EFFECT OF EXTRUSION ON THE FUNCTIONAL PROPERTIES OF CARROT PULP ADDED EXTRUDATES AND *IN-VITRO* BIOACCESSIBILITY OF LUTEIN AND BETA-CAROTENE

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ABSTRACT

EFFECT OF EXTRUSION ON THE FUNCTIONAL PROPERTIES OF CARROT PULP ADDED EXTRUDATES AND *IN-VITRO* BIOACCESSIBILITY OF LUTEIN AND BETA-CAROTENE

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In this study, effects of extrusion on the functional properties of carrot pulp added extrudates was investigated. Extrusion was performed at four different conditions. Two different temperature profile and two different screw speeds were investigated. Change in antioxidant activity, phenolic content, β-carotene and lutein content and their *in-vitro* bioaccessibilities after extrusion were investigated. Antioxidant activity and total phenolic content was determined by DPPH and Folin–Ciocalteu method, respectively. It was found that extrusion process significantly decreased ($p \leq 0.05$) the level of both the antioxidant activity and the total phenolic content, but the extrusion temperature and the shear rate did not cause a significant change ($p > 0.05$) within the ranges studied. Effect of conventional heating process on antioxidant activity and phenolic content was also investigated, where no significant changes were observed ($p > 0.05$).

The quantification of carotenoids was conducted with HPLC. It is investigated that extrusion process resulted in the loss of β-carotene and lutein content at specified conditions. Extrusion process at 130°C last zone temperature did not affect the lutein *in-vitro* bioaccessibility while increased the *in-vitro* bioaccessibility at 160°C ($p \leq 0.05$). *In-vitro* bioaccessibility of β-carotene increased after extrusion both at 130°C and 160°C last treatment zone temperatures.

Keywords: Extrusion, Functional Foods, *In-vitro* Bioaccessibility, Antioxidant Activity, Total Phenolic Content

ÖZ

EKSTRÜZYON İŞLEMİNİN HAVUÇ POSASI EKLENMİŞ ÜRÜNLERİN FONKSİYONEL ÖZELLİKLERİ VE LUTEİN VE BETA-KAROTENİN *İN-VİTRO* BİYOERİŞİLEBİLİRLİKLERİ ÜZERİNE ETKİSİ

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Bu çalışmada ekstrüzyonun havuç posası eklenmiş ürünlerin fonksiyonel özellikleri üzerine olan etkisi araştırılmıştır. Ekstrüzyon dört farklı koşulda uygulanmıştır. İki ayrı sıcaklık profili ve iki farklı vida hızı araştırılmıştır. Ekstrüzyon sonrası antioksidan aktivitedeki, fenolik miktarındaki ve beta-karoten ile lutein miktarları ve *in-vitro* biyoerişilebilirlliklerindeki değişim araştırılmıştır. Antioksidan aktivite ve fenolik içerik sırasıyla DPHH ve Folin–Ciocalteu metoduyla belirlenmiştir. Ekstrüzyon işleminin hem antioksidan aktivite seviyesini hem de toplam fenolik içeriğini önemli miktarda azalttığı ($p \leq 0.05$), ekstrüzyon sıcaklığı ile kayma hızının ise çalışılan aralıklarda anlamlı bir değişime ($p > 0.05$) sebep olmadığı bulunmuştur. Geleneksel ısıtma yönteminin antioksidan aktivite ve toplam fenolik içeriği üzerine etkisi de incelenmiş ve anlamlı bir değişim gözlemlenmemiştir ($p > 0.05$).

Karotenoid miktarının ölçümü HPLC ile gerçekleştirilmiştir. Ekstrüzyon işleminin belirli koşullar altında beta-karoten ve lutein miktarında kayba neden olduğu bulunmuştur. 130°C son bölge sıcaklığına sahip ekstrüzyon işlemi luteinin *in-vitro* biyoerişilebilirliğini etkilemezken 160°C *in-vitro* biyoerişilebilirliğini arttırmıştır ($p \leq 0.05$). β-karotenin *in-vitro* biyoerişilebilirliği ise her iki farklı sıcaklıktaki ekstrüzyon işleminden sonra artmıştır ($p \leq 0.05$).

