble in toxic organic solvents such as benzene, chloroform and methylene chloride. It decomposes easily with time. Carotenoids are susceptible to degradation due to high temperatures, oxidation and UV light, which further limit their application in the food industry (Varona *et al.*, 2013).

Lycopene stability depends on the particular food to has been added to, on the production process and the storage conditions (Abano *et al.*, 2012). It is also very unstable in crystal form regardless of concentration so storing the crystalline form is not recommended (Kun *et al.*, 2006).

When used as a food colorant, lycopene is stable in food stored at appropriate conditions such as storage at 4°C. (Abano *et al.*, 2012) reported that lycopene extract is stable for up to 37 months.

2.7   Lycopene bioavailability

Carotenoids are strongly bound to intracellular macromolecules in many foods and absorption may therefore be limited unless carotenoids are released from the food matrix. Processing tomatoes increases the lycopene content because the heat used in concentrating the juice or pulp makes lycopene more bioavailable.

Food processing breaks down cell walls and releases the lycopene from the fruit matrix (Su *et al.*, 2002). Cooking and crushing tomatoes (as in the canning process) and serving in oil-rich dishes (such as pasta sauce or pizza) greatly increases assimilation from the digestive tract into the bloodstream. Lycopene in tomato paste is four times more bioavailable than in fresh tomatoes (Lycocard, 2013). The physical disruption of cell structure in processed tomato products (tomato juice) and hence increased lycopene bioavailability compared with fresh tomatoes is shown in Figure 2-7 (Lycocard, 2013).
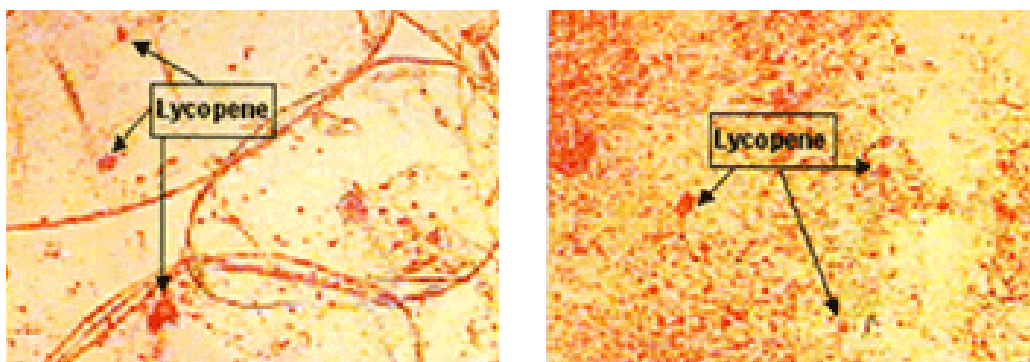
Figure 2-7: The physical disruption of the cell structure in processed tomato products (tomato juice, above right) compared to fresh tomatoes (left) (Lycocard, 2013).

Chopping and pureeing, which reduces the physical size of the food particles, also enhances lycopene bioavailability (Hadley *et al.*, 2002). Mechanical treatment with heat helps release lycopene from the tomato matrix due to thermal weakening and disruption of lycopene–protein complexes, rupturing of cell walls, and dispersion of crystalline carotenoid aggregates (Kun *et al.*, 2006).

2.8 Extracting lycopene

There is an increasing trend using food processing by-products as a source of functional food components. The literature generally agrees that lycopene is stable in tomato matrices during mild thermal treatments but during intense processing conditions or when lycopene is dissolved in oil or organic solvents, degradation and isomerization might occur rapidly (Colle *et al.*, 2010).

There are several different types of methods for extracting lycopene.

- **Solvent extraction**

Conventional spectrophotometric and HPLC assays for quantifing lycopene from plant tissue use organic solvents to extract the compound. Although the method is reliable, it is laborious, cumbersome and requires the use and disposal of organic solvents (Colle *et al.*, 2010). If the organic solvent used to extract the lycopene is toxic, then residual traces will make the extract unsuitable for human consumption (Zuknik, *et al.,* 2012).

Extraction with organic solvents is a well-established method in the food industry. As most tomato carotenoids are lipid- (fat) soluble, common organic solvents such

as dichloromethane, hexane, ethanol, acetone, ethyl acetate, petroleum ether, and mixtures of polar or nonpolar solvents in different ratios such as acetone-chloroform (1:2) and hexane-acetone-ethanol (2:1:1) have been tested for carotenoid extraction (Barba *et al.*, 2006a). The amount of lycopene extracted using hexane/acetone or hexane/ethanol is higher than when using methanol, dichloromethane or chloroform (Barba *et al.*, 2006a).

Food and Drug Administration have classified solvents into three categories:
- Class 1 solvents are highly toxic and harmful to the environment, and should not be used in the food industry. Examples include benzene and carbon tetra-chloride.
- Class 2 solvents are mildly toxic, and although they can be used under specific conditions in the pharmaceutical and food industries, there are strict limitations. Examples include hexane, methanol and chloroform.
- Class 3 solvents are acceptable in small residual percentages, and are used in the food and pharmaceutical industries. Examples include acetone, ethanol, ethyl acetate, propanol and propyl acetate (Ishida & Chapman, 2009).

- **Hydrostatic pressure processing**

High hydrostatic pressure processing (HPP) without heating can increase the yield of lycopene from tomato paste waste. Xi (2006) reported that the lycopene yield from high pressure treatment of tomato paste waste was much higher than from solvent extraction, with more lycopene extracted in 1 min using HPP than was extracted in 30 min using solvent extraction.

Kong *et al.* (2010) produced high purity lycopene from tomato by-products through pressurized extraction in equipment called "Extractor Naviglio". This extraction method use tap water with minimal organic solvent. The by-products can be used as livestock feed (Kong *et al.*, 2010).

- **Enzymatic treatment**

Using cellulase and pectinase enzymes increase yields of lycopene from tomato waste substantially. Using the optimal enzyme concentration and process time increased lycopene yield to twenty times then that achieved by using other methods (Kong *et al.*, 2010).

- **Supercritical fluid extraction (SCFE) with CO_2**

The literature suggests that supercritical fluid extraction (SCFE) of lycopene with carbon dioxide (CO_2) gives the best results (Choksi & Joshi, 2007). Many researchers have investigated SCFE for extracting lycopene from various by-products. Liquid CO_2 is used as the solvent, and can be pressurized or heated to the desired processing conditions. The matrix is packed into the extractor cell. After extraction, the extract is precipitated through a control valve (Tzia & Liadakis, 2003).

Supercritical CO_2 is an ideal solvent for extracting food components because it is non-toxic, non-flammable, safe, and inexpensive. Because lycopene is generally sensitive to light, heat, oxygen and acids, lycopene extracted from tomatoes by SCFE with CO_2 isomerizes and decomposes less than other methods. However, CO_2 lacks polarity and the ability to form specific solvent-solute interactions.

Adding a small amount of polar solvent such as water, ethanol, methylene chloride or hexane can greatly enhance the solvent power of CO_2. Solubility studies done over 50–80 °C and a 200–400 bars showed that these are suitable operating conditions for carotenoid extraction (Shi *et al.*, 2009).

The solvent extraction procedure has been used to extract lycopene from tomato skins. Maximum lycopene yield from tomato skin was achieved by using response surface methodology (Vagi *et al.*, 2007). The extraction yields and the amounts of lycopene depend on the experimental conditions. The product obtained by supercritical CO_2 extraction at 460 bar and 80°C contained the highest concentration of carotenoids with 90% of lycopene (Vagi *et al.*, 2007).

- **Ultrasonic extraction**

Lycopene extraction has also been done by ultrasonic assisted extraction (UAE) with response surface methodology (RSM). Sonication was found to enhance the efficiency of relative lycopene yield (enhancement of 26% extraction yield of lycopene in six replications in 40-min at 40°C and 70% v/w in the presence of ultrasound), lower the extraction temperature and shorten the total extraction time. This method enhanced the extraction yield of trans-lycopene by 75.93% compared to conventional extraction methods, and also minimised the degradation and

isomerisation of lycopene (Eh & Teoh, 2012b). Conventional methods of extraction require longer extraction times, higher extraction temperatures and a higher solvent to sample ratio for increased efficiency of extraction. Therefore, compared to the improved UAE of lycopene, conventional methods are more time-consuming and less efficient in terms of energy and solvent consumption.

- **Soxhlet extraction**

Soxhlet extraction is a simple and inexpensive method suitable for extracting a compound of low solubility from a solid mixture, and can extract more sample mass than most other extraction methods. This method, described by Soxhlet in 1879, is the most commonly used example of a semi-continuous method applied to extraction of lipids from foods. Oil and fat from solid material are extracted by repeated washing (percolation) with an organic solvent, usually hexane or petroleum ether, under reflux in a special glassware apparatus. The apparatus consists of a glass condenser, a cellulose extraction thimble with the sample, and a boiling flask containing the heated solvent. Solvent vapours are refluxed in the condenser and repeatedly wash over the solid sample to extract the desired compound into the flask with the solvent. Because the heat applied to the boiling flask keeps the system at a relatively high temperature, no filtration is required after extraction and the solvent can be reused.

However, the Soxhlet method is time-consuming compared with other methods, because the solvent needs to be heated to its boiling point before the compound can be extracted from the sample. This extended exposure to heat can result in thermal decomposition of thermo-labile target species. A conventional Soxhlet apparatus provides no agitation, which would help expedite the process. Large amounts of extract (solvent) are used for the evaporation-concentration process. If it is not recycled and reused, this can be wasteful. It is costly to dispose of extractant so it does not pose a threat to the environment (de Castro & Priego-Capote, 2010).

The Soxhlet method was selected for this research because of the low cost, multiple extractions with recycled solvent, controlled temperature and condensation conditions, and the system operates under vacuum. Recycling the solvent minimises the amount of solvent required. These features make the

Soxhlet extraction method most suitable for extracting lycopene from tomato paste in controlled laboratory conditions.

2.9 Immobilization

Immobilization technology involves trapping a material (solid, liquid or gas) within a semi-permeable and biodegradable matrix (Anal & Singh, 2007). The material being immobilized can vary from whole organisms or microbe cells to solutions of labile materials such as lycopene or drugs. The matrix is generally sourced from polymeric materials such as alginate and chitosan. The carrier selection is determined by the biological material being immobilized and environmental conditions.

When the material is held within the semi-permeable matrix, the effects of external factors such as pH, temperature fluctuations and light etc. are greatly reduced (Anwar *et al.*, 2009). Immobilization provides a solution to many of the problems associated with bioprocess technology and drug delivery. Another advantage is the cost effectiveness of downstream processing - immobilized cells are easier to separate and can be recycled.

2.9.1 Current methods for immobilization

Conventional methods to prepare lycopene microcapsules are spray-drying (Shu *et al.*, 2006), microwave-assisted, supercritical fluid extraction method (Blanch *et al.*, 2007) and ultrasonic emulsification (Guo *et al.*, 2014).

Spray drying is a popular way to encapsulate raw material. Shu *et al.* (2006) prepared lycopene microcapsules by spray drying using a wall system consisting of gelatine and sucrose. But lycopene showed some isomerization and the storage stability of products was poor at room temperature.

Blanch *et al.* (2007) prepared lycopene microcapsules by a supercritical fluid extraction method using α-β- and γ-cyclodextrins (CDs) as wall materials. The use of supercritical fluids in food products avoids using large amounts of organic solvents, but the costs of equipment and operation are high and the technological parameters need further research.

Ultrasonic emulsification is a better alternative with lower energy consumption and using less surfactant. It is gaining more interest in many industrial fields compared to other mechanical processes. Although ultrasonic waves are used mostly for liquid stirring and mixing, researchers have focused on also using this technique to prepare lycopene microcapsules. To enhance the storage stability of lycopene ultrasonic emulsification technics optimized by response surface methodology (RSM) experiment (Guo *et al.*, 2014).

2.10 Controlled release of lycopene

Lycopene is easily degradable but can be immobilized within gels. Gels are polymeric three-dimensional networks that swell on contact with water but do not dissolve. Their water absorption property enables gels to be used for food additives, and for immobilization and controlled release of active compounds. Gels have been used extensively as controlled release matrices in the food, medicine, agronomy and cosmetic industries. Most studies have been done on gels made from polymers. Gel-able native or tailored polysaccharides, which are generally non-toxic and highly biocompatible, are receiving increasing attention (Hernandez-Espinoza *et al.*, 2012).

Polysaccharide gels can be used as matrices for controlled release of functional components. Some polysaccharide networks can protect entrapped molecules while passing through the stomach and then release the molecules in the small intestine and colon during gel degradation. Studies using polysaccharide gels as controlled release matrices usually involve chitosan, alginate, starch derivatives and modified dextran (Hennink & van Nostrum, 2002; Hernandez-Espinoza *et al.*, 2012).

Encapsulation is used to entrap functional components in a carrier and protect against oxidation, isomerization, and degradation during storage. This method is also used to control the release of functional components into the body so the unstable constituents remain intact in the stomach and then release in the intestine over a range of physiological pH values (Figure 2-8).

Letter	Region	pH	Transit time
O	Oesophagus	7.0	10-14 sec
A	Stomach	1 -2.5	20-60 min
B	Proximal small intestine	6.15 - 7.88	2-3 hours
C	Distal small intestine	6.80 - 7.88	
D	Ascending colon	5.20 - 6.72	Highly variable depends on bowl evacuation
E	Descending colon	5.26 - 7.02	
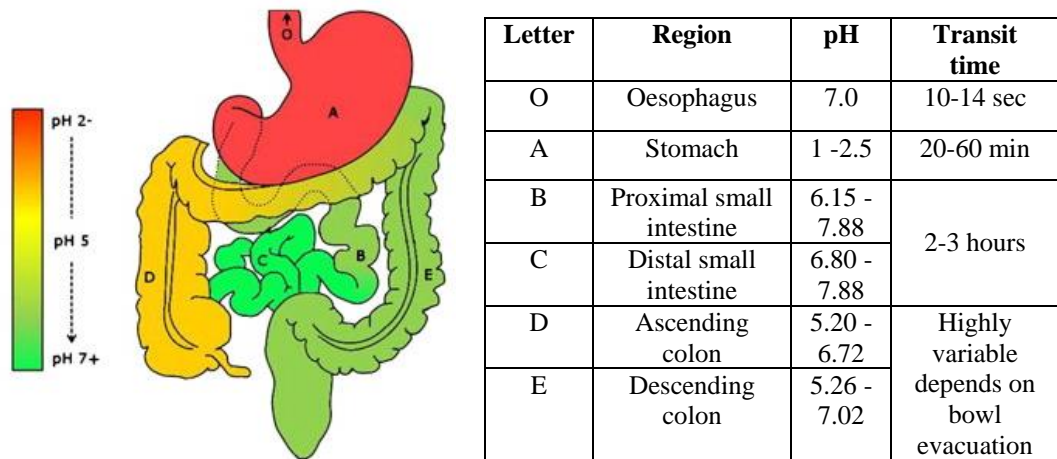
Figure 2-8 Characteristics of gastrointestinal (GI) tract showing the pH at the different parts (Cook *et al.*, 2012).
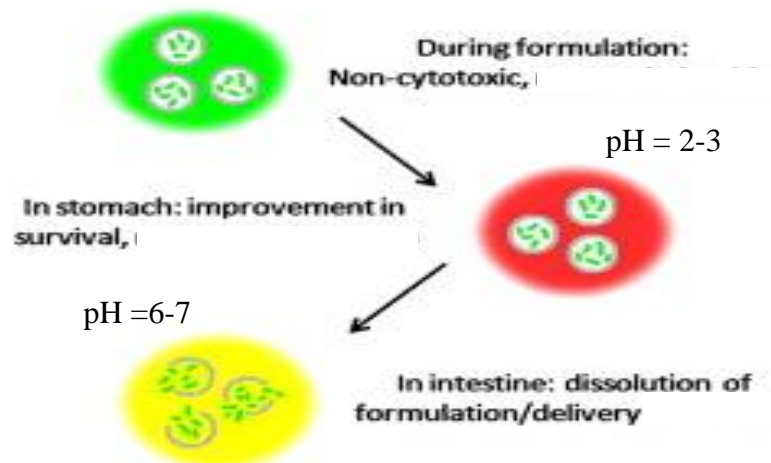
Figure 2-9 Release of lycopene in GI tract (intestine) (Cook *et al.*, 2012).

The release of encapsulated lycopene is affected by pH (Figure 2-9). There is no release in the acid environment of pH levels (2.0 to 3.5) the stomach and rapid release at the higher pH conditions (5.5 to 7.0) the intestine (Cook *et al.*, 2012).

2.11 Immobilization systems

Several gels and gelling agents are currently used to immobilize lycopene.

2.11.1 Hydrogels

Polymeric hydrogels are extremely hydrophilic and can undergo significant swelling when exposed to biological fluids. If the polymer matrix contains an entrapped drug, the particles are liberated when the matrix swells due to changes in pH and temperature. The release rate depends on the diffusion of biological fluids into the matrix (Lui *et al.*, 2011).

Hydrogel matrices are often used for controlled release of bioactive molecules, in particular pharmaceutical proteins, and for encapsulating living cells. For these applications, the gels often need to be able to degrade under specific physiological conditions (Hennink & van Nostrum, 2002).

- **Arabinoxylan gels**

Arabinoxylan gels are non-starch polysaccharides present in the cell walls of cereal endosperms. They are one of the main components of soluble and insoluble dietary fibres and have various health benefits. Arabinoxylans gels with different rheological and lycopene transport properties can be obtained by modifying the polysaccharide concentration from 3 to 4% (w/v). Lycopene slowly diffuses from arabinoxylan gels, indicating that these gels could be carriers for lycopene delivery to specific sites after network degradation. The possibility of modulating lycopene release from arabinoxylan gels makes these biomaterials potential candidates for the controlled delivery of biomolecules (Hernandez-Espinoza *et al.*, 2012).

- **Agar-agar**

Agar-agar is extracted from the cell membrane some species of red algae. It comprises a mixture of agarose and agar-pectin. It used as an ingredient in desserts.

- **Alginate gels**

Alginate is a polysaccharide with mannuronic and glucuronic acid residues and can be cross-linked by calcium ions at room temperature and physiological pH

levels. Therefore alginate gels are frequently used as a matrix for the encapsulation of cells and drugs. (Hennink & van Nostrum, 2002).

Alginate beads have been used extensively in microencapsulation because they are extremely easy to prepare on a lab-scale, the process is very mild, it can be conducted in sterile environments and virtually any ingredient can be encapsulated, whether it is hydrophobic, hydrophilic or sensitive to temperature. Commercially-available alginates have been used in the food and pharmaceutical industries as thickening, emulsifying and film forming agents for many years.

Sodium alginate ($NaC_6H_7O_6$) also known as 'algin' (sodium salt of alginic acid), is a natural gelling agent extracted from the cell walls of some brown seaweed species. The process involves drying the seaweed, followed by cleaning, boiling, gelling and pulverizing the extract to produce sodium alginate in the form of a light yellow powder. When dissolved in liquids, sodium alginate acts as a thickener, creating a viscous fluid. When sodium alginate is added drop wise to a calcium chloride solution, it forms gel-like beads (Goh *et al.*, 2012).

Structure of alginate beads

Alginate has two building blocks, α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues. These may have up to three different polymer configurations, held together by glycosidic linkages in randomised order (Figure 2-10) (Goh *et al.*, 2012). In the presence of divalent cations (Ca^{2+} or Mg^{2+}), alginate forms covalent bonds that produce a rigid gel formation. Surface polymerization occurs instantaneously. During prolonged exposure, divalent ions permeate through the matrix to solidify the structure. The randomised order of the different configurations provides a porous structure and is an ideal encapsulation method for reactions with high oxygen requirements (Anal & Singh, 2007; Goh *et al.*, 2012).
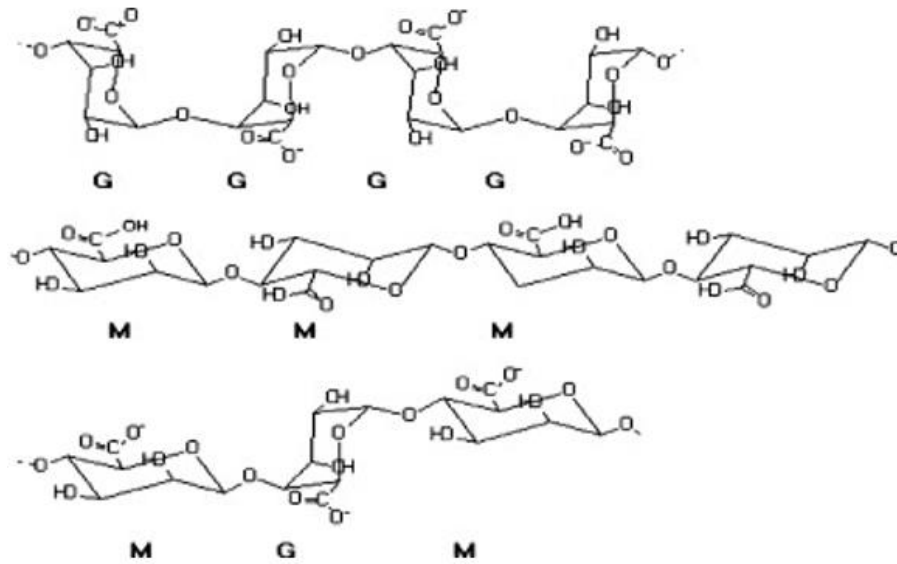
Figure 2-10 Components of alginates: G–G, M–M and M–G blocks linkage (Goh *et al.*, 2012).

Hydration of alginic acid produces a high-viscosity ''acid gel'' due to intermolecular binding. After gelation, the water molecules are physically entrapped inside the alginate matrix, but are still free to migrate. This is of great importance in many applications such as alginate gels for cell immobilization and encapsulation (Lahaye & Rochas, 1991; Tønnesen & Karlsen, 2002).

Alginates with a high guluronic acid (G) content produce stronger gels than alginates rich in mannuronate (M) due to the binding with the Ca^{+2} ion (Figure 2-11). The G residues exhibit a stronger affinity for divalent ions than the M residues (Tønnesen & Karlsen, 2002; Anal & Singh, 2007).
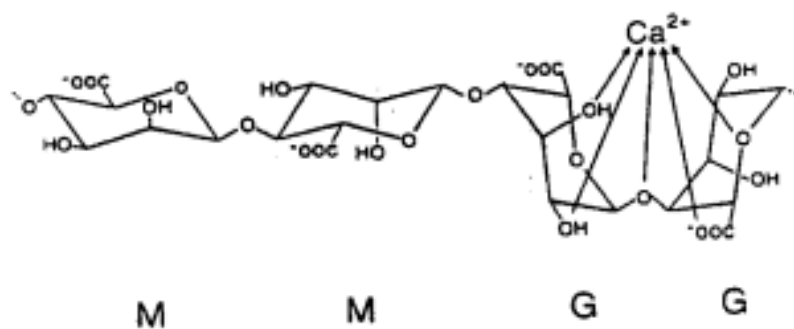
Figure 2-11 Probable binding mode between the calcium ion (Tønnesen & Karlsen, 2002).

Dietary effects of sodium alginate in humans

A study by Anderson *et al.* (1991) showed that ingesting sodium alginate had no significant effect on plasma biochemistry parameters, urinalysis parameters, blood glucose and plasma insulin concentrations. None of the volunteers in the study reported any allergic responses. The trial showed that ingesting sodium alginate at a high level for 23 days caused no abnormal effects. Enzymatic and other indicators of adverse toxicological effects remained unchanged (Anderson *et al.*, 1991).

Optimisation of alginate bead formation

The performance of the alginate bead depends on sodium alginate concentration, calcium chloride ($CaCl_2$) concentration, cell density and particle diameter (Anal & Singh, 2007). A low alginate concentration gives a weak matrix and allows leakage of essential metabolites. On the other hand, a high sodium alginate concentration may decrease the mass and oxygen transfer. Research has shown that sodium alginate concentrations of 2% - 4% are suitable for most types of bioconversions (Anal & Singh, 2007; Anwar *et al.*, 2009).

Blandino *et al.* (1999), investigated the effect of $CaCl_2$ concentration on beads structure and showed that using $CaCl_2$ concentrations greater than 0.3 M resulted in thicker membrane formation and subsequently reduced mass transfer. The $CaCl_2$ concentration used by other studies is typically between 0.1 M - 0.3 M (Blandino *et al.*, 1999; Anwar *et al.*, 2009).