Anahtar Kelimeler: Ekstrüzyon, Fonksiyonel Gıdalar, *In-vitro* Biyoerişilebilirlik, Antioksidan Aktivite, Toplam Fenolik Madde

To My Family

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BHA	Butylated Hydroxyanisol
BHT	Butylated Hydroxy-Toluene
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential Scanning Colorimeter
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
L/ D	Ratio of Barrel Length to Barrel Diameter
ORAC	Oxygen Radical Absorption Capacity
RVA	Rapid Visco-Analyzer
TEAC	Total Radical Trapping Antioxidant Parameter
TBHQ	Tert-Butylhydroquinone
TE	Trolox Equivalent
THF	Tetrahydrofuran

CHAPTER 1

INTRODUCTION

1.1. Extrusion Cooking

Extrusion cooking is a process in which processing of feed material is achieved under the combined effect of temperature, pressure and shear. In recent years, extrusion process draws interest in food industry especially in the production of snacks, cereals and pasta as due to the advantages it offers. While short residence time provides the quality of extrudates; opportunity to form variously shaped products creates appetizing foods. Moreover; too little waste production during extrusion makes it possible to overcome the environmental or government restrictions and also lowers the cost of final product (Riaz & Rokey, 2011a).

Food extrusion can be thought as a high temperature-short time process as barrel temperatures are adjusted to not to exceed 180°C and residence time in extruder usually changes in the range of 20-200 seconds (Martínez-Bustos *et al.*, 2012). This time- temperature combination is adequate to the physical changes or chemical reactions like starch gelatinization, protein denaturation, enzyme inactivation and destruction of microorganisms (Wolf, 2010) whereas formation of flavours or color change reactions may not take place mainly as a result of limited time (Chessari & Sellahewa, 2001; Martínez-Bustos et al., 2012).

A typical extruder is composed of engine, feeder, barrel, screw, die and control panel. In the barrel section, with the help of the screws' rotating action shear energy is generated. In consequence of the effect of shear energy and high temperature of extrusion, pressure inside the barrel rises and causes food material to undergo chemical and rheological changes. When leaving the barrel through a die the rapid pressure drop induces evaporation of water and expansion of extrudate, which result in a puffed and porous product. Die also gives the extrudate its final shape. The smaller the diameter of the hole on die, the higher the resistance that food material encounter at the exit, which increase the inside pressure and also causes more back flow of food material (Moscicki & Zuilichem, 2011).

With regard to screw number, extruders are classified as single screw extruders or twin screw extruders. Extruders whose barrel contains two screws with equal length named as twin screw extruders, whereas if only one screw is located it is called as single screw extruder. According to the configuration and rotation of screws, twin screw extruders are further grouped as intermeshing/non-intermeshing and co-rotating/counter-rotating, respectively. If the screws penetrate each other, the type of extruder is intermeshing twin screw extruder; otherwise it is non-intermeshing. Furthermore; if the screws rotate in the same direction, it is termed as co-rotating. The extruders with the screws rotate in opposite direction are named as counter-rotating twin screw extruders (Giles et al., 2004; Riaz & Rokey, 2011b). Main drawback of single screw extruders is poor mixing ability, for this reason additional blending may be required prior to feeding especially for multi-component food materials. Apart from mixing efficiency, twin screw extruders are superior to single screw extruders in terms of self-cleaning opportunity, high productivity and flexible feed material handling (Moscicki & Zuilichem, 2011; Riaz, 2001). Ingredients which have too high moisture content and are sticky that cannot be handled in single screw extruders can be processed in twin screw extruders. More complex design and high cost can be counted as adverse sides of twin screw extruders. Twin screw extruders are used both for the pet food industry and for human consumption such as in the production of breakfast cereals, pasta, potato pallets, candy, chocolate and snack foods (Berk, 2013).