The commonly-used encapsulation technique is based on dripping-gelation by ionotropic gelation and involves extruding a liquid through a nozzle (Figure 2-12) and allowing the droplet to harden in a suitable solution such as $CaCl_2$. Solidification of the droplet surface starts within milliseconds, where Ca^{2+} ions react with the negatively-charged polymer chains to form a three-dimensional rigid structure (Vemmer & Patel, 2013).
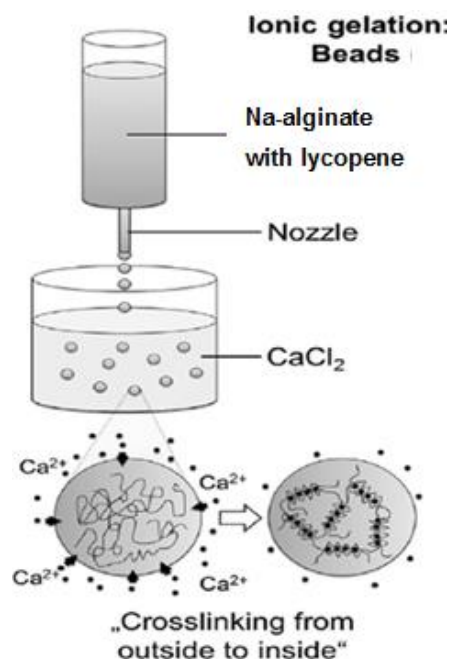
Figure 2-12: Encapsulation based on dripping, ionic gelation production of beads and cross linking (Vemmer & Patel, 2013).

Droplet size (diameter) is an important factor as it affects mass transfer rate. Fick's law of diffusion shows that mass transfer rate decreases when thickness of a barrier increases (Seader & Henley, 2006). Therefore, droplet size needs to allow mass transfer at a suitable rate for release of the immobilized material.

Calcium alginate ($C_{12}H_{14}CaO_{12}$) is formed when the sodium salt in sodium alginate is replaced with calcium. Calcium alginate gel beads are formed by calcium-induced ionotropic gelation of alginate (Kim *et al.*, 2008). Entrapment within insoluble calcium alginate gel is recognized as a rapid, nontoxic, inexpensive and versatile method for immobilising and encapsulating cells and labile compounds (Anwar *et al.*, 2009).

2.11.2 Chitosan gels

Chitosan is a biodegradable, natural polymer commonly used for cell immobilization. It is a copolymer of glucosamine and N-acetyl-glucosamine (Figure 2-13). The free amino groups allow chitosan to chelate with metal ions (Sinha *et al.*, 2004). Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units (Figure 2-14). It is obtained by deacetylation of chitin (Bhattarai *et al.*, 2010).
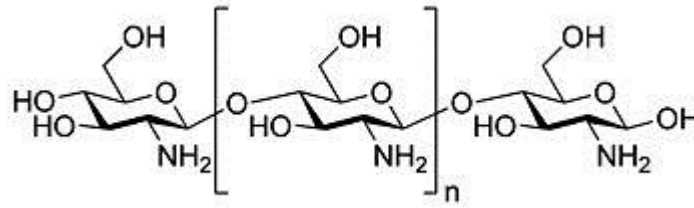
Figure 2-13: Structural configuration of chitosan (Cook *et al.*, 2012)
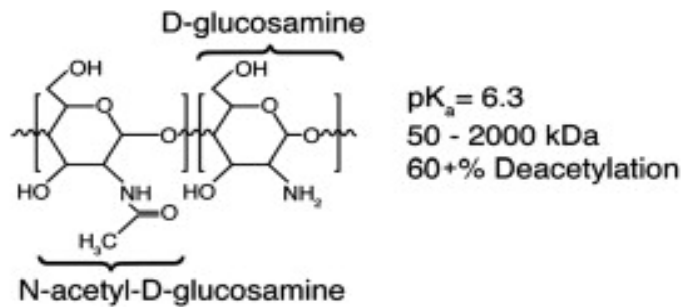
Figure 2-14: Chemical structure of chitosan (Bhattarai *et al.*, 2010)

Chitosan-based hydrogels are obtained by crosslinking the polymer with glycerol-phosphate disodium salt. In the presence of this salt, chitosan solutions remain liquid below room temperature but quickly gel when heated. The temperature for the sol–gel transition decreases with increased deacetylation (Hennink & van Nostrum, 2002; Bhattarai *et al.*, 2010). Applications of chitosan include flocculating agent, clarifying substance, thickening substance, coating material, wound-healing factor agent, and antimicrobial agent (García *et al.*, 2014).

Table 2-5: Characteristics of chitosan **(García *et al.*, 2014).**

Parameters	Percentage (dry basis)
Total solids	98.9
Insoluble material	0.99
Amino groups	7.5
Deacetylation degree	80

Due to its porous and non-toxic properties, chitosan is commonly used in the pharmaceutical industry for drug delivery (Croisier & Jérôme, 2013).

Because alginate beads are delicate and prone to shear damage and swelling, research on drug delivery has led to incorporating chitosan with the alginate to increase physical strength of the bead (Figure 2-15). When chitosan coats the surface of the bead, it forms cross links on the surface and within the bead, increasing the tensile strength while still maintaining porosity (Hari *et al.*, 1996; Kim *et al.*, 2008; Bowman *et al.*, 2011).
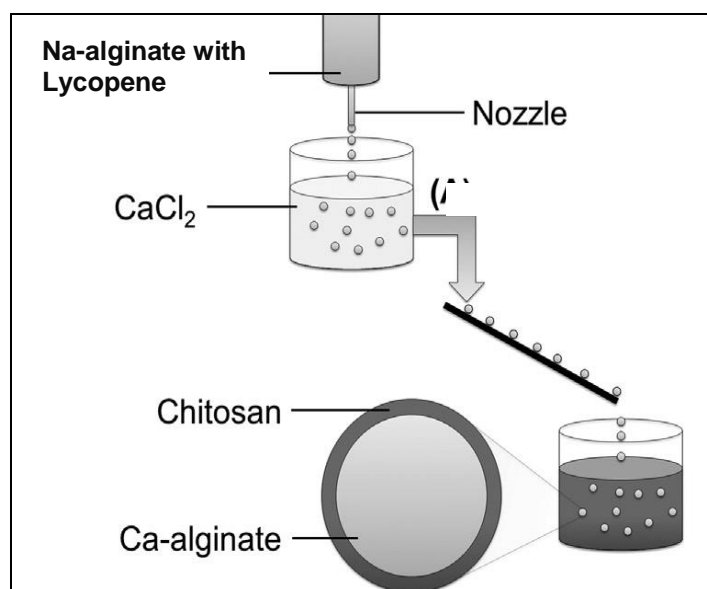
Figure 2-15: Ionic polymer coating of alginate beads with chitosan (Vemmer & Patel, 2013).

2.12 Drug-release

Several parameters determine drug release behaviour from alginate-chitosan microspheres (beads). These include concentration and molecular weight of the chitosan, effect of additives, pore size, density of cross linking (greater cross-linking decreases release rate) and diffusion of the compound from the bead to the external environment. Release rate decreases with increasing molecular weight of chitosan (Sinha *et al.*, 2004).
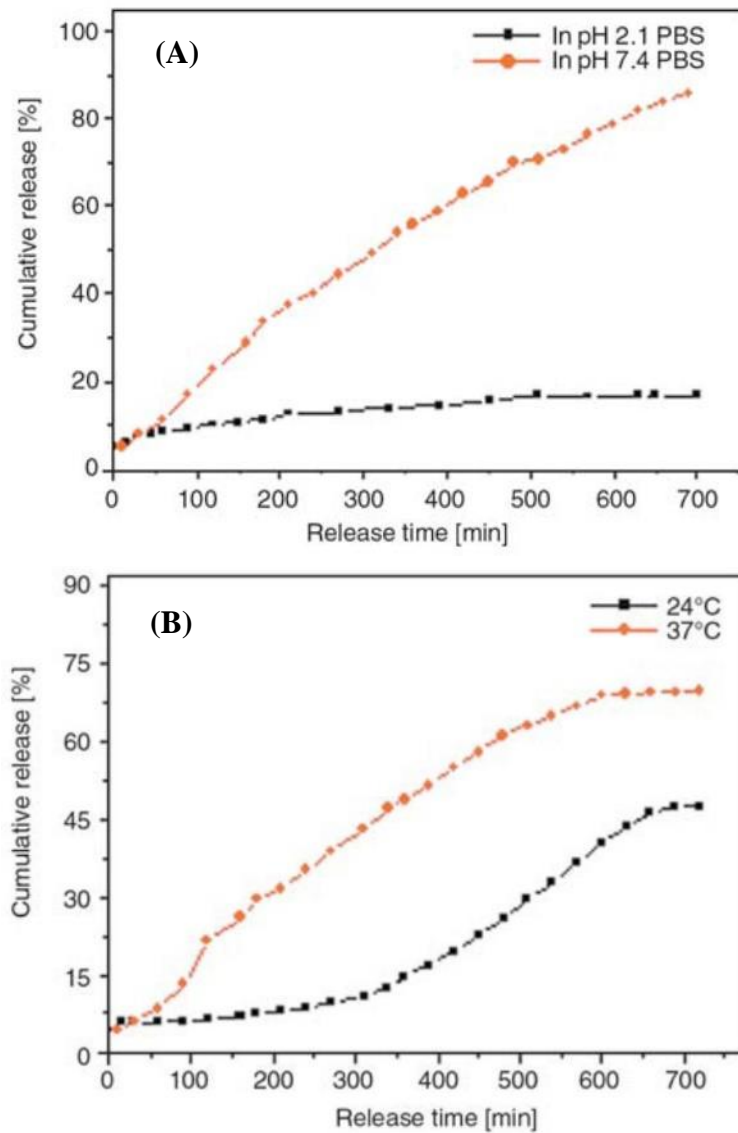
Figure 2-16: Effect of pH value and temperature on drug release from alginate beads with indomethacin. (A) pH at 37°C, and (B) temperatures at pH 7.4 PBS (Deng *et al.*, 2010)

Deng *et al.* (2010) investigated the effect of temperatures and pH on the swelling and drug-release behaviour of alginate-chitosan beads in phosphate buffer solutions (PBS). The pH values were simulated to gastric pH = 2.1 and intestinal fluids pH = 7.4 in the human body. A known amount of beads was immersed in a specific volume of the PBS and a UV-VIS scanning spectrophotometer used to measure absorbance and hence indicates the amount of the drug that had diffused from the beads (Lee *et al.*, 1997).

Release drug indomethacin from alginate-chitosan beads affected by pH and temperature, with a remarkable dependence on pH at 37°C. In pH 2.1 PBS, the drug was released from the beads as an initial burst (about 10%), after which there

was almost no change (Deng *et al.*, 2010) while release of the drug in pH 7.4 PBS continued steadily over time (Figure 2-16A).

The effect of temperature on drug-release from beads at pH 7.4 PBS showed release rate at 37°C was much higher than at 24°C (Figure 2-16B) (Deng *et al.*, 2010).

2.13 Objectives of this research

Today many nutritional supplements are available as capsules or gel-caps which release the active ingredients in a controlled manner. The aim of the present study is to develop a food-grade lycopene formulation for controlled release in humans.

The main focus of this research is to extract, purify, encapsulate and then determine the stability and release of the immobilized lycopene. Commercial tomato paste will be used as the starting material because it has high lycopene content.

The main method used for extracting lycopene will be Soxhlet extraction, using different solvents including petroleum ether, acetone, hexane, dichloromethane, ethyl acetate and ethanol. Solvents affect both extraction rate and efficiency, and trials will be done to identify the best solvent for lycopene extraction.

This will be followed by purification and analysis of lycopene in the extracts. The lycopene will be determined by TLC and HPLC. This will include identification of the optimal mobile phase for HPLC analysis.

The next objective is to immobilize the extracted lycopene and produce stable lycopene beads. This will be done by using sodium alginate, agar agar and calcium chloride to produce lycopene-immobilized alginate beads. To make the beads more robust they will be coated with chitosan solution, to limit lycopene degradation and improve stability.

Lastly, the release of lycopene from the manufactured beads will be determined at two pH values. The effects of temperature on the amount of lycopene released

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will also be investigated. Release of lycopene in different parts of the gastrointestinal (GI) tract will be simulated using various pH levels and temperatures.

The extraction, storage, handling, and analysis of lycopene must be carried out under controlled environmental conditions to minimize losses through oxidation or isomerization.

The following flow diagram is a summary of the proposed research:

Solvent extraction of lycopene from tomato paste ⟹ Extracted Lycopene

Immobilization of lycopene
Na-alginate + Lycopene + Agar

Lycopene Alginate beads
(30 min in CaCl$_2$ solution)

Encapsulation:
chitosan coating of lycopene beads

Evaluated release of lycopene at diffrent temperatures & pH
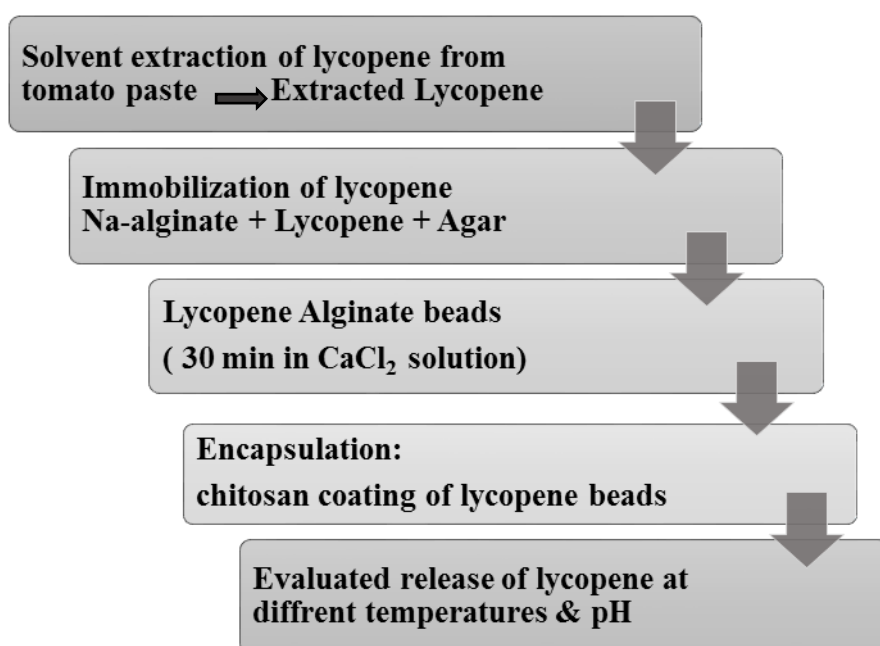
Chapter 3

Materials and Methods

To isolate lycopene (ψ,ψ-carotene) from tomato paste (*Lycopersicon esculentum)*, conventional solvent extraction was performed on tomato paste. Soxhlet extractions were also conducted to determine the amount of lycopene in freeze-dried tomato paste powder. This chapter summarizes the materials and methods used in this research. The experimental work was divided into the following sections:

- Selecting solvents and extracting lycopene
- Isolating and analysing the extracted lycopene
- Immobilizing the lycopene
- Encapsulating the lycopene and then evaluating release

A commercially available canned tomato paste (Leggo, Australia) was purchased from the local supermarket was used. The label indicates it is 99% concentrated tomato with no artificial colours, flavours or preservatives and has lycopene content was 48 mg / 100 g.

3.1 Selecting Solvents

The solvent used for extracting lycopene should have the following characteristics:

- Low cost.
- Low toxicity and considered safe for food use (GRAS).
- High affinity for extracting the target compound. This will depend on factors such as interfacial tension and viscosity.
- Stable during the extraction process.
- Low detrimental effect on the environment.
- Low flammability (for safety).

The following solvents or mixture of solvents were used to identify the best solvent for extracting lycopene from tomato paste. All solvents were analytical grade and purchased from Ajax Finechem Pty Ltd, New Zealand.

- n-Hexane 95% (C_6H_{14}) - flammable liquid, analytical grade

- Dichloromethane (CH_2Cl_2) - toxic, harmful by inhalation
- Ethyl acetate ($CH_3COOC_2H_5$) - flammable liquid
- Petroleum ether (BP 40-60°C) - flammable liquid, irritating to skin
- n-Hexane + ethyl acetate (1:1 by volume)
- Ethanol + ethyl acetate (1:1 by volume)
- n-Hexane + acetone + ethanol (2:1:1 by volume)
- n-Hexane + ethanol (4:3 by volume)

3.2 Extracting lycopene

Conventional solvent extraction was done using wet tomato paste. After opening the original packing and during analysis the sample was protected from heat, light and moisture uptake.

3.2.1 Extraction of lycopene and β-carotene from tomato paste

100 ml of solvent (1:1 acetone: petroleum ether) was added to 15 g (wet weight) samples of tomato paste in conical flasks and mixed using a glass rod. Petroleum ether with a low boiling point was used as it is non-polar and can dissolve most of the carotenoid residue.

The flasks were placed in a shaking water bath for 20-min at 35°C and 10 mL of distilled water was added. The samples were transferred to centrifuge tubes and centrifuged for 10-min at 4000 rpm. The samples separated into two layers (Figure 3-1), which were removed using a pipette.

To analyse the extracted lycopene from the tomato paste samples thin layer chromatography were used.

Figure 3-1: Centrifuged sample showing organic and aqueous layers

A second trial was also carried out with a tri-mixture of 50:25:25 (v/v/v) hexane: acetone: ethanol to extract lycopene and other carotenoids (β-carotenoids) from the tomato paste. 100 ml of tri-mixture solvent was added to 15 g (wet weight)

samples of tomato paste in conical flasks and mixed with a stirrer for 20-min at 35°C. Then stand for 10-min in a cooling water bath, the solution was separated into two layers.

Separating funnel was used to separate these layers. Upper organic layer was used to separate β-carotene and lycopene. Upper organic layer contains lycopene and β-carotene. The carotenoid mixture was applied to the silica column. Column chromatography is a two phase system, the stationary phase is a column of adsorbent and the mobile phase is a liquid eluent. The adsorbents, silica gel (the same ones used in TLC) were used. 20 g of silica gel mix with petroleum ether, swirl until obtained homogenous slurry. Then added silica mixture using a funnel into the burette to make a silica column.

The carotenoid mixture of sample (upper organic layer) was applied to the top of the column in a narrow band using petroleum ether to wash down the sides of the column. The eluent flows down through the column by gravity action. Petroleum ether with a low boiling point and dichloromethane was used to pass through the column to separate the β-carotene and lycopene (Figure 3-2).
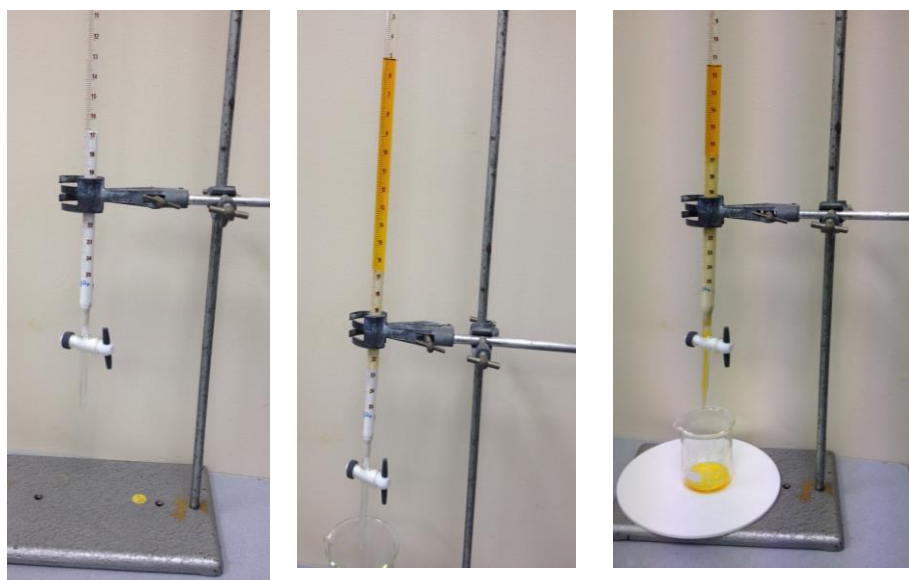
Figure 3-2: Column chromatography to separate β-carotene and lycopene

After petroleum ether were passed through the column (the carotenoids remain in a narrow band at the top of the column), the solvent was changed to 10:90 ratio dichloromethane: petroleum ether. The solvent is then switched to a 50:50 mixture

of dichloromethane: petroleum ether to speed up the elution of the lycopene band, which is collected in separate beaker. To confirm the lycopene and β-carotene the separated fraction samples run on thin layer chromatography.

3.3 Batch extraction

Batch solvent extraction was done, 5g wet tomato paste samples mixed with 10-mL of ethyl acetate and other samples were prepared separately with solvents dichloromethane, n-Hexane, petroleum ether and tri-mixture of (2:1:1, v/v/v) n-Hexane: acetone: ethanol. Then extracted lycopene was analysed with TLC and HPLC.

3.4 Soxhlet extraction

For this research, the Soxhlet extraction method was used to extract lycopene from dry tomato paste powder. The tomato paste sample was dried, ground into small particles and placed in a porous cellulose thimble for Soxhlet extraction. The flask with the solvent was heated, evaporated into the condenser, and condensed to a liquid, which trickled into the extraction chamber containing the sample.

3.4.1 Preparation of samples

Samples (approximately 25 g) were frozen by pouring liquid nitrogen on the tomato paste (Figure 3-3). The frozen samples were placed in a Labconco freeze dryer system (Figure 3-4) and then vacuum dried for 24 hours. After drying, each sample was ground to a powder using a mortar and pestle.

Figure 3-3: Pouring liquid nitrogen on tomato paste

Lycopene was extracted from dried samples by Soxhlet (solvent) extraction. To extract lycopene, duplicate, accurately weighed samples of dried tomato paste powder (approx. 5 g) were placed in cellulose extraction thimbles (Figure 3-4) (Whatman International Ltd., internal diameter 33 mm × external length 100 mm) and covered with glass wool. The thimbles were placed in the Soxhlet extraction unit (Figure 3-5) and extracted for 20 hours with 150 mL of one of the eight solvents.
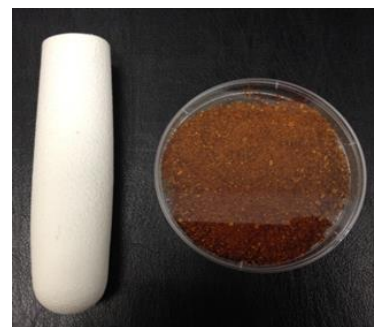
Figure 3-4: Labconco freeze drier system and tomato paste powder before extraction
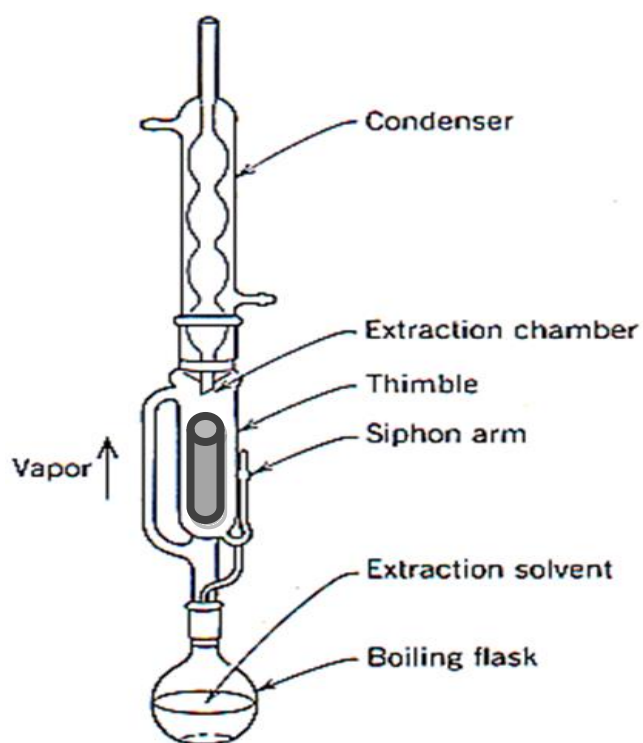
Figure 3-5 Set up of Soxhlet apparatus

Boiling chips were added to each solvent-filled boiling flask and the hot plate under each flask controlled to the boiling point of each solvent (Table 3-1).

Table 3-1: Boiling point of solvents used

Solvent	Boiling Point
n-Hexane	68°C
Ethyl acetate	77°C
Ethanol	78°C
Petroleum ether	40°C
Dichloromethane	40°C

Once the solvent was boiling at a steady rate, each boiling flask was covered with aluminium foil (Figure 3-6) to minimise heat loss.

Extractions were done in the dark to minimise lycopene degradation and run for 20 hours to allow several refluxes (Appendix 1).
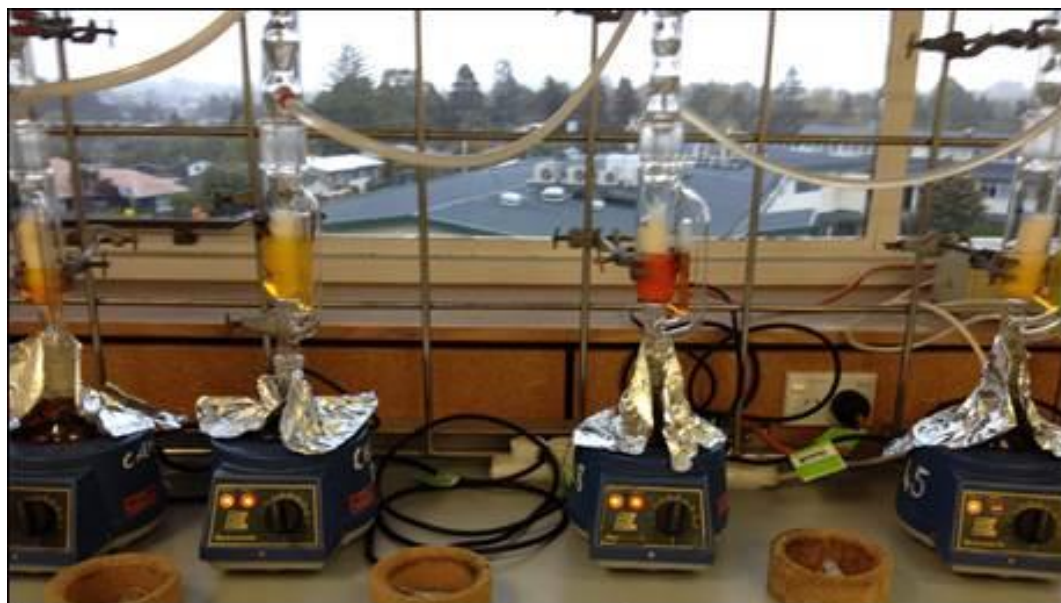
Figure 3-6: Soxhlet extraction with different solvents

The amount of solvent remaining in each boiling flask was measured to determine how much solvent evaporated during the extraction process. A known volume (approximately 2 ml) of the concentrate solvents were removed by pipette for lycopene analysis.

The remaining solvent was then evaporated in a vacuum rotating evaporator (Buchi Rotavapor R-124, Figure 3-7). An allowance was made for the sample that had been removed for lycopene determination. The dried concentrate containing the extracted lycopene was weighed.
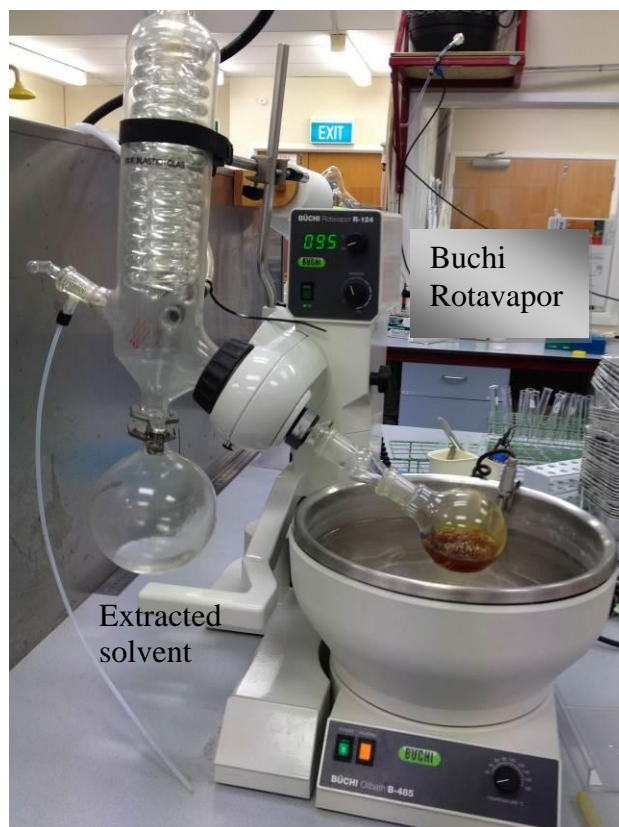
Figure 3-7: Buchi Rotavapor for evaporating solvent from the lycopene concentrate

3.5 Identification of lycopene

Lycopene in each sample was determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

3.5.1 Thin layer chromatography (TLC)

TLC Silica gel 60 F_{254} Aluminium plates were used for isolating and purifying extracted lycopene (carotenoids) for further study, including preliminary screening of extracts and comparison of samples with standards. Silica is used which is most commonly adsorbent for TLC of carotenoids. Silica plates (silica layers coated onto aluminium foil, easily cut into pieces of the desired size with scissors without damaging the layer) were used as adsorbent for the stationary phase.

To confirm purity of the extracted lycopene, TLC (Britton, 2008) was conducted with slight modifications. The TLC silica gel 60 F_{254} aluminium sheets (20×20 cm) were activated at 110˚C for 30 minutes and used as a stationary phase to determine the lycopene in each lycopene-extracted solvent. Two solvents were

used as the mobile phase on the TLC plates toluene-hexane (1:19 v/v) and 80:20 (v/v) of petroleum ether (low boiling point 60-90˚C) and acetone mixture.

20 µL of the extract was applied with a pipette as a spot on the TLC plate (Figure 3-8), and the plate was placed in the mobile phase, which rising up by capillary action. Carotenoids are unstable and sensitive to oxygen, light, heat and acids. Therefore all TLC procedures were carried out as rapidly as possible as the carotenoid zones faded rapidly due to oxidative breakdown. During TLC, the developing chamber was covered with foil paper (black paper or cloth could also be used) or kept in a dark cupboard, because carotenoids are sensitive to light.

Figure 3-8: TLC silica plate with drop of lycopene extract

The TLC results were photographed and documented, and the retention factor (R_f) (Appendix 1) for lycopene was calculated by the following formula:

Retention factor (R_f) = distance travelled by the compound
                                    distance travelled by the solvent front

A trial was also done in which a column for purifying the lycopene was packed with silica to separate the lycopene from other tomato paste pigments.

3.5.2 High performance liquid chromatography (HPLC)

The extracted solution of lycopene samples were analysed in an HPLC system consisting of a Waters 515 HPLC pump, Waters 996 photodiode array detector (PDA- UV/VIS detector) and Rheodyne 7725i manual sample injector with a 20-µL sample loop (Figure 3-9). The system was controlled with Waters Empower^{TM} 2 Chromatography software.
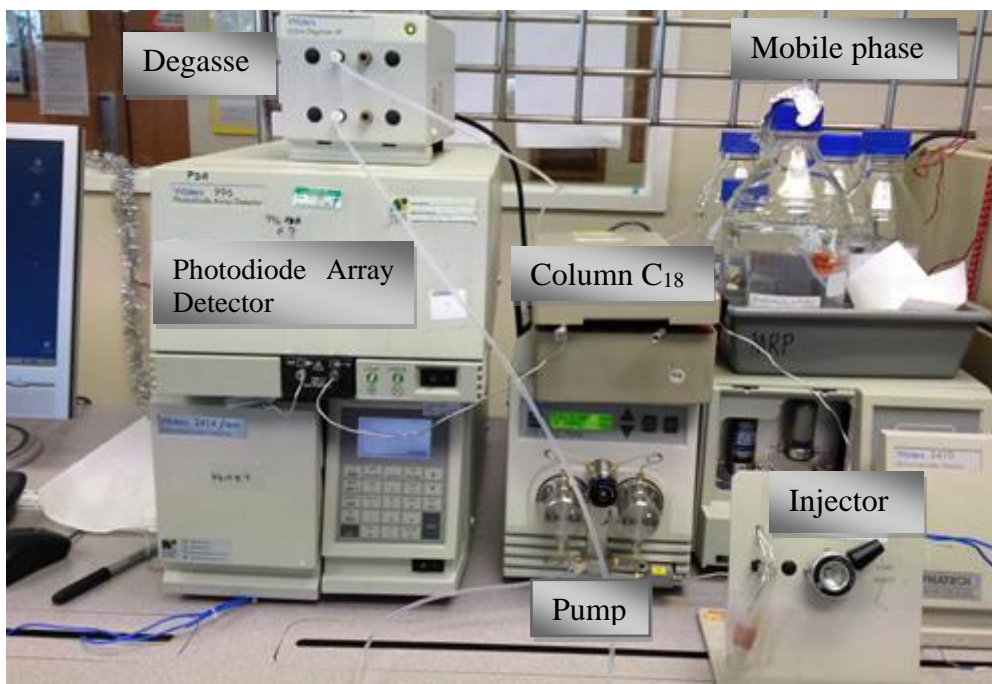
Figure 3-9: HPLC system for lycopene solution analysis.

The non-polar C-18 analytical chromatographic column "A Discovery BIO Wide Pore" C18 column, 25cm×4.6mm, 5μM particle size (Supelco Analytical HPLC Products) was used as the stationary phase for separating and determining non-polar hydrophobic lycopene molecules. The analysis was performed isocratically at a flow rate of 1mL min^{-1} at 22⁰C.

A Waters in-line Degasser AF was connected to degas the mobile phase. The solvent mobile phase in a reservoir was pumped at a specified flow rate. A high pressure pump was used to inject the sample into the continuously flowing mobile phase and run into the HPLC column. The flow diagram of the HPLC is as shown in Figure 3-10.
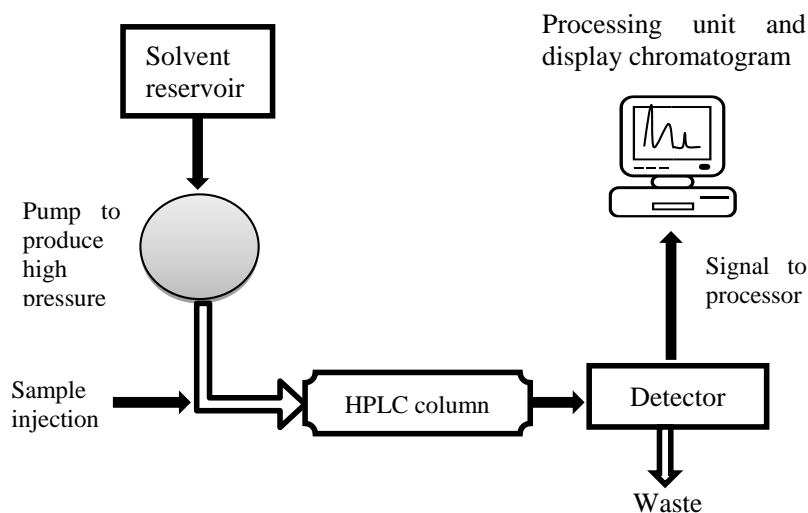
Figure 3-10: A flow diagram of HPLC

Selecting the mobile phase for HPLC

To identify the optimum mobile phase composition, two different solvents were used: 42:42:16 (v/v/v) acetonitrile: methanol: dichloromethane and 3:1 (v/v) acetonitrile: dichloromethane. The optimum composition was based on the chromatographic response factor.

Characterization of the lycopene extract was performed in isocratic mode. The sample injection volume (20 µl in the loop) was injected for 15 minutes into the mobile phase flowing at 1.0 mL/min. The lycopene was detected at a wavelength of 480 nm. The column was controlled at 22°C (room temperature) and 450-500 psi during analysis. Experiments were done at room temperature in the absence of direct sunlight.

3.6   Analysis of extracted lycopene

Lycopene was analysed using high performance liquid chromatography (HPLC) (C-18 analytical column was used) and ultraviolet-visible spectrophotometry (UV-Vis).

Preparation of standard samples for calibration

Appropriate dilutions of standard solution were prepared for analyses the extracted lycopene and all standards calibration done in duplicate.

A stock solution of commercially available pure lycopene (Sigma Aldrich) was prepared by dissolving 2 mg of lycopene in 10 mL dichloromethane (DCM) to give a 0.2 mg/mL (200 µg/ mL) (Figure 3-11) and then stock solution was diluted with dichloromethane to give 0 (blank), 1, 2.5, 5, 7.5, 10 and 20 µg/mL (Table 3-2). Duplicates aliquots of the standards and lycopene extracts samples were analysed by UV-Vis (480 nm) and HPLC.

As the conjugated-double-bond in carotenoids are unstable to light, oxygen, heat, acid, and alkaline conditions, particular attention must be paid when preparing and storing the sample and standard preparations. Standard stock solutions of lycopene in DCM were wrapped in aluminium foil and stored in the freezer at 20˚C. All analysis was done in duplicate and in the absence of direct sunlight.

Figure 3-11: stock solution

Table 3-2: Dilution of stock solution

Concentration µg/mL	Stock solution (SS) ml	Dichloromethane (DCM) ml
20	1	9
10	0.5	9.5
7.5	0.325	9.675
5	0.250	9.750
2.5	0.125	9.875
1	0.05	9.950
0	0	10

3.6.1 HPLC and UV-Vis analysis

A 20-µL aliquot of each dilution was injected into the HPLC column and data and chromatograms were collected using the chromatograph software. Quantitative analysis was based on peak area measurements. A calibration graph was prepared from the results. The linearity of calibration the curves (peak area vs. concentration) was checked over the lycopene concentration range of about 10-500 µg/ mL.

3.7　Immobilizing the lycopene

To immobilizing the lycopene as gel beads, the lycopene extract was mixed (dissolved) with sodium alginate solution. One hundred and fifty millilitre of sodium alginate solution 4% w/v (4 g sodium alginate in 100 mL) was prepared with distilled water and the solution was gently mixed to obtain homogeneity and eradicate air bubbles that may trapped in the beads. When this has been accomplished, the alginate solution was added to the lycopene extract in a 1:1 (v/v) ratio to obtain a final alginate concentration of 2% (w/v).

The solution was stirred with a magnetic stir for 20-30 min until the lycopene was emulsified and appeared creamy. Another aggregating enhancing polymer, agar-agar, was then added to enhanced alginate composition. This involves adding a 1% w/v agar solution (1 g agar-agar powder in 100 mL distilled water at 80°C) to form a 1:1:1 (v/v/v) lycopene-alginate-agar mixture.

Tests to form beads were done using a syringe with an 18-gauge hypodermic needle. A Gilson's Minipuls ® 3 peristaltic pump (Figure 3-12), designed for transferring fluids with a high level of speed control and at a low pulsation level, was used to add drops of lycopene-alginate-agar mixture into 0.2M calcium chloride ($CaCl_2$) solution at room temperature (20°C). Different sized capillary tubes (2-4 mm) were used to produce different sized beads. The diameters of the beads were measured with a scale.

Magnetic stirrers were used in the gelling solution to prevent the beads sticking together. Beads were left in the $CaCl_2$ solution for 30 min to harden. They were then separated by filtration and washed with deionized water.

Magnetic stirrer   Peristaltic pump
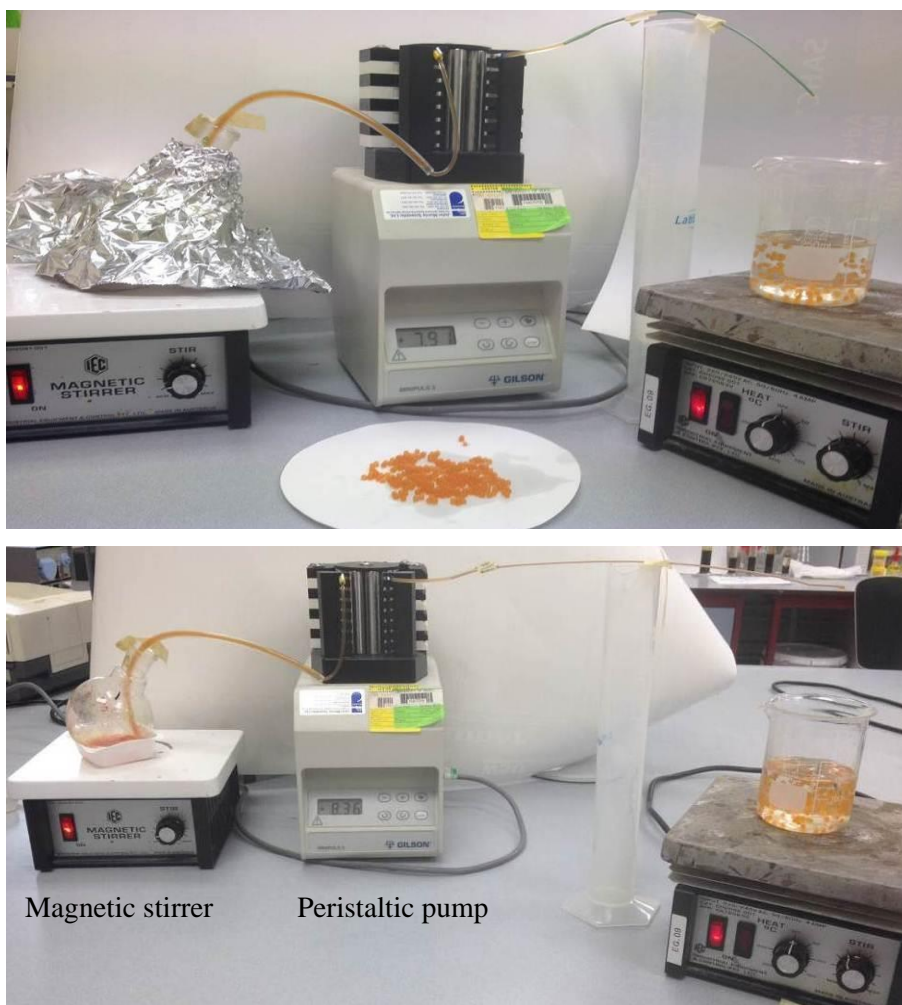
Figure 3-12: Gilson's MINIPULS peristaltic pump for making beads.

3.8 Encapsulating lycopene and evaluating release

Low molecular weight chitosan (Sigma-Aldrich) was used to make a 0.4% w/v chitosan solution (4 g chitosan in 100 mL 0.4% glacial acetic acid). The pH of the chitosan solution was 4.12.

The lycopene-alginate beads were immersed in 30 ml chitosan solution for 40 minutes to form chitosan-coated alginate beads. A scanning microscope with Nikon digital sight camera was used to determine the size of lycopene beads. The camera had a progressive resolution of 640×480, acq resolution of 2560 ×1920 (fine) and 24 bit capture depth with magnification lens. The software Image-pro plus 6.3 supports with Nikon digital camera was used to examine morphology of the beads under 6.4 and 16 times magnification.

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To determine the effect of pH and temperature on lycopene release, lycopene beads were re-suspended in phosphate buffer solution (PBS) with different pH values.

3.8.1 Controlled release of lycopene in the encapsulated beads

Preparing phosphate buffer solutions (PBS)

Buffer solutions with different pH values were prepared by mixing 0.2M disodium hydrogen phosphate and 0.1M citric acid solutions (Table 3-3).

The disodium hydrogen phosphate solution was prepared by dissolving 14.2 g disodium hydrogen phosphate (Na_2HPO_4) in 500 mL deionized water, and the citric acid solution was prepared by dissolving 14.5 g citric acid in 500 mL deionized water. The pH of the solution was measured with a digital pH meter model Cyberscan 100 [pH] (Eutech Instruments, Singapore).

Table 3-3: Preparing buffer solutions with different pH values

pH of buffer	Citric acid solution (0.1 M) (ml)	Disodium hydrogen phosphate solution (0.2 M) (ml)
2.23	99	1
3.16	70	30
4.5	45	55
6.6	18	82

To evaluate the effect of pH and temperature on lycopene release, 1 g of lycopene-alginate beads were immersed in 9 ml of phosphate buffer at pH 2.23 (pH in the stomach), 3.1, 4.5, and 6.6 (pH in the intestine) at 22°C (room temperature) and 37°C (body temperature). The beads were kept suspended by using magnetic stirrers (120 rpm). Mainly at two pH 2.2 (in stomach) and 6.6 (in intestine) results were recorded.

The lycopene contents in the liquid were measured at 10-minute intervals using a UV-Visible spectrophotometer (UV-1700 pharma Spec- Shimadzu) at 480 nm. Measurements of lycopene release at pH 2.2, 3.16, 4.5 were taken for 60 minutes (average gastric emptying time) and twice at pH 6.8 over 2 hour period the (average time food remains in the intestine). Tests were done in duplicates.

Chapter 4

Results and Discussion

This chapter discusses trials for investigating methods for extracting and immobilizing lycopene from commercially available tomato paste. Once the optimum solvent for extraction and the mobile phase for HPLC had been determined, extracted lycopene was immobilized in chitosan coated alginate beads and its release measured.

4.1 Extraction and isolation of lycopene

4.1.1 Preliminary trials

A trial was done with solvents to extract lycopene from commercial brand tomato paste. Lycopene was extracted using a 1:1 (v/v) petroleum ether and acetone mixture. When distilled water (10 mL) was added and the solution centrifuged, it separated into an upper organic layer containing the lycopene and a lower aqueous water layer (Figure 4-1). The presence of lycopene in the upper layer was confirmed by thin layer chromatography.
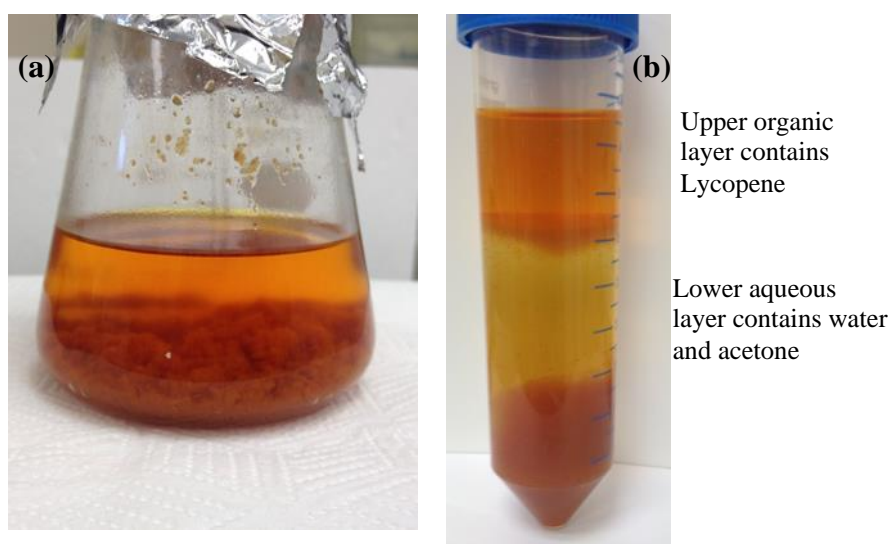

Figure 4-1: (a) Extraction with petroleum ether: acetone. (b) Layers after centrifuging upper organic, middle aquous layer and bottom is tomato pulp

The second trial was done using a method developed by Motilva *et al.* (2014) to extract lycopene and other carotenoids from tomato paste using a tri-mixture of

50:25:25 (v/v/v) hexane: acetone: ethanol. The tomato paste became light in colour after a 20-min, single step stirred extraction, indicating that the carotenoids had been extracted. The solution was separated into two layers in a separating funnel (Figure 4-2). The upper layer contained lycopene and carotenoids (β-carotene) which were further separated using a silica column (Figure 4-3) and thin layer chromatography.