1.2. Snack Foods

By the application of modern extrusion techniques, it has become widespread to market cereals as snacks so that snack foods are gaining popularity as much as breakfast cereals or corn flakes in recent years. Statistics confirm this as consumption of snack food rose threefold in the United State from 1977 to 1994 (Camire, 2001; Putnam & Gerrior, 1999). Moreover, US Department of Agriculture drew up a report in 2000 indicating that snack foods meet 20% of energy and some nutrient requirements of American children (Camire, 2001; USDA, 1999). As much comparable data is not available for other regions and nations, this statistics can give us a general view for the consumption trends for snacks. However; assumption of the snack foods as unhealthy acts as a barrier and limits the consumption of them (Camire, 2001; Dinkins, 2000). Therefore, fortification of snacks is a considerable parameter.

Cereals are used as major ingredients in extrudates because of good expansion ability as a result of high starch composition (Dar et al., 2014). Corn flour and corn grit are commonly used ingredients in the extruded snack food production; however, using corn as a single ingredient result in a snack with high glycemic index and unsatisfactory nutritional value. Therefore corn based snack foods need to fortify with fruit or vegetables as a minor ingredient (Brennan et al., 2013; Caltinoglu et al., 2014; Conti-Silva et al., 2012). Inferences of the surveys and health concern of public direct the researchers to enhance nutritional quality of snack foods and develop functional foods.

1.3. Functional Foods

Variations in the physical conditions, health status, lifestyle, culture and nutritional requirements of people prevents to form a standard foodstuff fit everyone and directs food scientists to produce food enriched with specific ingredients. For this reason, as well as providing basic nutritional requirements, the food products that have a positive impact on health or developed to be beneficial on physiological means are defined as functional foods (Diplock et al., 1999; Pang et al., 2012; Thompson & Moughan, 2008). Genetic engineering is one of the methods used in creating functional foods. Simpler and easier development of functional foods can be achieved via either addition of functional ingredients to food or alteration the composition of unprocessed food. Today, functional food market turns into a big industry worth annually 60 billion dollar and gradually increases its market presence (Consumer Reports on Health, 2007; Edlin & Golanty, 2012; Thompson & Moughan, 2008).

1.4. Carrot

Carrot is a kind of vegetable whom composition is dominated by water (86-90%) and remaining is composed of carbohydrate (7-10%), fiber (3%), protein (1%), ash (1%) and fat (0.2%). Fiber and carbohydrate contents are formed mainly from cellulose and simple sugars, respectively (Arscott & Tanumihardjo, 2010; Sharma et al., 2012a). Carrot contains adequate amounts of thiamin, riboflavin and niacin. Potassium is the dominant mineral in it (Nicolle et al., 2004). Carrot is also known for the rich beta carotene content which is responsible for the orange color (Arscott & Tanumihardjo, 2010). Carrot contains approximately 9700 μg β-carotene/100 g, 3140 μg α-carotene/100 g and 220 μg lutein/100 g (Stahl & Sies, 1999). High β-carotene composition is crucial as β-carotene is the precursor of Vitamin A. According to data given in 2002, approximately 140 million pre-school children and

7 million pregnant woman face vitamin A deficiency, thereupon up to 3 million of that population die in a year (Haddad et al., 2004). World Health Organization defines Vitamin A deficiency as a public health problem that affects 122 countries worldwide (Arscott & Tanumihardjo, 2010; World Health Organization, 2009). β-carotene content also contributes the antioxidant potency of carrot as phenolic compounds do. Carrots have antioxidant activity because of the phytonutrients and phenolic compounds they include. Chlorogenic acid is the dominant phenolic acid found in carrot (Sharma et al., 2012a; Zhang & Hamauzu, 2004).