Upper organic layer contains Lycopene
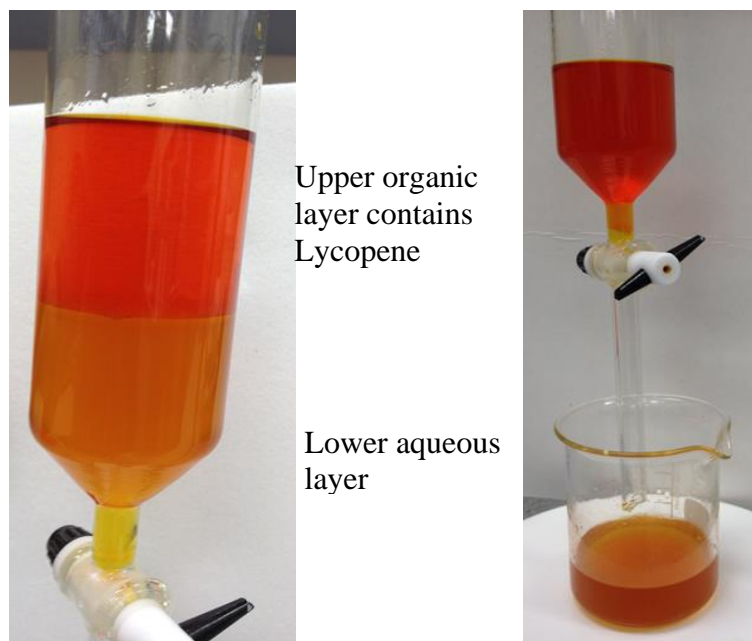
Lower aqueous layer

Figure 4-2: Separating two layers in separating funnel
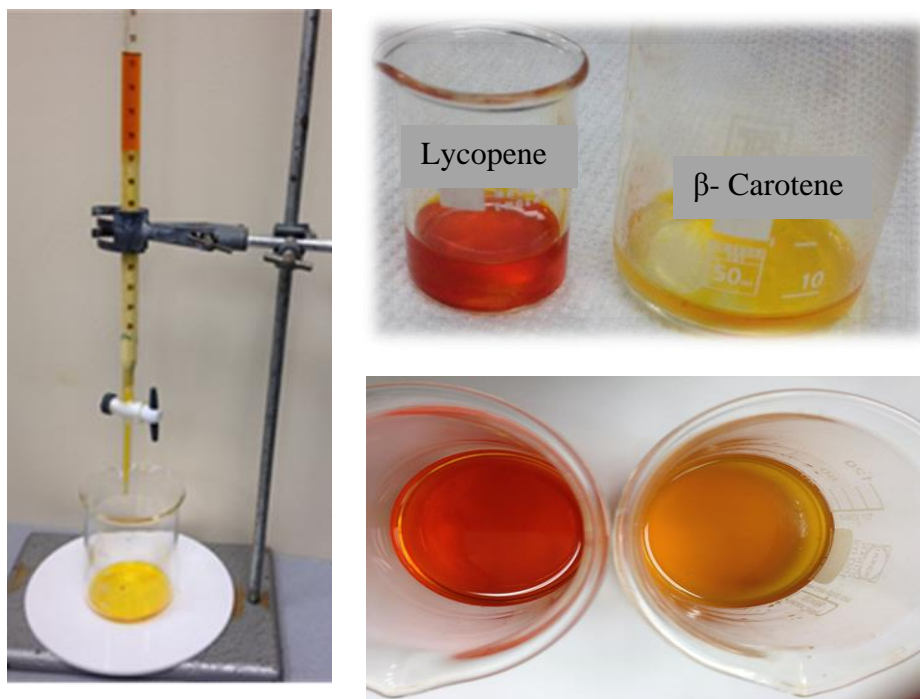

Lycopene

β- Carotene

Figure 4-3: Lycopene and β-Carotene separated by silica column chromatography. On the left is the silica column packed in a burette.

The colour of lycopene and β-carotene are due to the double bonds in their structure (lycopene has more double bonds than β-carotene). The β-carotene was separated as a distinct yellow band from the reddish-orange lycopene. The β-carotene (non-polar) was not as strongly bound to the silica and eluted before the lycopene. Lycopene gives a red-orange band and due to its high degree of unsaturation, eluted after yellowish β-carotene pigments. Thin-layer chromatography using silica gel plates confirmed the fractions were lycopene and β-carotene.

The lighter colour of the tomato paste after extraction (Figure 4-4) indicates that the tri-mixture 50:25:25 (v/v/v) hexane: acetone: ethanol gives better extraction than 1:1 (v/v) petroleum ether / acetone mixture.
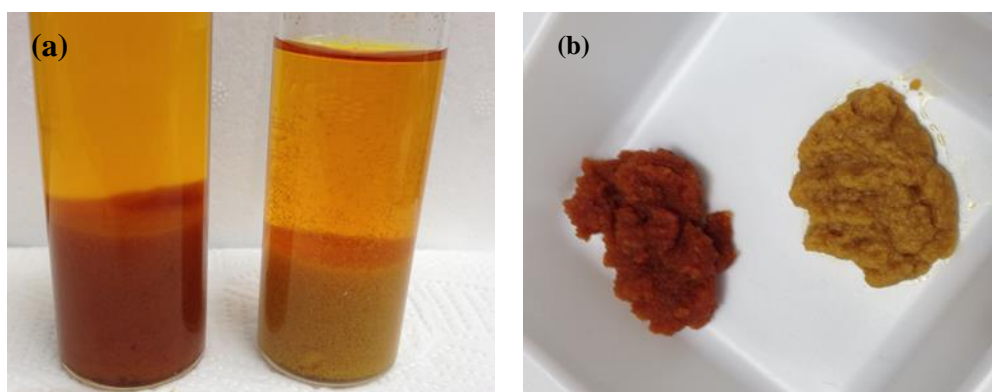
Figure 4-4: (a) Solvent phases after extraction with the tri-mixture (right) and petroleum ether: acetone (left) (b) After extraction pulp become lighter with the tri-mixture (right side) than petroleum ether: acetone (left)

Thin layer chromatography silica gel plates were used to isolate and purify extracted lycopene and β-carotene and compare the samples with standards lycopene. Silica is the most commonly used adsorbent for TLC of carotenoids. The β-carotene dicyclic (upper spot), γ-carotene (monocyclic) the middle spot and lycopene (acyclic) lower spot (Figure 4-5) appeared on the TLC plate. Separated carotenes can easily be distinguished by their characteristic colour. Because of its strong colour, lycopene is easily detected on TLC.
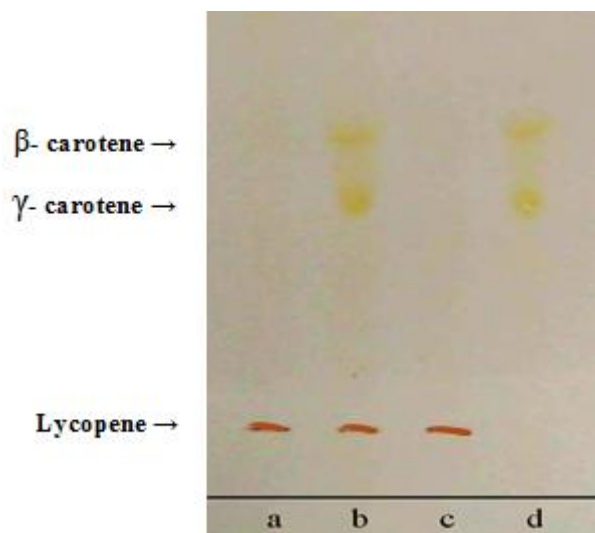
Figure 4-5: TLC of lycopene and carotenoids. TLC plates were developed with toluene: hexane (1:19, v/v). (a) Standard lycopene (Sigma). (b) Tri-mixture extract. (c) Separated lyopene and (d) carotenoids from silica column chromatography.

The eluate containing lycopene was evaporated at room temperature in the fume hood to produce a dry lycopene extract (Figure 4-6).
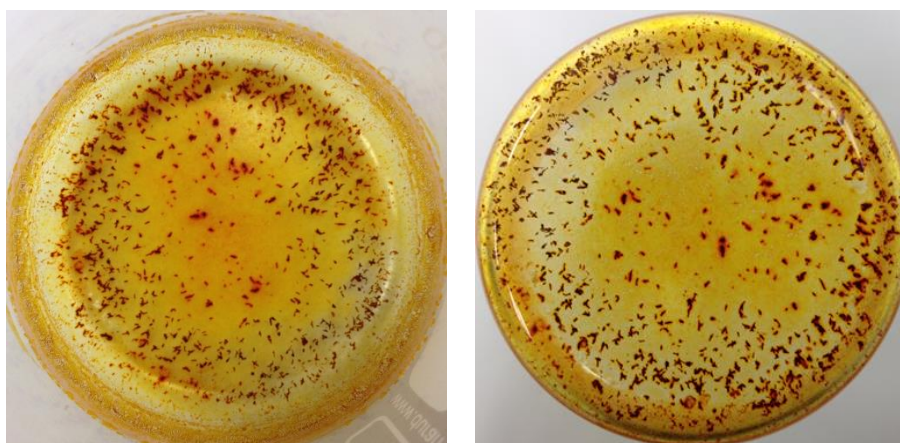
Figure 4-6: Lycopene extracted from tri-mixture extract

4.1.2 Determining Optimum Wavelength by UV/Vis

The UV-Vis absorption of lycopene extracted from the tri-mixture and a standard lycopene (Sigma New Zealand) was measured in the range 250 to 600 nm. The UV-Vis spectra of the lycopene standard had the same maximum absorption peaks at 455, 480, and 513 nm reported by Seifi *et al.*, (2013). Unfortunatelly, this spectra could not be printed so published data is given (Figure 4-7).
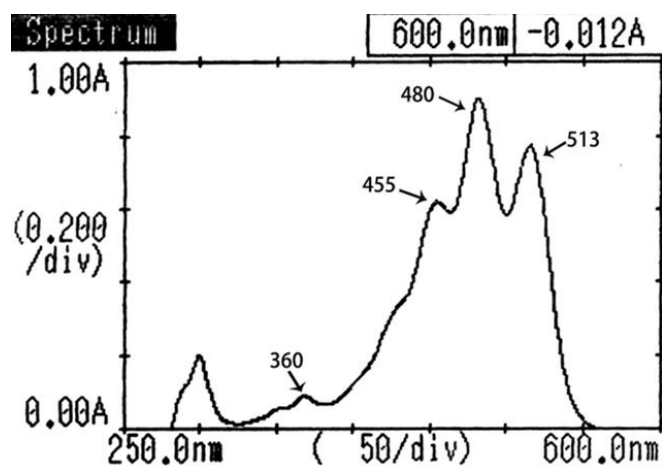
Figure 4-7: UV-Vis absorption spectra (250 to 600 nm) of 10μg/mL lycopene in dichloromethane (Seifi *et al.*, 2013)

Kun *et al.* (2006) also used UV-Vis to identify lycopene extracted from tomato paste using 2:1:1 hexane, ethanol, and acetone. The optical density of the hexane extract was measured at 480 nm.

4.1.3 Calibration of lycopene with UV-Vis

A calibration for lycopene was also done by UV-Vis spectra. The absorbance of lycopene standard (Sigma) was linear in the concentration range 1-10 μg/mL (Figure 4-8) and could be represented by the following equation:

Absorbance = 0.047 lycopene μg/mL +0.03 $R^2 = 0.9993$

When the calibration curve extended to 20 μg/ mL, the relationship was also linear (Figure 4-8). However the accuracy (as indicated by the R^2 value) decreased by 10%.

Absorbance = 0.054 lycopene μg/mL $R^2 = 0.994$

Therefore sample should be diluted to ensure the lycopene concentration is in the range of 1-10 μg/ mL.

A calibration for lycopene was also done by HPLC using the mobile phase 3:1 acetonitrile: dichloromethane. Absorbance of lycopene standard (Sigma) was linear in the range of 1-10 μg/mL (Figure 4-9). The peak area is measured by absorbance at 480 nm and represented by the following:
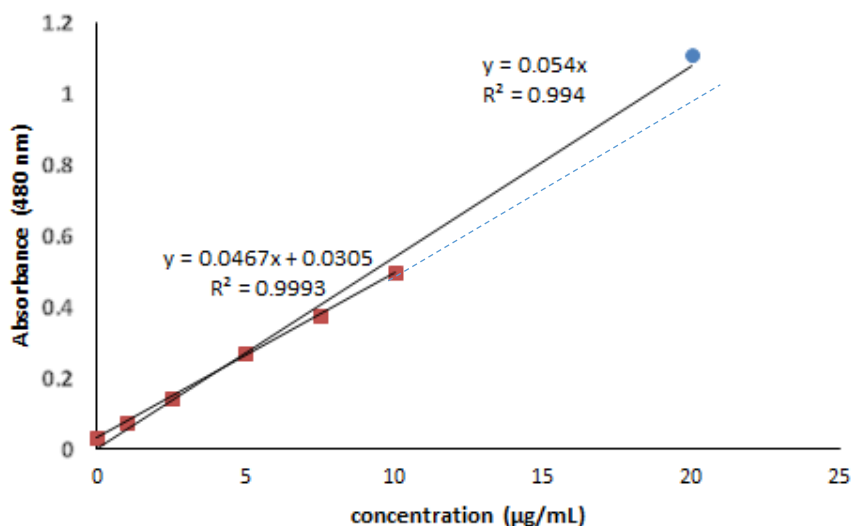
Figure 4-8: UV-Vis at 480nm Calibration curve for lycopene standard dissolved in dichloromethane
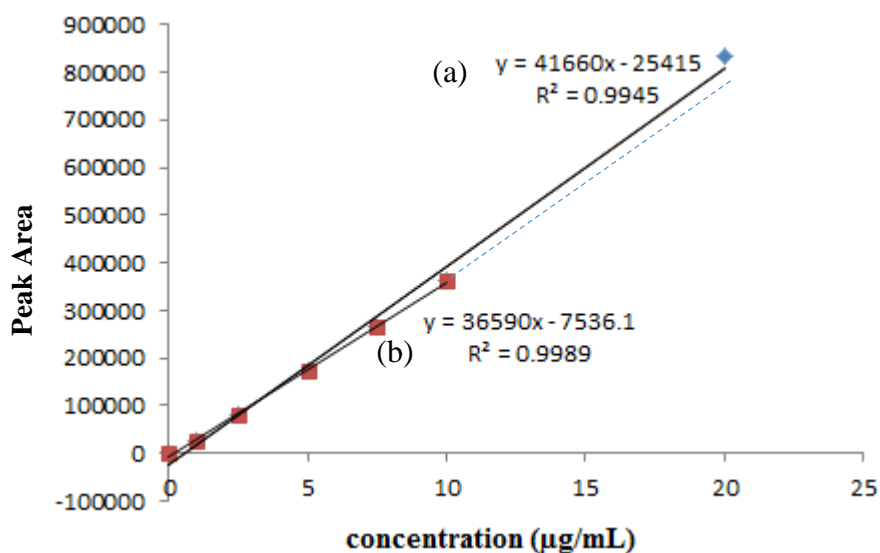
Figure 4-9: Calibration cure for lycopene by HPLC

Peak area = 361079 $\qquad$ $R^2 = 0.999$

When the calibration was extended to the concentration to 20 μg/ mL, the relationship was also linear (Figure 4-9). However the accuracy (as indicated by the R^2 value) decreased by 2%.

Peak area = 832318 $\qquad$ $R^2 = 0.994$

Y=41660×20 -25415 $\qquad$ Y (a) =807785

Y= 36590×10- 7536 $\qquad$ Y (b) =358364 $\qquad$ a/b = 2%

The spectrophotometric method could be used for routine analysis of lycopene, as it is more convenient, faster and less expensive than HPLC analysis and large numbers of samples can be processed in a relatively short time. The disadvantage of UV-Vis is that it cannot distinguish between trans and cis-isomers of lycopene. HPLC methods are reliable, can indicate the presence of contaminants, but they are also laborious and require use and disposal of organic solvents.

4.2 Batch solvent extraction

Batch extractions were done with five different solvents or solvent mixtures: ethyl acetate; dichloromethane; n-Hexane; petroleum ether; and tri-mixture of 2:1:1 (v/v/v) n-Hexane, acetone and ethanol.

Lycopene content of the extracted solvent samples were measured by thin layer chromatography and HPLC.

4.2.1 Thin layer chromatography (TLC)

Two different solvent combinations were used for the mobile phase for thin layer chromatography: 1:19 (v/v) toluene-hexane; and 8:2 (v/v) petroleum ether (bp 60-90°C) and acetone were used at room temperature for 20-min.

Lycopene when using a 1:19 (v/v) toluene-hexane mobile phase appears as a reddish orange band and the β-carotene fraction has a light yellowish band (Figure 4-10). When 8:2 (v/v) petroleum ether/acetone was used as the mobile phase, lycopene appeared as orange-yellow spot.

The crude carotenoid fraction gave a reddish orange spot ($R_f = 0.34$) and a yellow ($R_f = 0.56$) spot on TLC silica plates when using a mobile phase toluene-hexane.

Lycopene is an acyclic compound. These compounds tend to crystallize on TLC plates, resulting in poor resolution. Including toluene or dichloromethane in the mobile phase keeps these compounds in solution without affecting the chromatographic separation.
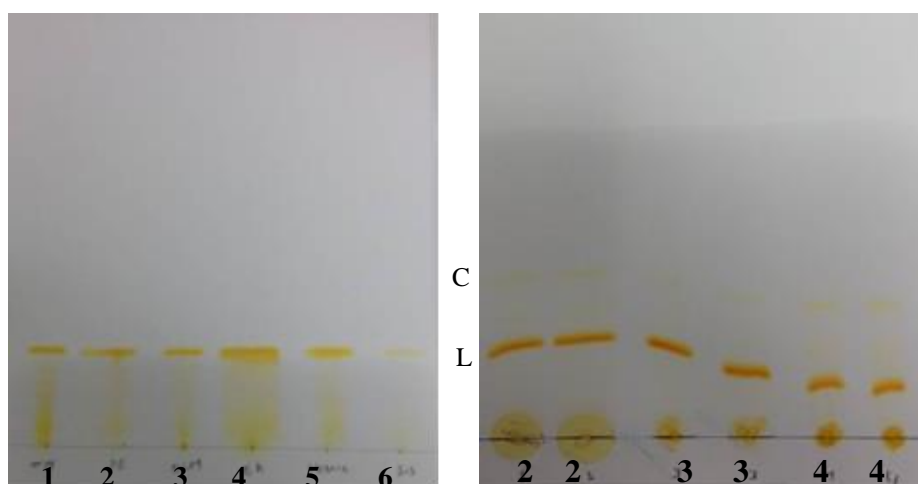
Figure 4-10: TLC of extracted lycopene, TLC silica gel plates were developed with, petroleum ether: acetone (80:20) and toluene: hexane (1:19, v/v) showing the presence of lycopene (1) tri-mixture (2)petroleum ether(3) dichloromethane (4) ethyl acetate (EA) (5) hexane (6) standard lycopene , (L) lycopene and (C) carotene

Thin layer chromatography analysis is cheaper than HPLC and multiple samples can be analysed simultaneously on the same plate under the same conditions. However, it is less accurate than HPLC, and only indicates the presence of lycopene, rather than the amount.

4.2.2 High performance liquid chromatography (HPLC) of lycopene

The structure and composition of the carotenoids influence their separation by HPLC, allowing qualitative and quantitative analysis. A photodiode array detector records the absorption spectrum of each component in the chromatogram.

HPLC analyses were done using a C18 column on standard lycopene and samples obtained by extraction from tomato paste with different combinations of solvents. Two different combinations of mobile phases for HPLC were initially used: 42:42:16(v/v/v) acetonitrile, methanol and dichloromethane; and 3:1 (v/v) acetonitrile and dichloromethane. The UV-Vis spectra for lycopene gave a maximum adsorption at 472 and 480 nm, which is the same as reported by Davis *et al.* (2003) and Roh *et al.* (2013).

The chromatogram of the standard lycopene shows the standard lycopene peak occurs at 7.5 minutes (Figure 4-11) using a mobile phase of 3:1 acetonitrile: dichloromethane.
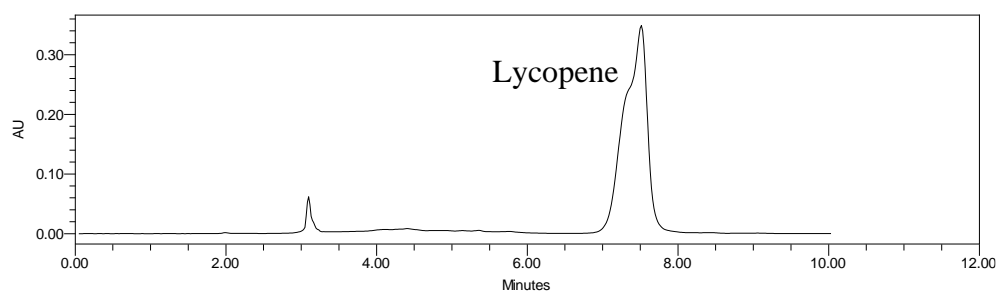
Figure 4-11: Chromatograph of standard lycopene in ethyl acetate, concentration 500 µg/mL with a mobile phase 3:1 acetonitrile: dichloromethane

4.2.2.1 HPLC calibration

For HPLC calibration, a mobile phase of 3:1 (v/v) acetonitrile: dichloromethane was used, and dilutions prepared from a stock solution of 500 µg/ml lycopene (Sigma) dissolved in ethyl acetate.

Calibration of the standard lycopene (Sigma Aldrich) was linear for 100-500 µg/mL (Figure 4-12) and can be expressed as

$$\text{Peak area} = 13451\text{x lycopene µg/mL} \quad R^2 = 0.9918$$

Figure 4-12: HPLC C18 calibration curve for lycopene standards dissolved in ethyl acetate. Running buffer was acetonitrile and dichloromethane

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4.2.2.2 HPLC results from batch lycopene extractions

Lycopene in the tomato paste was identified by comparing the retention times of pigment in the extraction mixture with the standard lycopene (Sigma Products) (Figure 4-11). Each peak represents a distinct component present in the sample.
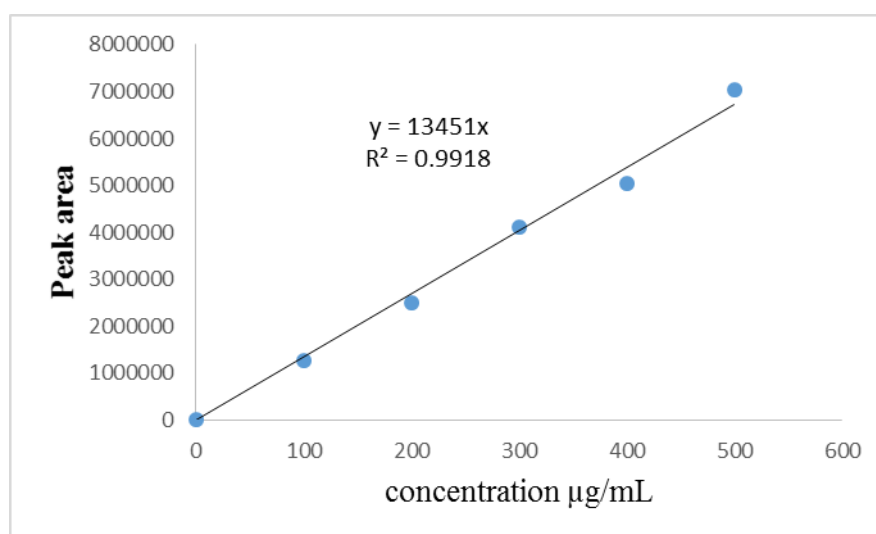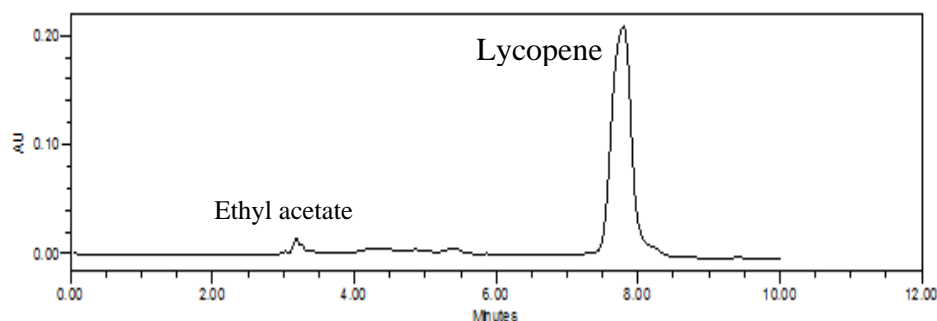

Figure 4-13: HPLC chromatogram of extract with ethyl acetate, HPLC condition were mobile phase: 3:1 (v/v) acetonitrile/dichloromethane at flow rate 1 mL/min; detection at 480 nm.

The first peak in the chromatograph obtained from the ethyl acetate extraction (Figure 4-13) is ethyl acetate. The lycopene peak was sharp and clearly defined. No other compounds or contaminants were detected. This chromatogram was similar to the chromatogram for the lycopene standard (Figure 4-11)

For the chromatogram for the dichloromethane, the first peak is dichloromethane and lycopene peak was irregular, (Figure 4-14) indicating the presence of cis-isomers and other carotenoid compounds.
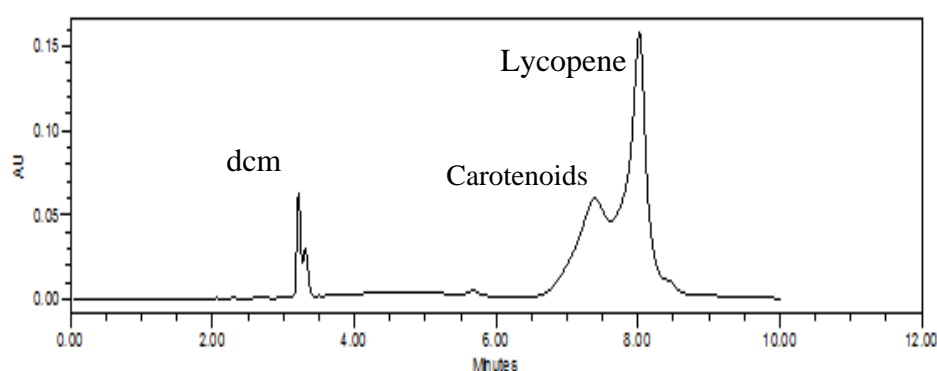
Figure 4-14: Chromatogram when tomato paste was extracted with dichloromethane (dcm).

Extracted lycopene using petroleum ether shows only a small amount of lycopene and other carotenoids. The first peak was petroleum ether and the second peak includes all carotenoids including a small peak for lycopene (Figure 4-15). Lycopene was eluted after the α- and β-carotenes.

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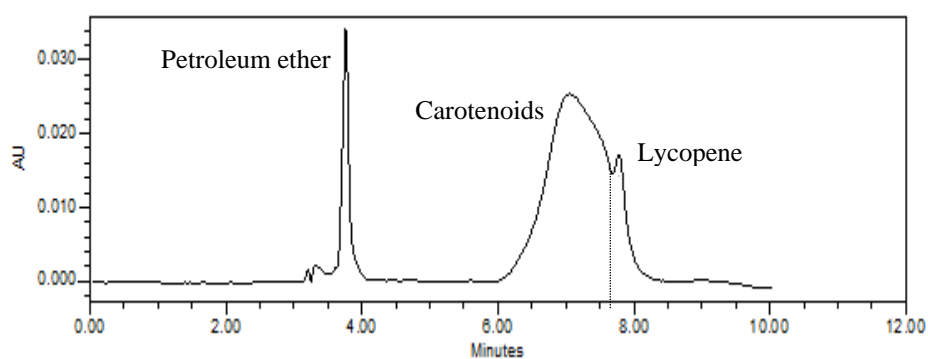
Figure 4-15: Chromatogram when tomato paste was extracted with petroleum ether

The chromatograph when lycopene was extracted from tomato paste with n-Hexane (Figure 4-16) has a broad lycopene peak which has a shoulder and can be resolved as two peaks.
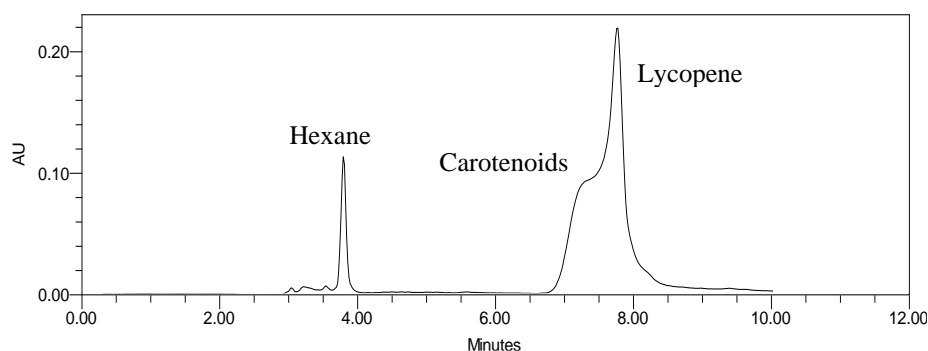
Figure 4-16: Chromatogram when tomato paste was extracted with n-Hexane

Lycopene extracted with a tri-mixture of 2:1:1 n-hexane, acetone, ethanol showed a large broad peak for lycopene (Figure 4-17) indicating that mixture of all-trans-lycopene, minor amounts of cis-isomers and other carotenoids including β-carotene had been extracted.
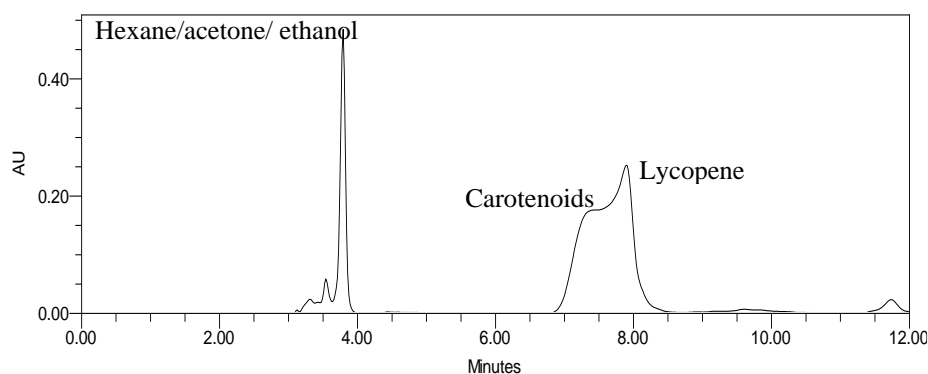
Figure 4-17: Chromatogram when tomato paste extracted with tri-mixture of 2:1:1n-Hexane: acetone: ethanol

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The HPLC retention time for lycopene for all extractions was 7.6-7.8 min. The retention times and peak areas for all solvents used for extracting carotenoids from tomato paste were reproducible (Table 4-1). Run times were short at 12 minutes and preparation was simple.

Table 4-1: Retention time and peak area of lycopene in extracted solvents in HPLC

Solvents	Retention time (minutes)	Peak area
Ethyl acetate	7.6	14483517
Tri-mixture	7.8	11035836
Dichloromethane	7.6	8681867
n-Hexane	7.6	8120308
Petroleum ether	7.6	1565527

The extraction yields for lycopene using different solvents are shown in Table 4-2. The colour of the solvent extract from tomato paste varied from light yellow to dark red. The yields were calculated by dividing measured lycopene by the reported lycopene content for the tomato paste supplied (48 mg per 100 g wet paste or 480 µg/ g).

Table 4-2: Batch solvent extraction results for 5 g tomato paste in 10 ml of solvent. Yield calculated based on labelled lycopene concentration of 480 µg/ g

Solvent	Peak Area (A)	Lycopene conc. (ug/ml)	Lycopene total mass (ug)	Extracted conc (ug/g wet tomato paste	% Yield
		(peak A /Y)	(conc. × V)	mass / wt.	
Ethyl acetate	14483517	1076.76	10767.61	2153.52	408.65
Dichloromethane	8681867	645.44	6454.44	1290.89	268.93
Petroleum ether	1565527	116.39	1163.87	232.77	48.49
Hexane/acetone/ ethanol (2:1:1)	6631793	493.03	4930.33	986.07	205.43
n-Hexane	8120308	603.70	6036.95	1207.39	251.54

The best extraction was from ethyl acetate at 2153 µg/g tomato paste which was 4.5 times greater than the stated concentration. In checking with the supplier, they also included tomato skin in the tomato paste, which has a lycopene concentration of up to 1200 µg/g. Rao and Agarwal (1999) report lycopene concentrations in tomato paste of up to 1500 µg/g. The higher yield could be because the C18 column did not resolve the lycopene from the other carotenoids in the extract, but the appearance of a shoulder or tail on the peak would have been expected, but was not observed for the ethyl acetate extract. Dichloromethane, hexane: acetone: ethanol, and n-hexane all gave similar results of 200 to 268 µg/g tomato paste, while petroleum ether gave very low extraction. From these results, it was concluded that ethyl acetate was the best solvent to use, and was used as the selected solvent for further extraction and immobilization of lycopene.

4.3 Soxhlet extraction

The extract from Soxhlet extraction of lycopene from tomato paste powder with ethyl acetate was reddish and a yellowish extract when petroleum ether was used (Figure 4-18). This indicated that ethyl acetate extracted all carotenoids including lycopene, but petroleum ether was less efficient.
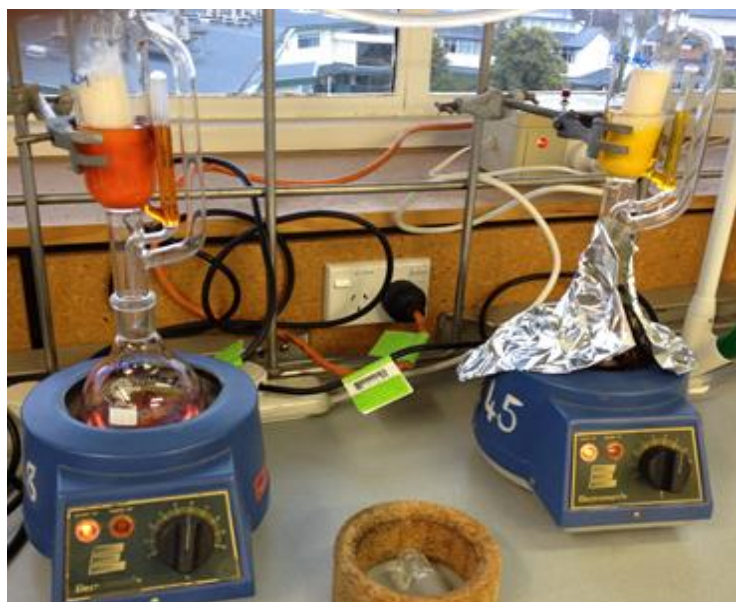

**Figure 4-18: Soxhlet extraction of lycopene from tomato paste with
ethyl acetate (left) and petroleum ether (right)**

Ethyl acetate (CH_3-COO-CH_2-CH_3) is a colorless liquid with a characteristic sweet smell. It is synthesized mainly via the classic Fischer esterification reaction of ethanol and acetic acid. Because of its low toxicity, it is used in pharmaceutical preparations, food additives and fragrances. Its boiling point is 77°C and it is miscible with water and ethanol. It has been approved for use in food products by the U.S. Food and Drug Administration and biodegrades completely into CO_2 and water (Ishida & Chapman, 2009).

After extraction the tomato paste powder had a lighter colour due to the removal of the carotenoids (Figure 4-19)
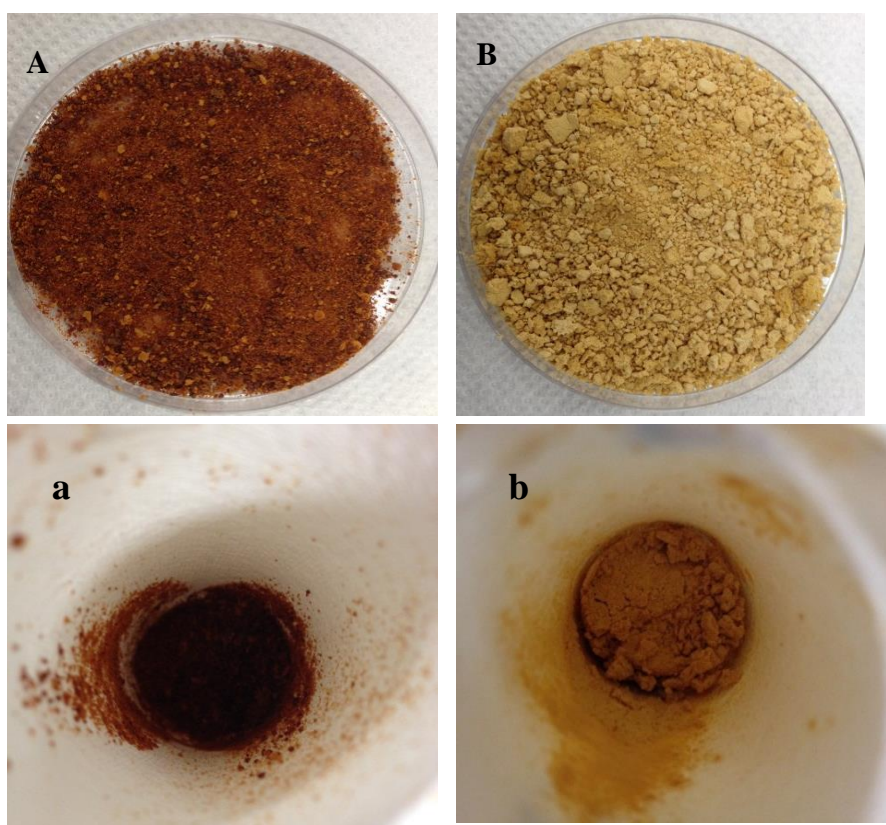
Figure 4-19: Tomato paste powder in petri-dish (A) and cellulose thimbles (a) before (A a) and after (B b) extraction with ethyl acetate.

4.3.1 Analysing and identifying extracted lycopene

The extracted solvents were evaporated in a Rotavapor evaporator. When the extracted material became a thick concentrate, it was transferred to a petri dish and left in a fume hood at room temperature, until the extract was completely dry. The colour of the extract was different and dependent on the solvents (Figure 4-20).

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The results show that ethyl acetate is an excellent solvent for extracting of trans–and cis-isomers of lycopene and β-carotene. It has low toxicity compared to the other organic solvents.

Petroleum ether

Petroleum ether
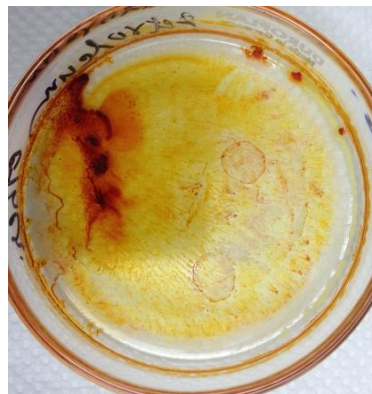
Hexane + ethanol
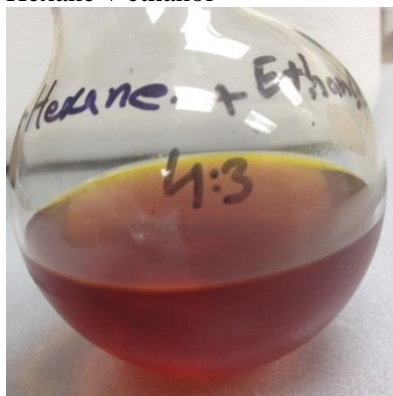
Hexane + ethanol
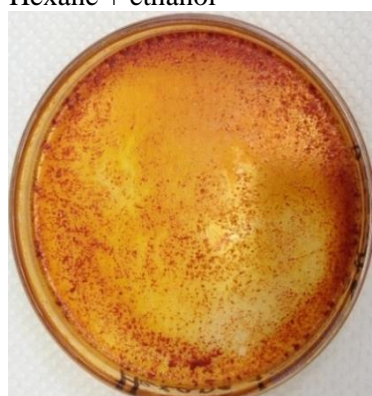
Hexane + ethyl acetate
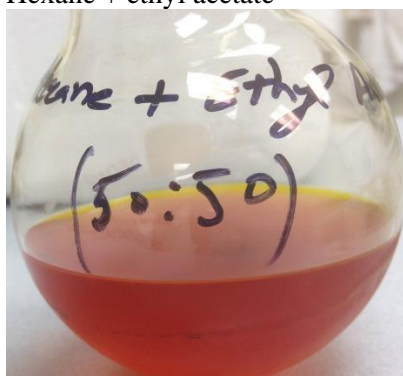
Hexane + ethyl acetate

Figure 4-20: Extracted lycopene solution from tomato paste before (left) and after the solvent evaporation (right)

Extracted material resulting from extraction using n-Hexane and dichloromethane became completely dry using a Rotavapor evaporator, without being transferred to petri dishes (Figure 4-21).
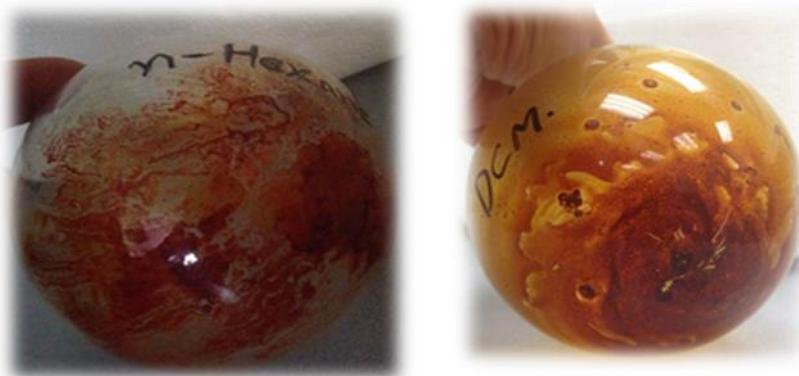
Figure 4-21: Extracted lycopene from tomato paste using n-Hexane and dichloromethane

The HPLC chromatogram of the product after Soxhlet extraction with ethyl acetate had a sharp peak for lycopene (Figure 4-22) using a mobile phase of 42/42/16 (v/v/v) acetonitrile: methanol: dichloromethane. Lycopene appears to have eluted earlier (retention time 5.7) than when a mobile phase of 3:1 acetonitrile: dichloromethane was used for the lycopene standard (which had a retention time of 7.6 minutes).
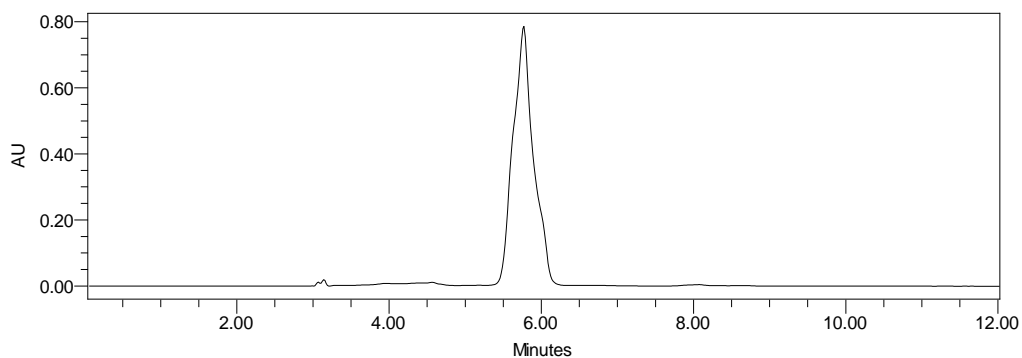
Figure 4-22: Chromatograph of Soxhlet extract of lycopene from tomato paste in ethyl acetate using a mobile phase: 42/42/16 (v/v/v) acetonitrile/methanol/dichloromethane

Chromatogram for ethyl acetate extracts of tomato paste powder along with retention times are shown in Figure 4-23.
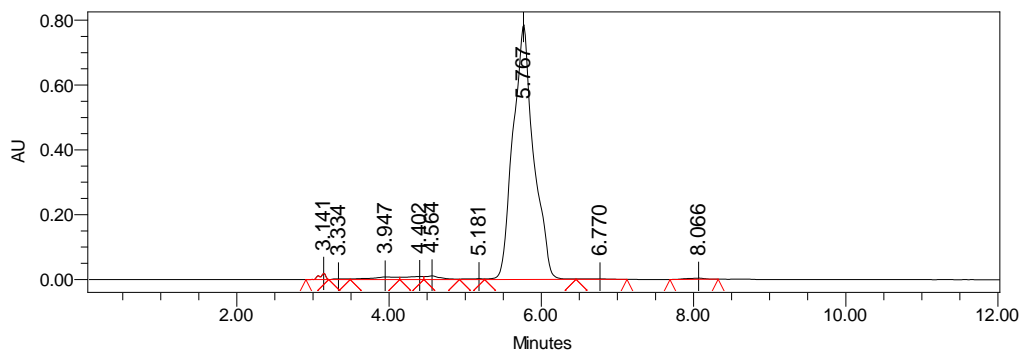
Figure 4-23: Chromatograph of lycopene in ethyl acetate showing integration and retention times (mobile phase of 42/42/16 (v/v/v) acetonitrile/methanol/dichloromethane)

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Comparing the area of the lycopene peak with standard compounds, lycopene recovery was highest for ethyl acetate. Yields from the Soxhlet extraction are shown in Table 4-3.

Table 4-3: Soxhlet solvent extraction results for 5 g tomato paste powder in 150 ml of solvent. Yield calculated based on labelled lycopene concentration of 480 µg/ g

Solvent	Volume of solvent (ml) after extraction	Peak area	Concentration (µg /ml)	Mass lycopene (mg)	Extracted concentration (µg/g wet tomato paste)	Yield %
n-Hexane	100	9842522	223.11	22.31	880.00	183
Dichloromethane	120	8784720	199.13	23.90	949.17	197
Ethyl Acetate	104	14754163	334.44	34.78	1391.10	289
Petroleum ether	100	4442094	100.69	10.07	397.41	82
Ethyl Acetate: Hexane(1:1)	110	4580452	103.83	11.42	453.25	94
Ethyl Acetate: Ethanol 1:1)	120	10148578	230.04	27.61	1103.81	229
Hexane: Acetone: Ethanol (2:1:1)	120	9891179	224.21	26.91	1075.13	223
Hexane: ethanol (4:3)	110	8742460	198.17	21.80	858.42	178

All other solvents and solvent mixtures n-Hexane, dichloromethane, petroleum ether, (1:1) ethyl acetate: n-Hexane, (1:1) ethyl acetate: hexane, (2:1:1) n-hexane: acetone: ethanol and (4:3) n-Hexane: ethanol resulted in peak integration as other compounds and carotenoids were also extracted (Figure 4-24 and Figure 4-25).
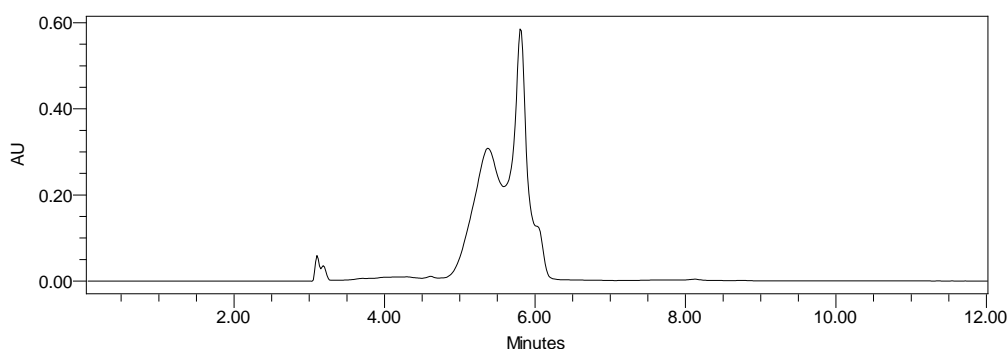
Figure 4-24: Chromatogram of Soxhlet extract of tomato paste powder with dichloromethane (mobile phase of 42/42/16 (v/v/v) acetonitrile/methanol/dichloromethane)
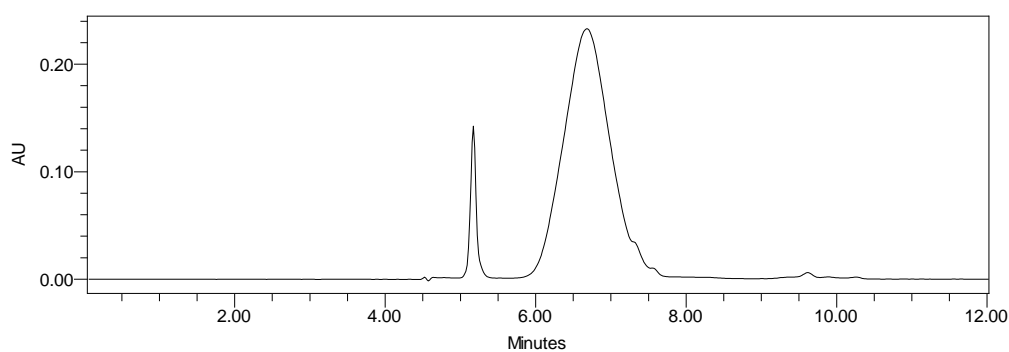
Figure 4-25: Chromatogram of Soxhlet extract of tomato paste powder with n-Hexane mobile phase of 3:1 Acetonitrile: dichloromethane)

4.4 Discussion

The extracted concentration of lycopene was greater than that stated on the label on the tomato paste. It is possible that the lycopene and other carotenoids in the tomato paste became more bioavailable due to prolonged heating during Soxhlet extraction, or that the C18 column and conditions used were not able to resolve the lycopene from the other carotenoids.