1.5. Antioxidants

A great majority of lipids in food materials are in the form of fatty acids esters and glycerol. As a result of the reaction of fatty acid esters with oxygen, lipid deterioration and fat rancidity take place. Other than fat rancidity, oxidation also results in nutritional reduction, undesirable flavor formation and color change in foods. Antioxidants are the molecules that contribute to extension of the shelf life of food substance by lessening these negative impacts of oxygen (Miková, 2001; Yanishlieva-Maslarova, 2001). As well as preventing the deleterious effect in foods, some of the antioxidants are to be believed to be beneficial on human body by preventing the harmful effects of free radicals, nucleic acid damage and also helps to fight the cardiovascular disease to some extent (Molyneux, 2004; Pisoschi & Negulescu, 2011; Pokorný & Schmidt; 2001).

Although it is possible to gain foods antioxidant property with the addition of synthetic antioxidants like BHT, BHA and TBHQ, safety concerns and governmental restrictions limit the usage of them, which results in a growing interest on the natural antioxidants (Balasundram et al., 2006; Frankel, 1998; Pokorný & Schmidt; 2001; Wanasundara & Shahidi, 1998). There are several natural antioxidants found in foods such as phenolic acids, carotenoids, flavonoids, tocopherols, benzoic acid derivatives, etc. As there is a wide range of compounds that show antioxidant property and as the antioxidant activity changes according to the several factors such

as polarity, solubility and temperature; a standard method determines the certain antioxidant activity or antioxidant capacity of a single compound is not available (Litescu et al., 2010; Ou et al., 2002; Pisoschi & Negulescu, 2011). Antioxidant activity and antioxidant capacity are two different terms related to each other. Antioxidant activity refers to the reaction rate of a single antioxidant with free radical, however; antioxidant capacity is a more general term, which regards the reaction between the antioxidant solution includes the mixture of antioxidant compounds and free radical (Litescu et al., 2010; Prior & Cao, 1999). Generally accepted antioxidant activity determination methods are based on reaction between the antioxidant and organic radical then followed by the observation its absorbance spectrophotometrically. DPPH (2,2-diphenyl-1-picrylhydrazyl) method, ABTS method, TEAC (Total Radical Trapping Antioxidant Parameter) assay, ORAC (oxygen radical absorption capacity) assay, FRAP (ferric reducing antioxidant power) assay can be counted as the most common antioxidant activity determination methods (Litescu et al., 2010; Pisoschi & Negulescu, 2011).

1.6. Phenolic Compounds

Phenolic compounds involve a large variety of structures from simple phenolic acids to polymer polyphenols. They are available in plants and play a role in enzymatic browning, color and flavor of fruits (Cheynier, 2012). Their anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic and anti-oxidant properties make them beneficial for health (Balasundram et al., 2006; Middleton et al., 2000). Vanillin, gallic acid, tannins, flavonoids and hydroxycinnamic acids can be counted as examples for the natural phenolic compounds (Balasundram et al., 2006; Middleton et al., 2000). Butylated hydroxyanisol (BHA) and butylated hydroxy-toluene (BHT) are the most common synthetic phenolic compounds used for their antioxidant property (Yanishlieva-Maslarova, 2001).

For the quantification of phenolics both spectrophotometric and chromatographic techniques are available. High performance liquid chromatography (HPLC) and gas chromatography (GC) are the main chromatographic techniques can be used for the separation and quantification of phenolics. If not a specific phenolic group is searched, the Folin–Ciocalteu method is the most widely used procedure for the total phenolic content determination (Naczk & Shahidi, 2004).

1.7. Carotenoids

Carotenoids are the colored lipophilic compounds function as pigments in fruits and vegetables (Rodríguez-Bernaldo de Quirós, & Costa, 2006; Su et al., 2002). Their lipophilic nature gives them a soluble characteristic in organic solvents and makes them insoluble in water (Rodriguez-Amaya, 2001). Carotenoids synthesized in plants are obtained by animal and humans via diet (Rodríguez-Bernaldo de Quirós, & Costa, 2006). Nowadays, more than 600 carotenoids have been identified (Shegokar & Mitri, 2012).

According to their structures carotenoids classified into the two main groups namely, carotenes and xanthophylls. Carotenes also called as hydrocarbon carotenoids are formed only from carbon molecules, whereas xanthophylls or oxy-carotenoids also contain oxygen. The specific examples of hydrocarbon carotenoids are β-carotene ($C_{40}H_{56}$) and lycopene ($C_{40}H_{56}$). Lutein ($C_{40}H_{56}O_2$) and zeaxanthin ($C_{40}H_{56}O_2$) are classified as oxy-carotenoids (Donhowe & Kong, 2014; Rodríguez-Bernaldo de Quirós, & Costa, 2006; Su et al., 2002; Yonekura & Nagao, 2007).

Beta-carotene is an orange colored carotenoid found in red-orange fruits and vegetables like carrot. As humans are not be able to synthesize Vitamin A, getting the sufficient amounts in the diet is important. Beta-carotene, alpha-carotene, gamma-carotene and beta-cryptoxanthin are the precursor vitamin A carotenoids, meaning that they are in inactive form to Vitamin A. Among them, beta-carotene comes to the forefront with its symmetrical structure, which result in the better

conversion to vitamin A and makes β-carotene having the highest vitamin A activity (Donhowe & Kong, 2014; Grune et al., 2010; Handelman, 2001; Shegokar & Mitri, 2012; Yonekura & Nagao, 2007).

Lutein is mainly found in human serum and also located in retina on eyes. It is beneficial for vision function and skin health (Bone et al., 1997; Handelman, 2001; Shegokar & Mitri, 2012). Lutein protects the eye by filtering the blue light and also contributes to prevent diseases like age-related macular degeneration and cataract (Kalariya et al., 2012; Kijlstra et al., 2012). Green leafy vegetables are the main source of lutein. Recommended daily amount of lutein is 5-12 mg. (Shegokar & Mitri, 2012).

During food processing or storage period of foodstuffs, loss of carotenoids may be seen due to several reasons such as enzymatic degradation, thermal destruction, oxidation, prolong exposure to light, being in contact with acid and isomerization from more stable trans form to cis-isomers (Rodriguez-Amaya & Kimura, 2004; Rodriguez-Amaya, 1997,1999,2002).

Although it is possible to determine carotenoid content by spectrophotometric methods, chromatographic techniques are more widely used as isomers of carotenoids, especially cis and trans isomers of β-carotene cannot be separated spectrophotometrically (Godoy & Rodriguez-Amaya, 1994; Schierle et al., 2004). HPLC is the widely used method for the quantification of carotenoids. In the HPLC analysis, separation and determination of carotenoids depend on the comparison of retention times with the authentic standards. As retention times change with type of column, mobile phase and wavelength; selection of these three parameters is crucial. C-18 and C-30 reverse phase columns are the most common carotenoid columns that are able to identify different carotenoids; however, C30 column is superior as it can also separate the cis-trans isomers of carotenoids (Takaichi, 2000, 2014).

1.8. Bioavailability & Bioaccessibility

Carotenoids play critical roles in the biological functions by means of their health promoting effects that contributes to fighting of diseases like cancer or cardiovascular diseases. As mammals cannot synthesize carotenoids, humans should obtain appreciable amount of carotenoid through diet (Astorg, 1997; Krinsky, 1993; Palmero et al., 2014). However; to be able to take advantage of carotenoids' favorable effect, digestion and absorption of maximum amount of carotenoids poses crucial importance as well as consuming them via diet. This concept introduces two key terms; bioavailability and bioaccessibility (Verrijssen et al., 2014). Bioaccessibility represents the amount of nutrients released from food matrix and ready for intestinal absorption in pursuit of digestion. Bioavailability is defined as the actual amount of the nutrients absorbed into the circulation system and ready for body utilization (Amorim-Carrilho et al., 2014; Colle et al., 2010; Duchateau & Klaffke, 2008; Palafox-Carlos et al., 2011; Parada & Aguilera, 2007; Sensoy, 2013; Van Loo-Bouwman et al., 2014).