In other research, a C30 reversed phase column was used to separate the two lycopene peaks, as a cis isomer peak followed by the all-trans peak (Clinton *et al.* (1996) (Figure 4-26). The data in that research shows a predominance of the cis-isomers and the possibility that multiple geometric isomers of lycopene were extracted. Therefore C30 columns would be more effective in the separation of isomer mixtures of lycopene and carotenoids.
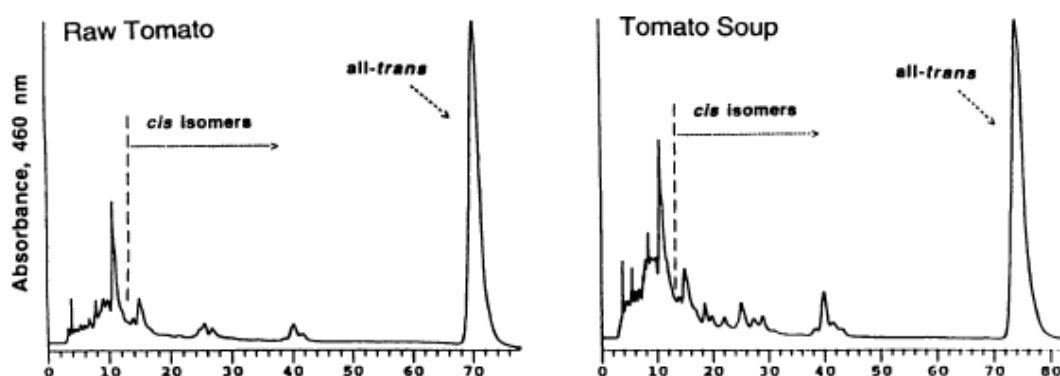
Figure 4-26: Chromatograms illustrating cis and trans isomers of lycopene in fresh tomato & tomato soup, HPLC used a C 30 reversed phase column (Clinton *et al.*, 1996).

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Lycopene exists as different isomers such as 15-cis-, 13-cis-, 9-cis-, and all-trans-lycopene. The predominant isomer is all-trans in fresh tomato (Figure 4-27), but is isomerized from trans to the cis-form by heat, light and some chemical reactions (Clinton *et al.*, 1996). The concentration of the all-trans isomer decreases under heat used when manufacturing tomato paste, and the concentration of cis-isomer first increases (due to isomerization of trans-isomer to the cis-form) but then decreases after two hours.
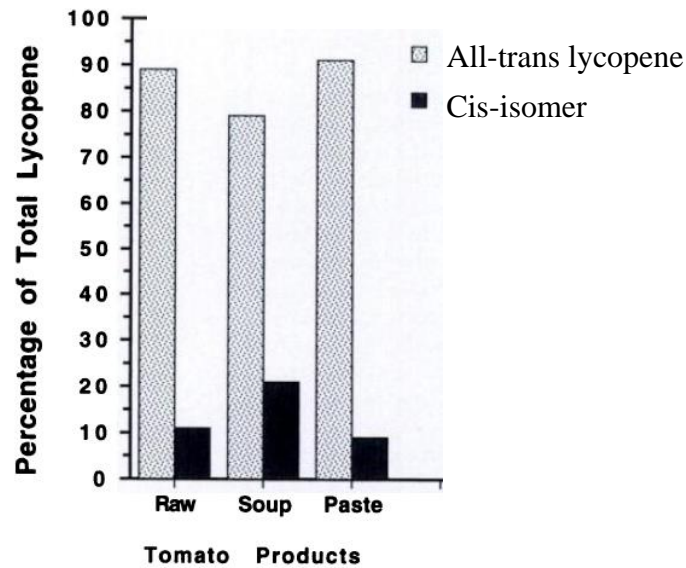
Figure 4-27: The proportion of cis & trans lycopene in tomato products (Clinton *et al.*, 1996).

Published research showed that all-trans lycopene accounts for 79 to 91% and cis lycopene isomers for 9 to 21% of total lycopene in tomatoes, tomato paste, and tomato soup (Clinton *et al.*, 1996; Yeum *et al.*, 1996). The distribution of trans and cis-isomers of lycopene depends on the solvent and the process temperatures (Figure 4-28 and Figure 4-29).
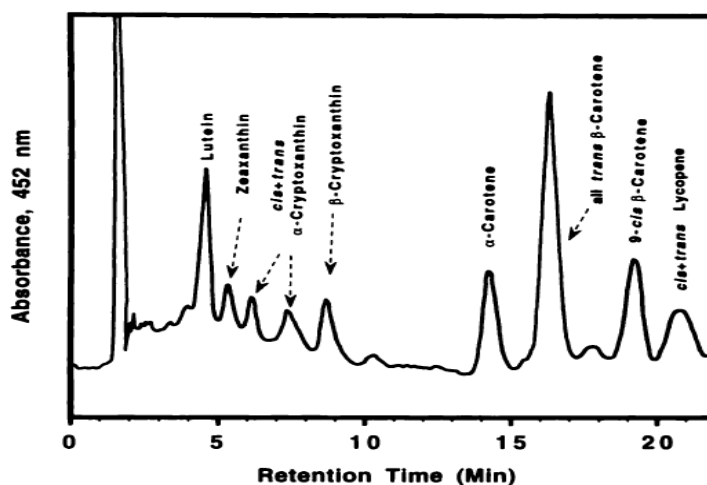
Figure 4-28: HPLC separation of carotenoids using a C30 reverse phase column (Clinton *et al.*, 1996)
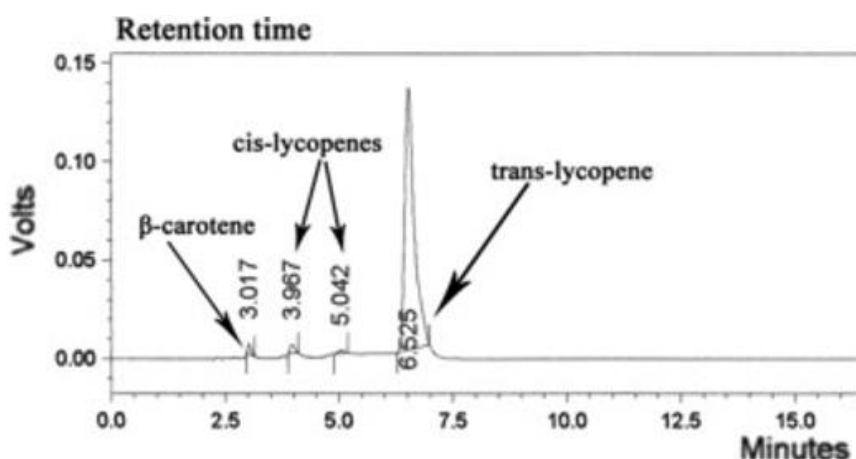
Figure 4-29: C30 RP-HPLC column chromatograph of tomato extract using isocratic mobile phases of methanol/MTBE/ethyl acetate (30:60:10, v/v/v) at 501 nm with flow rate of 1.2 mL/min (Eh & Teoh, 2012a)

Barba *et al.* (2006a) used a μBondapack C18 column for HPLC analysis of a carotenoid standard solution and tomato extract using hexane/acetone/ethanol for extraction. The resulting chromatographs are shown in Figure 4-30 and Figure 4-31 (Barba *et al.*, 2006a). The C18 column (300 mm × 2 mm, 10 μm pore size) was used with a μBondapack C18 pre-column (20 mm × 3.9 mm, 10 μm pore size). The mobile phase was methanol/ACN 90:10 (v/v) + Tri-ethylamine (TEA) 9 μM, and the flow rate was 0.9 ml/min. At a column temperature of 30°C and detection wavelength of 475 nm, reliable results were obtained. The peaks of lycopene and β-carotene in the standard solution were found to be 5.18 and 6.03 (retention time) respectively, while those in the tomato extract were 5.18 and 6.18 respectively (Barba *et al.*, 2006a).

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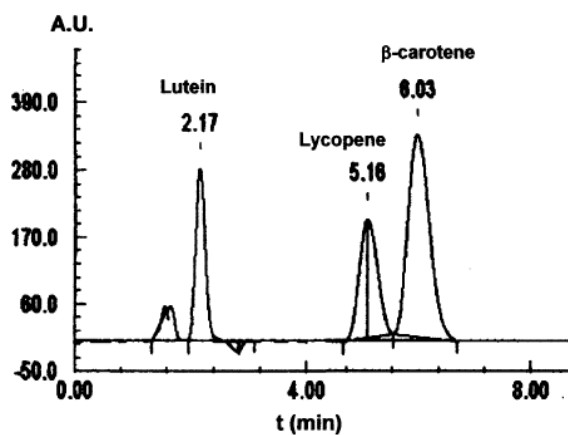
Figure 4-30: µBondapack C18 column chromatogram of carotenoid standard solution (Barba *et al.*, 2006a).
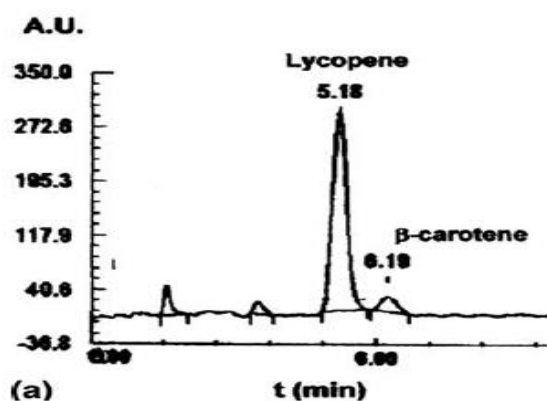
Figure 4-31: C18 column chromatograph of tomato extract (Barba *et al.*, 2006a).

A pre-column as described by Barba *et al.* (2006) was not used in this research. The analysis was performed isocratically at a flow rate of 1mL min^{-1} at 22°C. The lower operating temperature, as well as using a different mobile phase (42:42:16 acetonitrile: methanol: dichloromethane), would also contribute to differences in the results (Figure 4-32 and Figure 4-33).
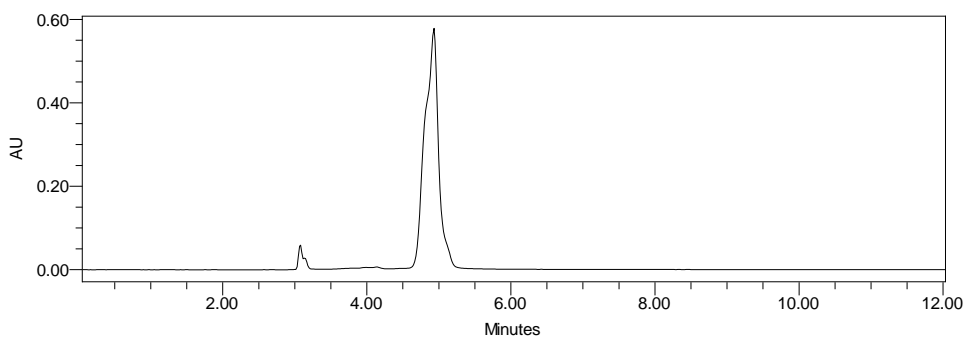
Figure 4-32: Standard lycopene stock solution in DCM with mobile phase 42:42:16 (v/v/v) Acetonitrile: methanol: dichloromethane

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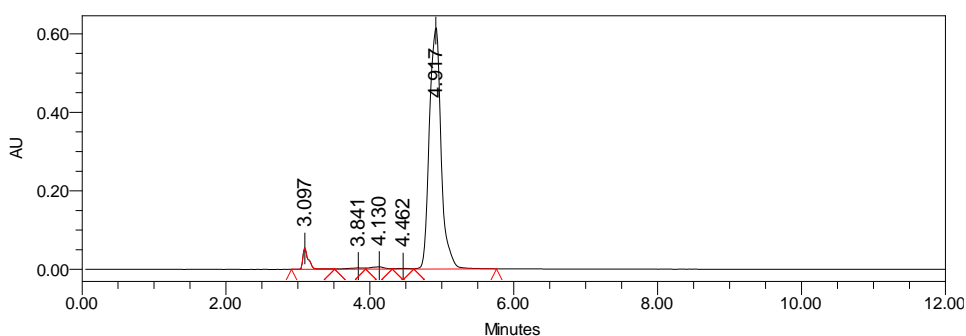
Figure 4-33: Standard lycopene in DCM with integration, mobile phase consisting of 42:42:16 Acetonitrile: methanol: dichloromethane.

4.5 Encapsulation

When extracted lycopene was mixed with 4% sodium alginate solution it was not completely dissolved and a separate solvent layer formed, because the organic solvents were immiscible in the alginate. This was partly expected as alginate is a polysaccharide dissolved in a polar solution, while the ethyl acetate is only soluble in water up to 8.3 g per 100 ml. A 1% agar-agar solution was added to the alginate solution, which enabled the solvent solution to mix with the alginate and make the lycopene-alginate-agar mixture.

To identify the optimum sodium alginate concentration for immobilization, sodium alginate with varying concentration ranging from 1-4% was used to acquire beads with greater stability. The beads were prepared using 0.2 M $CaCl_2$ solutions as describe earlier in chapter 4. With the 1 and 2% alginate solutions, the beads were not properly formed and they were dissolving in the $CaCl_2$ solution. This was because there was insufficient alginate to form strong cross links to produce a stable gel. Beads of sufficient strength were formed when a 4% sodium alginate concentration was used giving more stiffness and shape to the beads.

The beads were coated with chitosan to improve their stability in acidic pH. Because alginate is an anionic polymer with carboxyl end groups, it forms strong complexes with poly-cations such as chitosan, resulting in a stronger more stable bead.

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4.5.1 Bead analysis

A scanning microscope (Image-pro plus 6.3 supports with Nikon digital camera) was used to examine morphology of the beads at 6.4 and 16 times magnification lens (Figure 4-34). The Image-Pro plus 6.3 programs was connected with the camera so that results could be observed and saved.

The chitosan/alginate-agar beads were spherical with a smooth texture size of 2-4 mm (Figure 4-35).
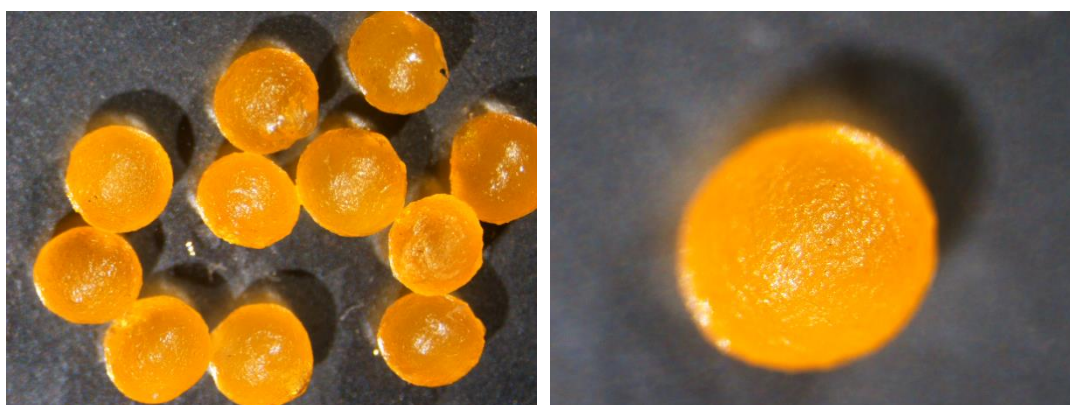
Figure 4-34: Lycopene alginate-agar-chitosan beads under magnification.

Figure 4-35: Chitosan coated lycopene alginate-agar beads

4.6 Evaluation of lycopene release

The release of lycopene from the beads was evaluated to determine the effect of the chitosan coating, sodium alginate concentration, pH levels and temperature.

4.6.1 Effect of chitosan coating

Beads with and without chitosan coating were kept in containers under the same conditions. After a few days, it was observed that the beads without the chitosan coating decreased in size and elasticity indicating they were drying out, while the beads with the chitosan coating did not shrink and maintained their soft texture. The chitosan coating protected the contents of the bead against drying. This is because a chitosan-alginate complex is water insoluble. From other research, the properties of chitosan-alginate films vary when prepared with chitosan of different molecular weights. Films prepared with low-molecular-weight chitosan (Mv 1.30 $\times$ 10^5) were twice as thin, transparent, and 55% less permeable to water vapour than films prepared with high-molecular-weight chitosan (Mv 10×10^5). It was inferred the low-molecular weight chitosan reacted more completely with the sodium alginate (Mv 1.04×10^5) than chitosan of higher molecular weight (Yan *et al.*, 2001; Sinha *et al.*, 2004; Struszczyk, 2004).

4.6.2 Effect of pH and temperatures

After beads had been immersed in a buffer solution, lycopene release was determined by measuring absorbance of the solution at 10 min intervals. 1 gm of lycopene alginate–agar beads (containing 182.1 μg per g of beads) was used in 9 ml of buffer solution.

Release of lycopene from uncoated beads at pH 6.83 (the pH in the intestine) was most rapid for the first 20 minutes. Release rate was greater at 37°C than at 25°C (Figure 4-36 and Figure 4-37). By 60 minutes, almost same amount of lycopene had been released regardless of temperature. This was because alginate is a polysaccharide and is susceptible to degradation and dissolving at elevated temperatures.
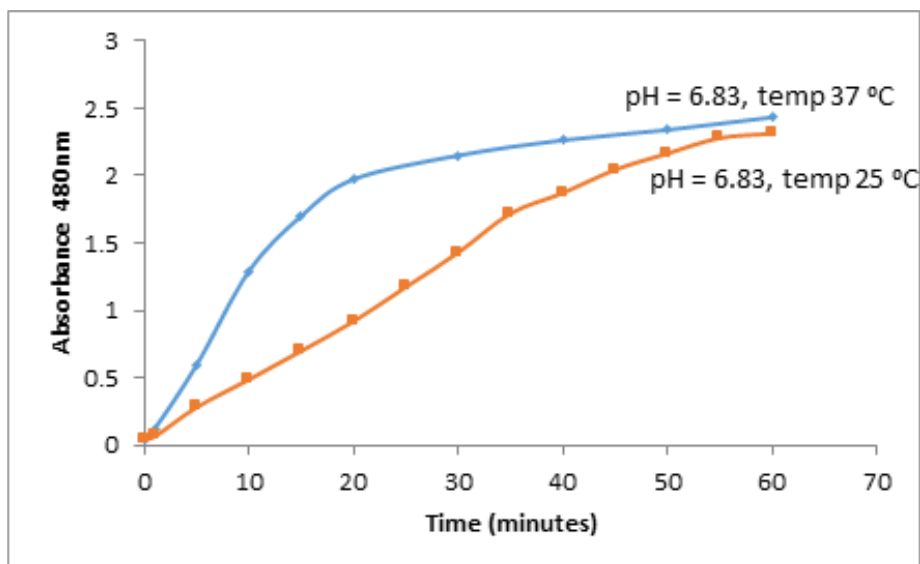
Figure 4-36: Effect of temperature on release from 4mm alginate-beads without chitosan coating, at pH 6.83

Effect of pH (pH 4.4 and pH 3.3) on lycopene release from 3mm lycopene-alginate beads without chitosan coating at 25°C is shown in Figure 4-37. The release is slower at pH 3.3 at 25°C.
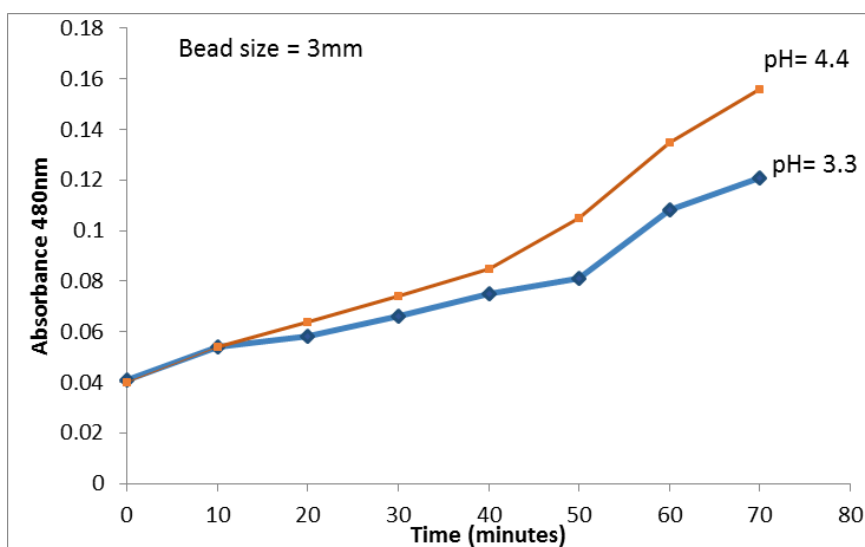
Figure 4-37: Effect of pH (pH 4.4 and pH 3.3) on release from 3mm lycopene-alginate beads without chitosan coating at 25°C

Lycopene release was more efficient at a pH of 6.60 (intestinal pH) at 37°C (body temperature) whereas very little was released at pH 2.36 (as in the stomach) at 25°C (room temperature) (Figure 4-38).
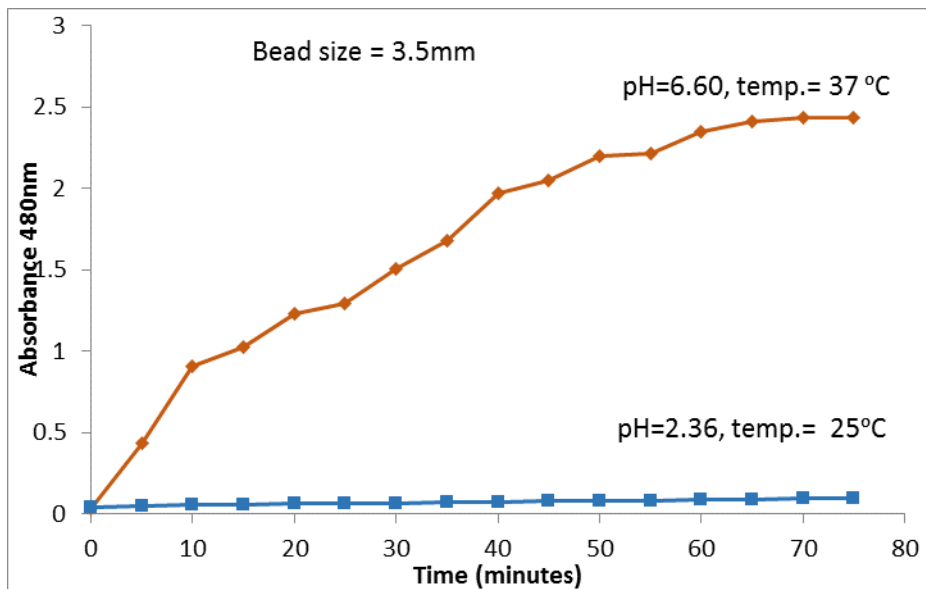
Figure 4-38: Graph of release of lycopene in different pH (2.36 and 6.60) and temperature 25°C and 37°C

Beads kept at the lower pH and temperature was still intact after one hour whereas beads kept at the higher pH (in intestine) and temperature was dissolved after 1-hour (Figure 4-39).

| 1g beads in PBS Not dissolved at pH 2.36 temp.25°C after 1 hour | Completely dissolved at pH 6.6 temp.37°C after 1 hour |
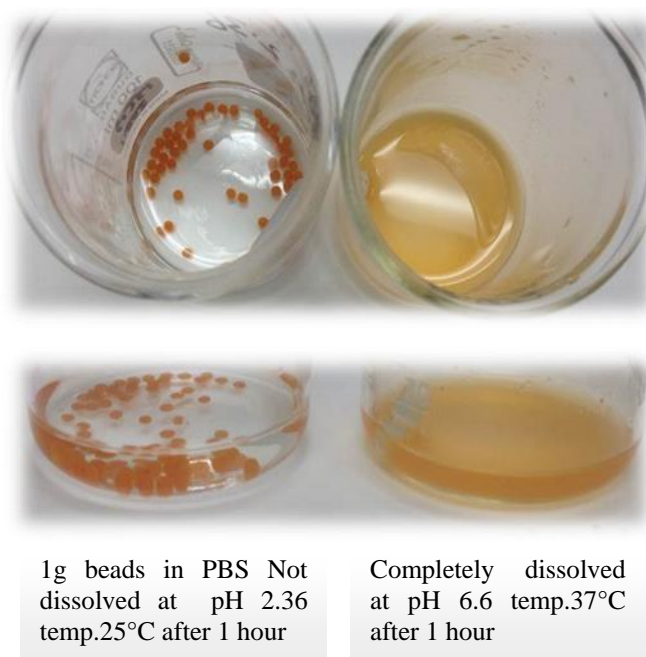
Figure 4-39: Comparison of lycopene release from alginate-chitosan coated lycopene beads at pH 2.36, 25°C and pH 6.60, 37°C

Lycopene release was tested at pH 2.23 and pH 6.83 at 25°C and 37°C. The results shows release was greater at 37°C than 25°C for both pH levels (Figure 4-40 and Figure 4-41).

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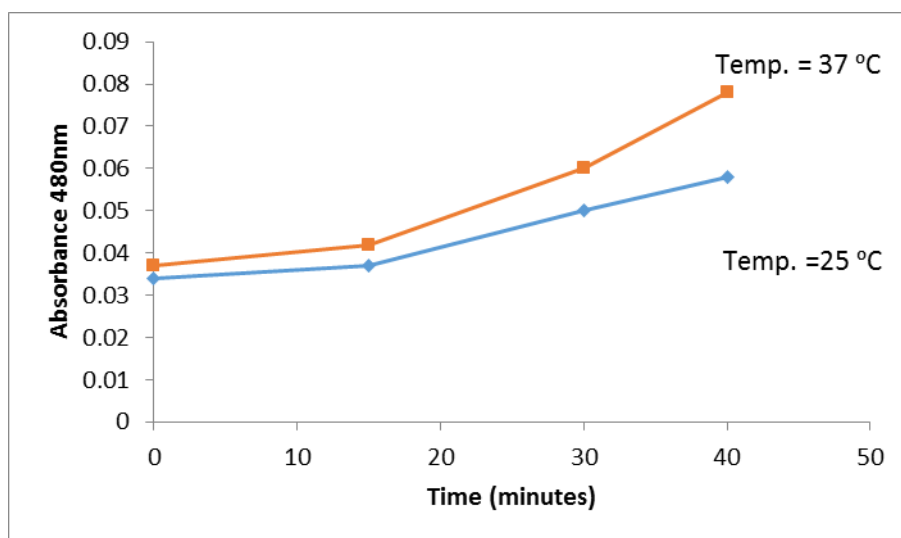
Figure 4-40: Lycopene-alginate-chitosan coated beads release at pH 2.23 and different temperature
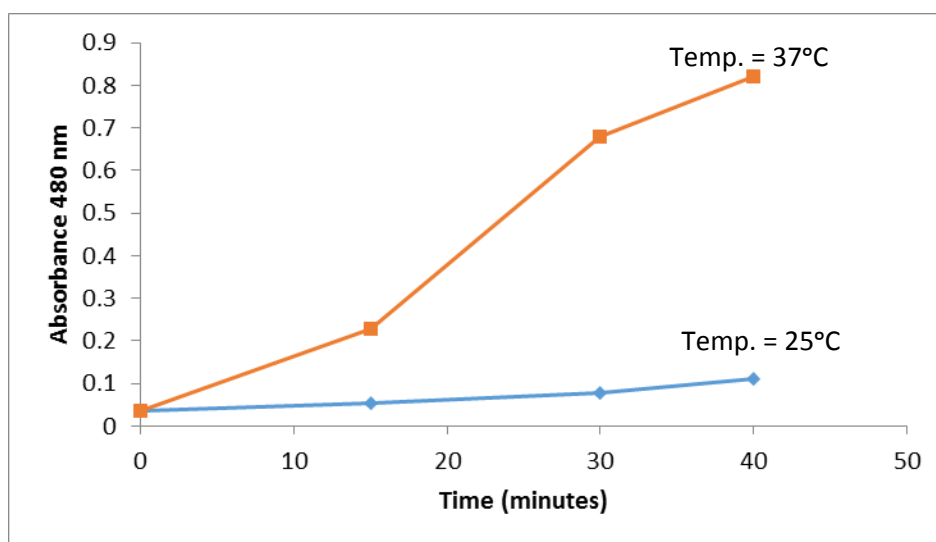
Figure 4-41: Lycopene-alginate-chitosan coated beads release at pH 6.83 and different temperatures

Lycopene release from chitosan-coated beads at 37°C was higher at pH 6.60 than at pH 2.36. Chitosan-coated alginate beads reduced the diffusion rate of the encapsulated substance and had maximum stability and lycopene retention under the simulated gastrointestinal pH 6.60 of 37°C (Figure 4-42).
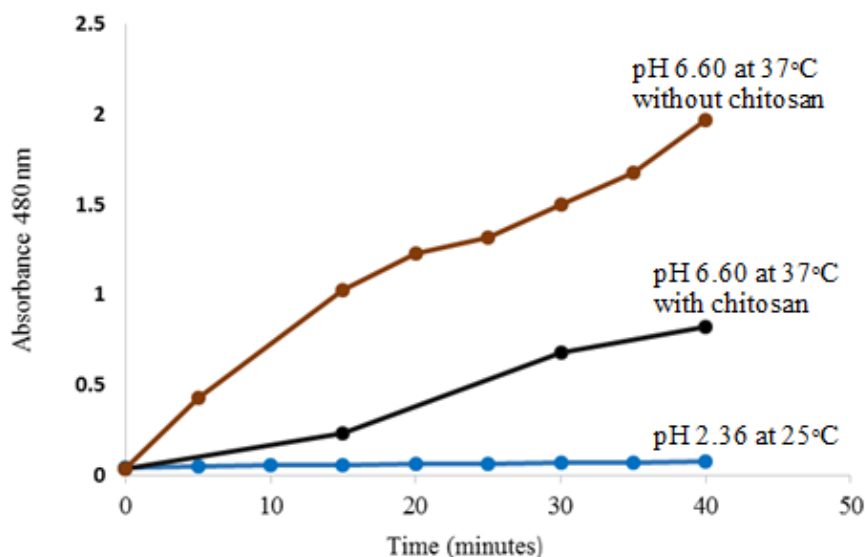
Figure 4-42: Lycopene release from coated and uncoated chitosan beads at 37°C and 25°C

Alginate–chitosan complexes can be useful in oral drug delivery systems because alginate alone dissolves at high pH such as in the intestine, and shrinks at lower pH levels (as in the stomach). Chitosan is insoluble at higher pH levels and dissolves when the pH is lower. Therefore a chitosan coating enables the alginate to pass through the acidic stomach and reach the intestine before its contents are released (Takka & Gürel, 2010).

When mixed, the carboxyl residues of alginate and the amino groups of chitosan ionically interact to form the polyelectrolyte complex. Complexes of chitosan with alginate reduce porosity of alginate beads and decrease leakage of the encapsulated drugs. The alginate beads without a chitosan coating shrank after a few days, whereas beads with a chitosan coating did not change. The breakdown of chitosan at low pH levels is prevented by the alginate network because alginate is insoluble under low pH conditions, as it was also mentioned by George & Abraham (2006).

A variety of synthetic and natural polymers has been studied as drug carriers, have capitalized on the many hydrophobic and hydrophilic components and the polymer–polymer, polymer–drug, polymer–solvent, or polymer–physiological medium interactions that can occur. While there are almost limitless combinations of materials to explore, engineers are restricted by material biocompatibility, toxic by-products and manufacturing cost. Lycopene extracted from tomato paste, incapsulated in alginate/agar and chitosan, represents a simple and easy method of controlled lycopene delivery that is non-toxic.

Chapter 5

Conclusions and Recommendations

5.1 Isolation of lycopene

Conventional solvent extraction methods were used to extract lycopene from tomato paste. Soxhlet extraction was the selected method for this research because it requires less solvent and shorter extraction times than other methods. And it is suitable for the extraction of lycopene from tomato paste.

Different solvents were tested. Ethyl acetate had the best extraction and efficiency. The HPLC chromatogram had a sharp peak for lycopene, indicating no other components or contaminants were present. Other organic solvents extracted other compounds as well as lycopene. Ethyl acetate is favoured because of its low cost, low toxicity, and agreeable odour.

Analysis of extracted samples by thin layer chromatography showed the presence of lycopene as well as α and β-carotenoids. HPLC was done to determine the concentration and purity of extracted lycopene. The optimal mobile phase for HPLC analysis was found to be 3:1 (v/v) acetonitrile: dichloromethane. It gave a good chromatographic response and efficient separation of lycopene within a short retention time.

A non-polar C18 chromatographic column was used as the stationary phase, but the resulting chromatograph did not have separate peaks for cis and trans isomers of lycopene, and carotenoids. Lycopene extracted from tomato paste was greater than that stated on the label, possibly because the lycopene in the tomato paste became more bioavailable due to heating during Soxhlet extraction. Results obtained indicated that lycopene content was greater than 1500 μg/g dry matter (as it mention in litrature review table 2-2). Urbonaviciene *et al.* (2012) reported the concentration of lycopene in blanched tomato skin as 135 mg/100 g (1350 μg/g), whereas the label on the tomato paste claimed lycopene 48 mg/100 g.

5.2 Encapsulation of lycopene

A pH and temperature-sensitive hydrogel bead of Ca-alginate and chitosan was prepared as a carrier for lycopene delivery. Sodium alginate, agar, calcium chloride and chitosan were used in the preparation. The dissolving behaviour of the beads indicated they were sensitive to the external pH and temperature.

The chitosan-coated lycopene-alginate beads were more stable than alginate immobilized lycopene without chitosan. Lycopene release from the beads was affected by pH and temperature. The release rate was much faster at higher pH levels (such as in the intestine) than at lower pH levels (such as in the stomach). Release was also faster at 37°C (normal human body temperature) than that at 24°C (room temperature).

Alginate shrinks at low pH levels (such as the gastric environment) and the encapsulated drugs are not released. In gastric fluids, the hydrated sodium-alginate is converted into a porous, insoluble alginic acid layer. Once the beads pass into the higher pH of the intestinal tract, the alginic acid layer is converted to a soluble viscous layer. This pH dependent behaviour of alginate can be exploited to customize the release profiles of lycopene-containing beads. Having an intact matrix and lycopene being diffused out through the pores will be a suitable approach for lycopene delivery. The possible dissolution of alginate at higher pH levels is prevented by the chitosan coating which is stable at higher pH ranges.

These results indicate that Ca-alginate chitosan coated beads possess potential applications in drug delivery systems controlled by pH and temperature. The entrapment of lycopene in polymeric networks could be a viable way to reduce lycopene oxidation.

5.3   Recommendations

Tomato peels, usually eliminated during tomato processing as by-product, are a valuable source of carotenoids. Wastes from processing tomato products contain the carotenoid rich skins and the seeds, and are available in large quantities. Extracting lycopene from tomato processing wastes is economical and these by-products could become a cheap source or lycopene, carotenoids and/or natural oils for formulating new nutraceuticals, pharmaceuticals, and cosmetic products.

More research is needed to improve current methods of extraction and encapsulation of lycopene. Extraction could be improved by using renewable feedstock such as tomato peels and processing waste, thus reducing the amount of waste generated. Safer solvents such as ethyl lactate, which is biodegradable, should be used for the extraction.

Improved HPLC analysis of lycopene and alpha and beta carotenoids could be carried out using a C30 column. Unlike the C18 column used in this research, the C30 column can successfully separate the cis and trans isomers of lycopene, and therefore enable more efficient isolation. Clinton *et al.* (1996) reported it is possible to separate 12 to 13 geometric lycopene isomers with the C30 column.

In this research encapsulation of lycopene for controlled drug release was achieved with lycopene-calcium-alginate beads coated with chitosan. The lycopene was stabilised and protected from oxidation. Controlled release of lycopene from these beads was optimal in the pH and temperature conditions of the human intestine. More research is needed to investigate the large-scale production of such beads for the pharmaceutical industry.

References

Abano, E.E., Ma, H., & Qu, W. (2012). Influence of combined microwave-vacuum drying on drying kinetics and quality of dried tomato slices. *Journal of Food Quality, 35*(3), 159-168.

Agarwal, S., & Rao, A.V. (2000). Tomato lycopene and its role in human health and chronic diseases. *Canadian Medical Association Journal, 163*(6), 739-744.

Altan, A., McCarthy, K.L., & Maskan, M. (2008). Evaluation of snack foods from barley-tomato pomace blends by extrusion processing. *Journal of Food Engineering, 84*(2), 231-242.

Anal, A.K., & Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science & Technology, 18*(5), 240-251.

Anderson, D.M.W., Brydon, W.G., Eastwood, M.A., & Sedgwick, D.M. (1991). Dietary-effects of sodium alginate in humans. *Food Additives and Contaminants, 8*(3), 237-248.

Antioxidant. (2011). In *The American Heritage Dictionary of the English Language*. Boston, MA: Houghton Mifflin Harcourt.

Anwar, A., Qader, S.A.U., Raiz, A., Iqbal, S., & Azhar, A. (2009). Calcium alginate: a support material for immobilization of proteases from newly isolated strain of Bacillus subtilis KIBGE-HAS. *World Applied Sciences Journal, 7*(10), 1281-1286.

Ausich, R.L., & Sanders, D.J. (1999). Patent No. US5858700 A. Process for the isolation and purification of lycopene crystals.

Baldermann, S., Ropeter, K., Kohler, N., & Fleischmann, P. (2008). Isolation of all-trans lycopene by high-speed counter-current chromatography using a temperature-controlled solvent system. *Journal of Chromatography A, 1192*(1), 191-193.

Barba, A.I.O., Hurtado, M.C., Mata, M.C.S., Ruiz, V.F., & de Tejada, M.L.S. (2006a). Application of a UV-vis detection-HPLC method for a rapid determination of lycopene and beta-carotene in vegetables. *Food Chemistry, 95*(2), 328-336.

Barba, A.I.O., Hurtado, M.C., Mata, M.C.S., Ruiz, V.F., & Tejada, M.L.S.d. (2006b). Application of a UV−vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chemistry, 95*(2), 328-336.

Basu, A., & Imrhan, V. (2007). Tomatoes versus lycopene in oxidative stress and carcinogenesis: conclusions from clinical trials. *European Journal of Clinical Nutrition, 61*(3), 295-303.

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews, 62*(1), 83-99.

Blanch, G.P., Ruiz del Castillo, M.L., del Mar Caja, M., Pérez-Méndez, M., & Sánchez-Cortés, S. (2007). Stabilization of all-trans-lycopene from tomato by encapsulation using cyclodextrins. *Food Chemistry, 105*(4), 1335-1341.

Blandino, A., Macias, M., & Cantero, D. (1999). Formation of calcium alginate gel capsules: Influence of sodium alginate and CaCl< sub> 2</sub> concentration on gelation kinetics. *Journal of Bioscience and Bioengineering, 88*(6), 686-689.

Borguini, R.G., & Torres, E. (2009). Tomatoes and tomato products as dietary sources of antioxidants. *Food Reviews International, 25*(4), 313-325.

Bowman, R.G., Rueb, C.J., Finney, J.M., Hendrickson, W.A., Rao, C.S., Bentley, N.M., & Herreid, R.M. (2011). Patent No. US20110020519 A1. Encapsulation of oxidatively unstable compounds.

Cámara, M., de Cortes Sánchez-Mata, M., Fernández-Ruiz, V., Cámara, R.M., Manzoor, S., & Caceres, J.O. (2013). Lycopene: A review of chemical and biological activity related to beneficial health effects. In R. Atta ur (Ed.), *Studies in Natural Products Chemistry* (Vol. 40, pp. 383-426): Elsevier.

Chiu, Y.T., Chiu, C.P., Chien, J.T., Ho, G.H., Yang, J., & Chen, B.H. (2007). Encapsulation of lycopene extract from tomato pulp waste with gelatin and poly(gamma-glutamic acid) as carrier. *Journal of Agricultural and Food Chemistry, 55*(13), 5123-5130.

Choksi, P.M., & Joshi, V.Y. (2007). A review on lycopene - extraction, purification, stability and applications. *International Journal of Food Properties, 10*(2), 289-298.

Choudhary, R., Bowser, T.J., Weckler, P., Maness, N.O., & McGlynn, W. (2009). Rapid estimation of lycopene concentration in watermelon and tomato puree by fiber optic visible reflectance spectroscopy. *Postharvest Biology and Technology, 52*(1), 103-109.

Clinton, S.K., Emenhiser, C., Schwartz, S.J., Bostwick, D.G., Williams, A.W., Moore, B.J., & Erdman, J.W. (1996). cis-trans lycopene isomers, carotenoids, and retinol in the human prostate. *Cancer Epidemiology Biomarkers & Prevention, 5*(10), 823-833.

Colle, I.J.P., Lemmens, L., Tolesa, G.N., Van Buggenhout, S., De Vleeschouwer, K., Van Loey, A.M., & Hendrickx, M.E. (2010). Lycopene degradation and isomerization kinetics during thermal processing of an olive oil/tomato emulsion. *Journal of Agricultural and Food Chemistry, 58*(24), 12784-12789.

Cook, M.T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V.V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release, 162*(1), 56-67.

Croisier, F., & Jérôme, C. (2013). Chitosan-based biomaterials for tissue engineering. *European Polymer Journal, 49*(4), 780-792.

Davis, A.R., Fish, W.W., & Perkins-Veazie, P. (2003). A rapid spectrophotometric method for analyzing lycopene content in tomato and tomato products. *Postharvest Biology and Technology, 28*(3), 425-430.

de Castro, M.D.L., & Priego-Capote, F. (2010). Soxhlet extraction: Past and present panacea. *Journal of Chromatography A, 1217*(16), 2383-2389.

Deng, K.L., Zhong, H.B., Tian, T., Gou, Y.B., Li, Q., & Dong, L.R. (2010). Drug release behavior of a pH/temperature sensitive calcium alginate/poly(N-acryloylglycine) bead with core-shelled structure. *Express Polymer Letters, 4*(12), 773-780.

EFSA, E. (2006). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission related to 2, 2-bis (4-hydroxyphenyl) propane (Bisphenol A). *EFSA J, 428*, 1-75.

Eh, A.L.-S., & Teoh, S.-G. (2012a). Novel modified ultrasonication technique for the extraction of lycopene from tomatoes. *Ultrasonics Sonochemistry, 19*(1), 151-159.

Eh, A.L.S., & Teoh, S.G. (2012b). Novel modified ultrasonication technique for the extraction of lycopene from tomatoes. *Ultrasonics Sonochemistry, 19*(1), 151-159.

Food-info. (2013). *Lycopene*. Retrieved from http://www.food-info.net/uk/caro/lycope ne.htm

García, M., Casariego, A., Diaz, R., & Roblejo, L. (2014). Effect of edible chitosan/zeolite coating on tomatoes quality during refrigerated storage. *Emirates Journal of Food and Agriculture, 26*(3), doi: 10.9755/ejfa. v26i3. 16620.

George, M., & Abraham, T.E. (2006). Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review. *Journal of Controlled Release, 114*(1), 1-14.

Goh, C.H., Heng, P.W.S., & Chan, L.W. (2012). Alginates as a useful natural polymer for microencapsulation and therapeutic applications. *Carbohydrate Polymers, 88*(1), 1-12.

Gouin, S. (2004). Microencapsulation: industrial appraisal of existing technologies and trends. *Trends in Food Science & Technology, 15*(7–8), 330-347.

Goula, A.M., & Adamopoulos, K.G. (2012). A new technique for spray-dried encapsulation of lycopene. *Drying Technology, 30*(6), 641-652.

Guo, H., Huang, Y., Qian, J.Q., Gong, Q.Y., & Tang, Y. (2014). Optimization of technological parameters for preparation of lycopene microcapsules. *Journal of Food Science and Technology-Mysore, 51*(7), 1318-1325.

Hadley, C.W., Miller, E.C., Schwartz, S.J., & Clinton, S.K. (2002). Tomatoes, lycopene, and prostate cancer: Progress and promise. *Experimental Biology and Medicine, 227*(10), 869-880.

Hari, P., Chandy, T., & Sharma, C.P. (1996). Chitosan/calcium–alginate beads for oral delivery of insulin. *Journal of Applied Polymer Science, 59*(11), 1795-1801.

Helmenstine, A.M. (2014). *Biochemistry of Lycopene*. Retrieved 25 February, 2014, from http://chemistry.about.com/cs/biochemistry/a/aa050401a.htm

Hennink, W.E., & van Nostrum, C.F. (2002). Novel crosslinking methods to design hydrogels. *Advanced Drug Delivery Reviews, 54*(1), 13-36.

Hernandez-Espinoza, A.B., Pinon-Muniz, M.I., Rascon-Chu, A., Santana-Rodriguez, V.M., & Carvajal-Millan, E. (2012). Lycopene/arabinoxylan gels: Rheological and controlled release characteristics. *Molecules, 17*(3), 2428-2436.

Hussain, S.P., Hofseth, L.J., & Harris, C.C. (2003). Radical causes of cancer. *Nature Reviews Cancer, 3*(4), 276-285.

Hyman, J.R., Gaus, J., & Foolad, M.R. (2004). A rapid and accurate method for estimating tomato lycopene content by measuring chromaticity values of fruit puree. *Journal of the American Society for Horticultural Science, 129*(5), 717-723.

Ishida, B.K., & Chapman, M.H. (2009). Carotenoid extraction from plants using a novel, environmentally friendly solvent. *Journal of Agricultural and Food Chemistry, 57*(3), 1051-1059.

Ishida, B.K., Ma, J., & Chan, B. (2001). A simple, rapid method for HPLC analysis of lycopene isomers. *Phytochemical Analysis, 12*(3), 194-198.

Kim, W.T., Chung, H., Shin, I.S., Yam, K.L., & Chung, D.H. (2008). Characterization of calcium alginate and chitosan-treated calcium alginate gel beads entrapping allyl isothiocyanate. *Carbohydrate Polymers, 71*(4), 566-573.

Kong, K.W., Khoo, H.E., Prasad, K.N., Ismail, A., Tan, C.P., & Rajab, N.F. (2010). Revealing the power of the natural red pigment lycopene. *Molecules, 15*(2), 959-987.

Kun, Y., Lule, U.S., & Xiao-Lin, D. (2006). Lycopene: Its properties and relationship to human health. *Food Reviews International, 22*(4), 309-333.

Lahaye, M., & Rochas, C. (1991). Chemical structure and physico-chemical properties of agar. *Hydrobiologia, 221*(1), 137-148.

Lee, K.Y., Park, W.H., & Ha, W.S. (1997). Polyelectrolyte complexes of sodium alginate with chitosan or its derivatives for microcapsules. *Journal of Applied Polymer Science, 63*(4), 425-432.

Lui, F., McConnell, E., & Pygall, S. (2011). *Update on Polymers for Oral Drug Delivery*. Shawbury, UK: iSmithers.

Lycocard. (2013). *Lycopene and Human Health*. Retrieved 25 June, 2013, from http://www.lycocard.com/index.php/lyco

Lycopene. (2012). *Mayo foundation for medical education and research*. Retrieved 25 June, 2013, from http://www.mayoclinic.com/health/lycopene/NS_patient-lycopene/DSECTION=evidence

Lycopene. (2013). Retrieved 25, June, 2013, from http://www.pierrechimica.com/lycopene_en/produzione.php

Mantzouridou, F., & Tsimidou, M.Z. (2008). Lycopene formation in *Blakeslea trispora*. Chemical aspects of a bioprocess. *Trends in Food Science & Technology, 19*(7), 363-371.

Mein, J.R., Lian, F., & Wang, X.D. (2008). Biological activity of lycopene metabolites: implications for cancer prevention. *Nutrition Reviews, 66*(12), 667-683.

Motilva, M.J., Macia, A., Romero, M.P., Labrador, A., Dominguez, A., & Peiro, L. (2014). Optimisation and validation of analytical methods for the simultaneous extraction of antioxidants: Application to the analysis of tomato sauces. *Food Chemistry, 163*, 234-243.

Olempska-Beer, Z. (2006). *Lycopene from Blakeslea trispora: Chemical and Technical Assessment (CTA)*. Retrieved 17 June, 2013, from http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/67/lycopene_trispora.pdf

Organic Herb Inc. (2012). *Tomato Extract Lycopene*. Retrieved 26 June, 2013, from http://www.organic-herb.com/Product/Item/75.html

Periago, M.J., Rincón, F., Agüera, M.D., & Ros, G. (2004). Mixture approach for optimizing lycopene extraction from tomato and tomato products. *Journal of Agricultural and Food Chemistry, 52*(19), 5796-5802.

Perkins-Veazie, P., Collins, J.K., Pair, S.D., & Roberts, W. (2001). Lycopene content differs among red-fleshed watermelon cultivars. *Journal of the Science of Food and Agriculture, 81*(10), 983-987.