Beginning from ingestion to entrance body circulation, food material faces mainly two steps; digestion and absorption. Digestion starts mechanically in the mount and continues in the esophagus, stomach and small intestine, respectively. It includes breaking down food material into smaller pieces with the help of digestive juices and enzymes. Crushing food material makes the functional compounds more easily absorbed in the small intestine. In the course of absorption; some nutrients, water and minerals are entered into the lymph and transported into the blood stream through the walls of small intestine (Norton et al., 2007; Sensoy, 2013).

In the particular case of absorption of carotenoids, carotenoids are released from disrupted food matrix through the cell wall. Apart from cell wall, location of carotenoids inside the chromoplast organelle is another factor that affects the release of carotenoids. This step proceeds with solubilization of carotenoids in oil droplets in the stomach and micelle formation. Thus, oil-reach nature facilitates the solubility of carotenoids. Then, absorbable carotenoids are taken into small intestine. For the

provitamin-A carotenoids, conversion of vitamin A into retinol takes place in this step. Finally carotenoids secreted into lymph and blood to join the circulation system (Castenmiller & West, 1998; Palmero et al., 2013, 2014; Rodriguez-Amaya, 2010; Van Loo-Bouwman et al., 2014). Therefore, bioaccessibility and bioavailability of carotenoids depend on both food-related factors such as type and location of carotenoid, destruction of food matrix, presence of other compounds like fat and fiber, food processing method and human-related factors like genetic background and general health status of human (Erdman et al., 1993; Furr & Clark, 1997; Pugliese et al., 2014b; Rodriguez-Amaya, 2010).

Quantification of bioavailability/bioaccessibility can be achieved through two different procedures namely; *in-vivo* and *in-vitro* methods. *In-vivo* approach for bioaccessibility estimation is based on the animal or human studies. However; human modelled studies are not always satisfactory as human-related factors like genetic and health condition of test subjects may affect the subjectivity of results. Moreover; if the studies done on animals, plausibility of the results remains questionable as it is controversial that whether animal models fit completely to human metabolism or not. Additionally, even if animals are accepted to meet human models exactly, cost and ethical considerations still remain as challenges to *in-vivo* studies (Carbonell-Capella et al., 2014; Fernández-García et al., 2009; Parada & Aguilera, 2007; Pugliese et al., 2014b; Sensoy, 2013; Van Loo-Bouwman et al., 2014). Therefore, to gain information about bioaccessibility/bioavailability, *in-vitro* approaches are more commonly used in the literature. *In-vitro* bioaccessibility procedure includes the modeling gastric and intestinal digestion with artificial membranes in the laboratory conditions. Provided that the digestion simulation is followed by Coco-2 cells (polarized human colon carcinoma cells) uptake procedure, bioavailability quantification can be estimated (Carbonell-Capella et al., 2014; Parada & Aguilera, 2007; Sensoy, 2013). *In vitro* methods can be applied either static or dynamic approach. In static approach the chemical digestion stimulated but mechanical forces and fluid motion are not included. In this manner, dynamic *in-vitro* approach can characterize *in-vivo* conditions and therefore; is superior to static model (Amorim-Carrilho et al., 2014; Ferrua & Singh, 2010).