Ranveer, R.C., Patil, S.N., & Sahoo, A.K. (2013). Effect of different parameters on enzyme-assisted extraction of lycopene from tomato processing waste. *Food and Bioproducts Processing, In press*.

Rao, A.V., & Agarwal, S. (1999). Role of lycopene as antioxidant carotenoid in the prevention of chronic diseases: A review. *Nutrition Research, 19*(2), 305-323.

Rao, V. (2013). *Lycopene and human health*. Retrieved 25 June, 2013, from http://www.lycocard.com/index.php/lyco_pub/prevention/

Rath, S. (2009). *Lycopene extract from tomato Chemical and Technical Assessment (CTA)*. Retrieved 27 May, 2013, from http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/71/lycopene_extract_from_tomato.pdf

Reboul, E., Borel, P., Mikail, C., Abou, L., Charbonnier, M., Caris-Veyrat, C., Goupy, P., Portugal, H., Lairon, D., & Amiot, M.J. (2005). Enrichment of tomato paste with 6% tomato peel increases lycopene and beta-carotene bioavailability in men. *Journal of Nutrition, 135*(4), 790-794.

Roh, M.K., Jeon, M.H., Moon, J.N., Moon, W.S., Park, S.M., & Choi, J.S. (2013). A simple method for the isolation of lycopene from *Lycopersicon esculentum. Botanical Sciences, 91*(2), 187-192.

Sandmann, G., Albrecht, M., Schnurr, G., Knorzer, O., & Boger, P. (1999). The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli. Trends in Biotechnology, 17*(6), 233-237.

Sathish, T., Udayakiran, D., Himabindu, K., Lakshmi, P., Sridevi, D., Kezia, D., & Bhojaraju, P. (2009). HPLC method for the determination of lycopene in crude oleoresin extracts. *Asian Journal of Chemistry, 21*(1), 139-148.

Schierle, J., Bretzel, W., Bühler, I., Faccin, N., Hess, D., Steiner, K., & Schüep, W. (1997). Content and isomeric ratio of lycopene in food and human blood plasma. *Food Chemistry, 59*(3), 459-465.

Science, F. (n.d.). *Tomato*. Retrieved 10 June, 2013, from http://foodscience.wikispaces.com/Tomato

Seader, J.D., & Henley, E.J. (2006). *Separation Process Principles* (2nd ed.). Chichester, UK: Wiley.

Seifi, M., Seifi, P., Hadizadeh, F., & Mohajeri, S.A. (2013). Extraction of Lycopene from Tomato Paste by Ursodeoxycholic Acid Using the Selective Inclusion Complex Method. *Journal of Food Science, 78*(11), C1680-C1685.

Shi, J., Khatri, M., Xue, S.J., Mittal, G.S., Ma, Y., & Li, D. (2009). Solubility of lycopene in supercritical CO2 fluid as affected by temperature and pressure. *Separation and Purification Technology, 66*(2), 322-328.

Shi, J., & Le Maguer, M. (2000). Lycopene in tomatoes: Chemical and physical properties affected by food processing. *Critical Reviews in Biotechnology, 20*(4), 293-334.

Shi, J., & Maguer, M.L. (2000). Lycopene in tomatoes: chemical and physical properties affected by food processing. *Critical Reviews in Food Science and Nutrition, 40*(1), 1-42.

Shimada, H., Kondo, K., Fraser, P.D., Miura, Y., Saito, T., & Misawa, N. (1998). Increased carotenoid production by the food yeast Candida utilis through metabolic engineering of the isoprenoid pathway. *Applied and Environmental Microbiology, 64*(7), 2676-2680.

Shu, B., Yu, W., Zhao, Y., & Liu, X. (2006). Study on microencapsulation of lycopene by spray-drying. *Journal of Food Engineering, 76*(4), 664-669.

Singh, P., & Goyal, G.K. (2008). Dietary lycopene: Its properties and anticarcinogenic effects. *Comprehensive Reviews in Food Science and Food Safety, 7*(3), 255-270.

Sinha, V.R., Singla, A.K., Wadhawan, S., Kaushik, R., Kumria, R., Bansal, K., & Dhawan, S. (2004). Chitosan microspheres as a potential carrier for drugs. *International Journal of Pharmaceutics, 274*(1–2), 1-33.

Strati, I.F., & Oreopoulou, V. (2011). Effect of extraction parameters on the carotenoid recovery from tomato waste. *International Journal of Food Science and Technology, 46*(1), 23-29.

Struszczyk, H. (2004). Alginate and chitosan fibers for medical uses. In F. Wallenberger & N. Weston (Eds.), *Natural Fibers, Plastics and Composites* (pp. 95-104). Boston, MA: Springer.

Su, Q., Rowley, K.G., & Balazs, N.D.H. (2002). Carotenoids: separation methods applicable to biological samples. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 781*(1-2), 393-418.

Takka, S., & Gürel, A. (2010). Evaluation of Chitosan/Alginate Beads Using Experimental Design: Formulation and In Vitro Characterization. *AAPS PharmSciTech, 11*(1), 460-466.

Tønnesen, H.H., & Karlsen, J. (2002). Alginate in Drug Delivery Systems. *Drug Development and Industrial Pharmacy, 28*(6), 621-630.

Tzia, C., & Liadakis, G. (2003). *Extraction Optimization in Food Engineering*. New York: Marcell Dekker.

U.S. Food & Drug Administration. (2003). *Lycopene (CAS: 502-65-8)*. Retrieved 1 July, 2013, from http://www.pierrechimica.com/lycopene en/pdf/Trasparenza%20US%20FDA.pdf

Urbonaviciene, D., Viskelis, P., Viskelis, J., Jankauskiene, J., & Bobinas, C. (2012). Lycopene and beta-carotene in non-blanched and blanched tomatoes. *Journal of Food Agriculture & Environment, 10*(2), 142-146.

Vagi, E., Simandi, B., Vasarhelyine, K.P., Daood, H., Kery, A., Doleschall, F., & Nagy, B. (2007). Supercritical carbon dioxide extraction of carotenoids, tocopherols and sitosterols from industrial tomato by-products. *Journal of Supercritical Fluids, 40*(2), 218-226.

Varona, S., Braeuer, A., Leipertz, A., Martin, A., & Cocero, M.J. (2013). Lycopene solubility in mixtures of carbon dioxide and ethyl acetate. *Journal of Supercritical Fluids, 75*, 6-10.

Vemmer, M., & Patel, A.V. (2013). Review of encapsulation methods suitable for microbial biological control agents. *Biological Control, 67*(3), 380-389.

Wang, C.Y., & Chen, B.H. (2006). Tomato pulp as source for the production of lycopene powder containing high proportion of cis-isomers. *European Food Research and Technology, 222*(3-4), 347-353.

Wang, G.S., Grammel, H., Abou-Aisha, K., Sagesser, R., & Ghosh, R. (2012). High-level production of the industrial product lycopene by the photosynthetic bacterium *Rhodospirillum rubrum*. *Applied and Environmental Microbiology, 78*(20), 7205-7215.

Wikipedia: Blakeslea trispora. (2013). Retrieved 26 June, 2013, from https://en.wikipedia.org/wiki/Blakeslea_trispora

Xi, J. (2006). Effect of high pressure processing on the extraction of lycopene in tomato paste waste. *Chemical Engineering and Technology, 29*(6), 736-739.

Yan, X.-L., Khor, E., & Lim, L.-Y. (2001). Chitosan-alginate films prepared with chitosans of different molecular weights. *Journal of Biomedical Materials Research, 58*(4), 358-365.

Yeum, K.J., Booth, S.L., Sadowski, J.A., Liu, C., Tang, G.W., Krinsky, N.I., & Russell, R.M. (1996). Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *American Journal of Clinical Nutrition, 64*(4), 594-602.

Appendix

1. Definitions

Reflux:

A reflux has been defined as the point where enough solvent has collected in the Soxhlet cylinder to be siphoned back into the boiling flask. The extraction chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask.

Retention factor

The measures how far a compound (spot) has moved up in a silica chromatography plate under known conditions and can be use a quick identification method.

2. Calibration of lycopene

Mobile phase: Acetonitrile + dichloromethane (3:1)

Stock solution of lycopene**:** 2.5 mg lycopene in 5 ml of ethyl acetate = 500 µg/ml

Stock solution conc.   = 500µg/ml

Lycopene (Sigma) powder   = 2.5mg

Density   = 1 g/ml

Ethyl acetate solvent   = 5 mL

Total volume   = 5mL
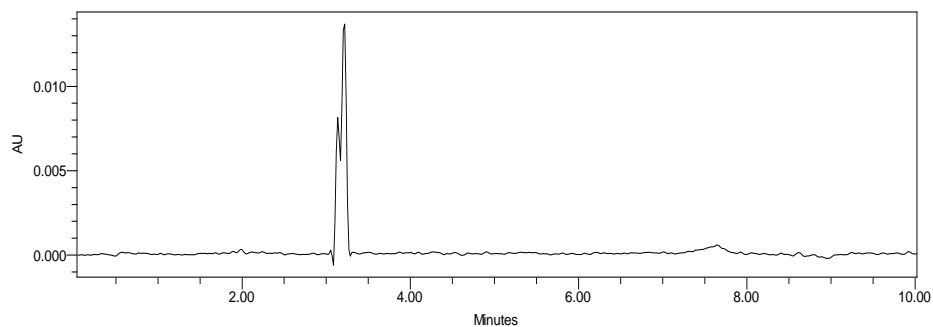
3. HPLC Chromatograph for lycopene standards

Figure A 1: HPLC chromatogram of blank ethyl acetate
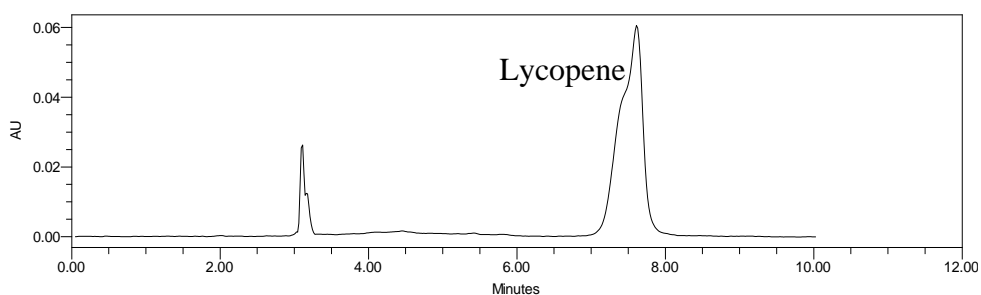
100 µg/mL stock solution of lycopene in Ethyl acetate (EA)
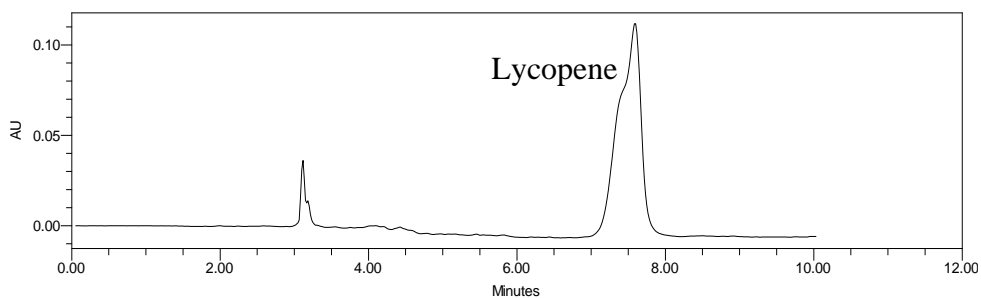

Figure A 2: Chromatogram of standard lycopene in ethyl acetate, concentration 100 µg/mL

Retention Time	Area	Height	Start Time	End Time
7.612	1275573	60241	6.878	8.178

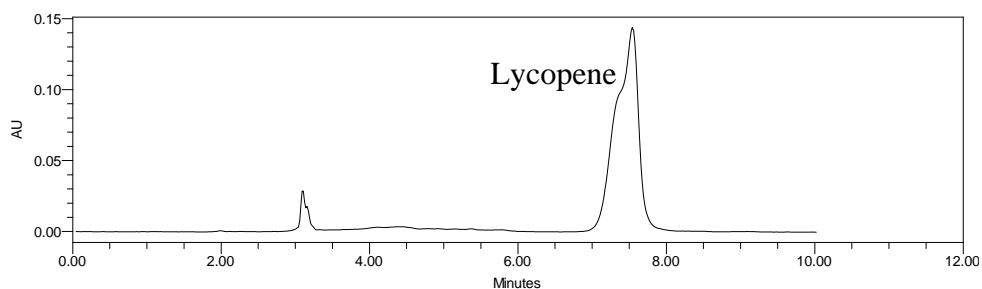### 200 µg/mL stock solution of lycopene in Ethyl acetate

Figure A 3: Chromatograph of standard lycopene in ethyl acetate, concentration 200 µg/mL

Retention Time	Area	Height	Start Time	End Time
7.591	2495002	118725	6.800	8.233

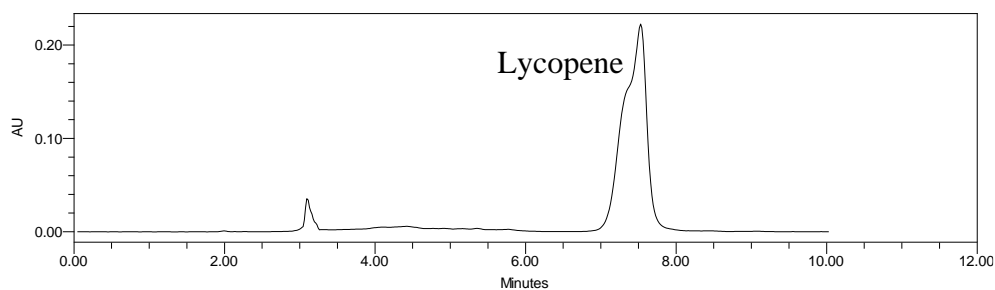**300 μg/mL stock solution of lycopene in Ethyl acetate**

Figure A 4: Chromatograph of standard lycopene in ethyl acetate, concentration 300 μg/mL

Retention Time	Area	Height	Start Time	End Time
7.527	4118710	221897	Lycopene.728	8.228

400 μg/mL stock solution of lycopene in Ethyl acetate
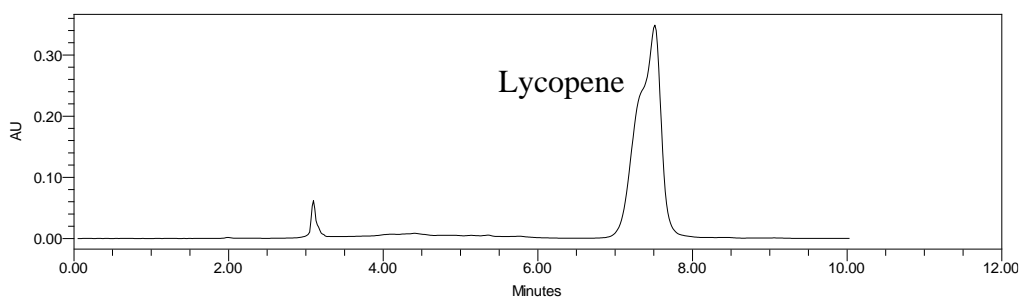

Figure A 5: Chromatograph of standard lycopene in ethyl acetate, concentration 400 μg/mL

Retention Time	Area	Height	Start Time	End Time
7.497	5031553	241782	6.712	8.162

500 μg/mL stock solution in Ethyl acetate

Figure A 6: Chromatograph of standard lycopene in ethyl acetate, concentration 500 μg/mL

Retention Time	Area	Height	Start Time	End Time
7.511	7046984	346614	6.832	7.982

4. Batch solvent extraction

Mobile phase: 3:1 (v/v) Acetonitrile:dichloromethane (acn:dcm)

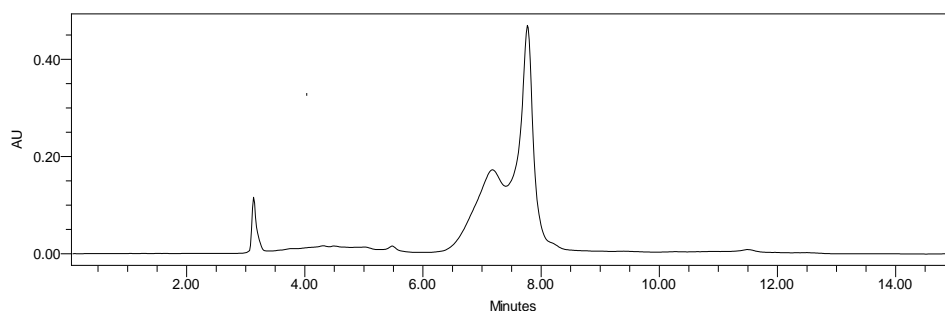**Solvent extraction** of 5g tomato paste with 10 ml Dichloromethane (DCM)

Figure A 7: Chromatogram when tomato paste was extracted with dichloromethane (acn:dcm)

Retention Time	Area
7.716	8681867

Ethyl Acetate: 5g Tomato paste in 10 ml ethyl acetate
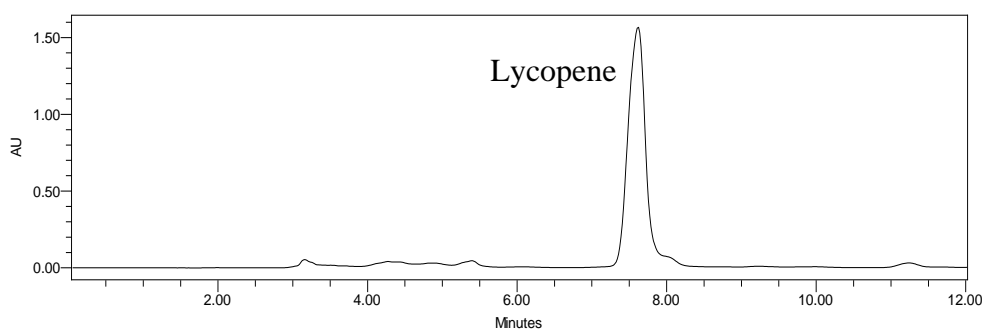
Figure A 8: Chromatogram when tomato paste was extracted with ethyl acetate (acn:dcm)

Retention Time	Area	Height
7.616	14443517	1560355

Petroleum ether: 5g Tomato paste in 10 ml petroleum ether.

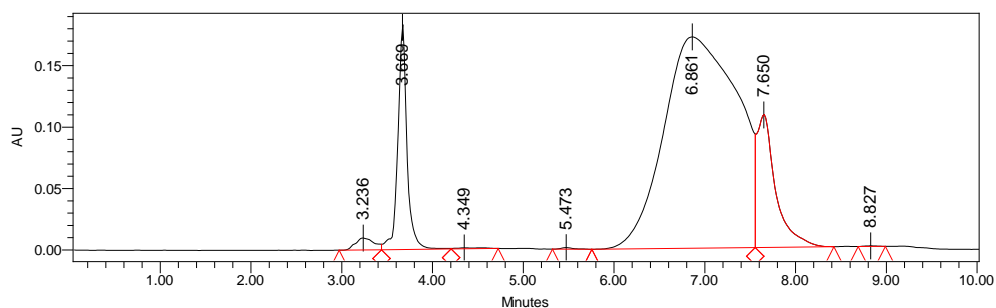**Figure A 9: Chromatogram when tomato paste was extracted with petroleum ether**

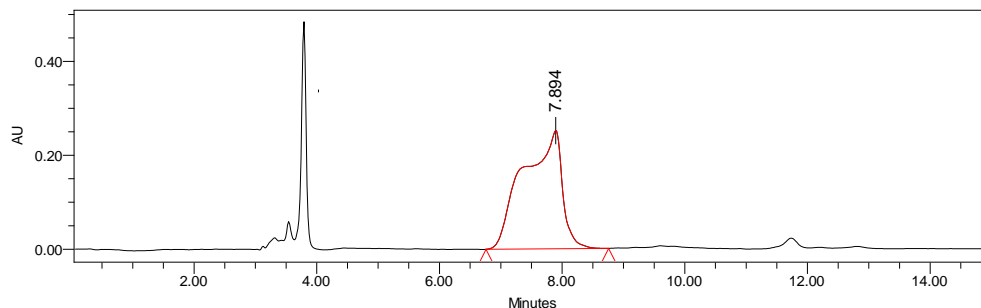| Retention Time	Area	Height	Start Time	End Time
6.861	9651955	172004	5.755	7.555
7.650	1565527	108286	7.555	8.422

Trimixture of Hexane +Acetone +Ethanol (2:1:1)

Figure A 10: Chromatogram when tomato paste was extracted with tri-mixture (acn:dcm)

Retention Time	Area	Height	Start Time	End Time
7.894	11035836	251889	6.758	8.758

Hexane: 5 g tomato paste in 10 ml of n-Hexane (with integration)

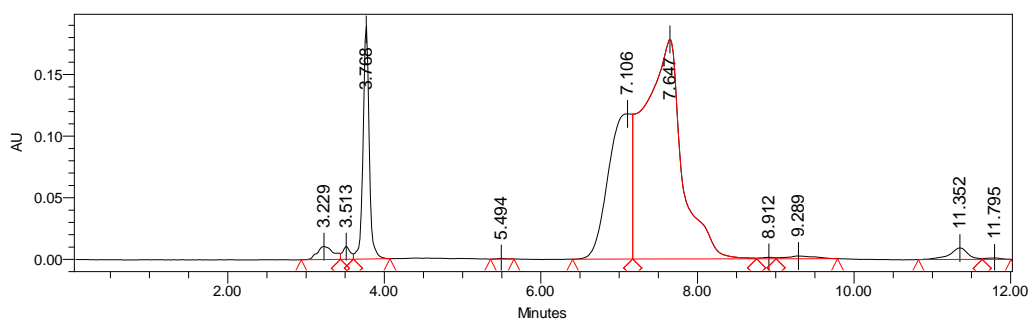**Figure A 11: Chromatogram when tomato paste was extracted with hexane (acn:dcm)**

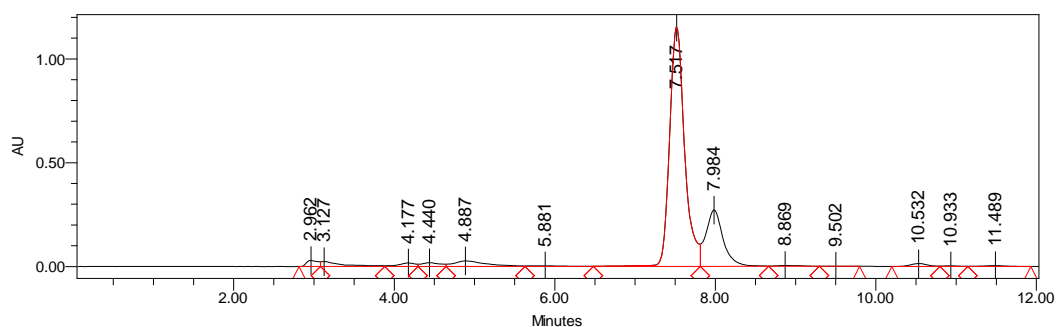| Retention Time	Area	Height	Start Time	End Time
7.647	6044021	178676	7.172	8.755

Ethyl acetate+ ethanol

Figure A 12: Chromatogram when tomato paste was extracted with ethyl acetate+ ethanol (acn:dcm)

Retention Time	Area	Height	Start Time	End Time
7.517	14964901	1155190	6.480	7.813
7.984	4088440	272097	7.813	8.663

5. Gilson's Minipuls Description

The Minipuls pump can be used for application:

- Transferring solutions, emulsions, suspensions, and gases at up to 200°C.
- Pumping liquids through chromatographic systems against a back pressure of up to 0.5 MPa (72.5 psi)
- Continuously sampling the components of a production process
- Formation of gradients (concentration, pH, etc.).

The Minipuls peristaltic pump manual control by front panel operation.

Speed Control Module

A general view of a Minipuls 3 (before the head is installed) indicates (Figure A13)

The front panel has a keypad and a display

1. **Slower:** Decrease the pump speed
2. **Faster:** Increase the pump speed
3. **Rabbit** Set speed to 48 rpm for priming
4. **Forwards:** Start the pump clockwise
5. **Backwards:** Start the pump counter-clockwise
6. **Stop:** To stop the pump

Figure A 13: Minipuls 3 (before the head is installed)

Standard flow rates (1, 2, 4, or 8 channels): 0.3 µL/min–30 mL/min at a maximum pressure of 0.5 MPa.

High flow rates (2 or 4 channels): 1 mL/min–220 mL/min at a maximum pressure of 0.3 MPa