1.9. Starch Gelatinization

Starch is carbohydrate molecule composed of amylose and amylopectin units. Main sources of starch can be counted as potatoes, wheat and corn (Souza & Andrade, 2002). Starch is widely consumed in human diet after being exposed to food processing techniques which may result in the gelatinization of starch during heating and retrogradation during cooling (Wang & Copeland, 2013). The main effect of extrusion on the starch is gelatinization, which includes the swelling and losing the crystalline structure of starch molecule and formation of a viscous paste (Berk, 2013; Kitabatake & Doi, 1992). Starch gelatinization is crucial as extend of it influences the digestivity (Wang & Copeland, 2013). Enzymes can show higher activity on readily gelatinized starch (Berk, 2013). To investigate the starch characterization several methods have been applied such as, Differential Scanning Colorimeter (DSC), Brabender Viscoamylograph (BV) or Rapid Visco-Analyzer (RVA). DSC instrument is widely used to determine starch gelatinization in food industry as it offers fast and precise results and requires small amount of sample. With the help of DSC method it is possible to determine the gelatinization temperature and transition enthalpies (Péret et al., 1998).

CHAPTER 2

MATERIAL & METHODS

2.1. Material

Corn grit was obtained from Teknik Tarım (Manisa, Turkey) and carrot was bought from local market (Ankara, Turkey). Moisture content of raw materials were measured (AND MX-50, Japan) and carrot pulp was prepared in a Cuisinart (U.S.) blender. Corn grit and carrot pulp combined in a ratio of 4:1 (w/w) to adjust the final moisture content to %25 ± 0.36. To make carrot pulp uniformly distributed in corn grit, they were mixed for 15 min (Kitchen Aid, Ariston, USA). Carrot pulp added corn grit was stored at 4°C overnight to reach to equilibrium and then brought to room temperature prior to extrusion.

2.2. Methods

2.2.1. Extrusion

In the study, co-rotating twin screw extruder with four heating zones (Feza Gıda Müh. Makine Nakliyat ve Demir Tic. Ltd. Şti., İstanbul) was used. Extruder has 25:1 L/D ratio and 3 mm die diameter. Flow rate of the feed was kept constant at 36 g/min during extrusion. Extrusion was performed with four different conditions shown in the Table 1. Samples were taken when the actual temperature varied only ±2ºC from the set treatment zone and die temperatures. Extrudates were left at room

temperature to cool down and then kept in black plastic bags at -20°C for further analysis.

Table 1 – Process variables during extrusion process.

Conditions	Temperature Distribution in Barrel (°C)				Die Temperature (°C)	Screw Speed (rpm)
	T1	T2	T3	T4		
1	80	90	100	130	120	125
2	80	90	100	130	120	225
3	80	105	130	160	130	225
4	80	105	130	160	130	125

2.2.2. Conventional Heating

Feed mixture composed of corn grit and carrot pulp and had a moisture content of %25 ± 0.36 was baked at 160°C and 200°C for 10 minutes in the drying oven (Bindr, Germany). Baked feed were left at room temperature to cool down and then kept in black plastic bags at -20°C for further analysis.

2.2.3. Antioxidant Activity

The procedure described by Anton et al., (2009) was used to calculate antioxidant activity of the samples as Trolox equivalent with DPPH test based on the reduction of DPPH radical. For the extraction of antioxidants 10 ml of 80% acetone was added to the 0.40±0.01 g of grounded sample. After 2 hours of extraction at 250 rpm, solution was centrifuged at 4000xg at 25°C for 10 min and supernatant was collected. Prior to use, supernatant was filtered through a 0.45 µm syringe filter (PTFE, 25 mm). 200 µl of filtered extract was reacted with 3.8 ml of DPPH (Sigma-

Aldrich, Germany) solution ($6.34x10^{-2}$ mM in methanol). Absorbance of the samples at 517 nm was measured after 60 minute incubation in the dark. Absorbance of 200 µl acetone:water (80:20, v/v) in 3.8 ml DPPH solution against a methanol blank was recorded as control. Trolox (Sigma-Aldrich, Germany) standard curve was prepared in the range of 0-16 µM.

2.2.4. Total Phenolic Content

Total phenolic content was determined by Folin-Ciocalteau method that is based on the reduction of Folin–Ciocalteu reagent, as described by Anton et al., (2009). 0.40±0.01 g of grounded sample was extracted with 10 ml of acetone:water (80:20, v/v) mixture. Extraction was completed on a rotary shaker (JeioTech-Multichannel Stirrer, MS-52 M) at 250 rpm during 2 hours. The extracted sample centrifuged (Sigma, 2-16 PK, Germany) at 4000xg for 10 minutes. Supernatants were collected and filtered through a 0.45 µm syringe filter (PTFE, 25 mm). 200 µl of filtered extract was reacted with 1.5 ml of freshly 10-fold diluted Folin–Ciocalteu reagent (Merck, Germany). Extract was allowed to react with Folin–Ciocalteu reagent at room temperature for 5 minutes, and then 1.5 ml of Na_2CO_3 solution (60 g/L) was added. After a 90 minute incubation period, sample was transferred to the a glass cuvette and its absorbance was measured at 725 nm against blank, extraction solvent, in a spectrophotometer (Shimadzu, UV-1700 Pharma Spec, Japan). The standard curve was prepared with gallic acid (Sigma-Aldrich, Germany) in the range of 0-600 mg/L and the results were expressed as mg gallic acid/g dry sample weight.

2.2.5. β-Carotene & Lutein Content

Carotenoid content of samples before and after extrusion process were investigated by HPLC. Authentic standards of β-Carotene & Lutein carotenoids were obtained from Sigma Aldrich (Germany). All the solvents used in the HPLC analysis were HPLC grade and obtained from Merck (Germany).

Extraction of Carotenoids: Extraction of carotenoids was carried out according to the method offered by Cemeroğlu (2010). 15±0.01 g grinded sample was weighed in a 100 ml beaker and 45 ml distilled water was added to provide rehydration at +4°C for overnight. Rehydrated sample was homogenized (Wise-Tis,Witeg-Germany) at 13500 rpm for 2 minutes. 0.2 g $CaCO_3$ was added to reduce acidity and 80 ml of extraction solvent, that was composed of acetone:hexane:tetrahydrofuran (50:30:20, v/v/v) which contained 0.1% BHT, was added on 5±0.001 g homogenated sample. Extraction was completed in a rotary stirrer (JeioTech-Multichannel Stirrer, MS-52 M) at 250 rpm for 20 min. At the end of this time sample became thoroughly colourless. Extracted samples were centrifuged (Sigma, 2-16 PK, Germany) at 9500xg at +4°C for 20 minutes to separate hexane phase. Hexane phase was transferred into the glass tubes and dried under nitrogen evaporator (Biotage, TurboVap-LV, USA) at 38°C. Dried samples were kept as frozen at -20°C for further HPLC analysis.

HPLC Analysis: Carotenoid separation was accomplished by reverse phase HPLC system by using combined method of O'Connell et al. (2007) and O'Sullivan at al. (2010). Dried samples were dissolved with 250 µl tetrahydrofuran (THF) and tubes were washed with known amount of mobile phase. Resulted extracts were transferred into a 2 ml amber vials after filtration through 0.45 µm single use cellulose filter (Syringe Filter, PTFE 25 mm) and then injected to the HPLC (Thermo Scientific, Finnigan Surveyor). For the analysis reversed phase C-18 column (250x4.6 mm, 5µm particle size, Inertsil ODS-2, GL Sciences Inc., USA) was used. As a mobile phase mixture of acetonitrile:methanol:dichloromethane (75:20:5, v/v/v) containing 4.5 mmol/L BHT, 10 mmol/L ammonium acetate and 3,6 mmol/L triathylamine was

used. Mobile phase degassed by ultrasonic agitation for 15 minutes before injection to HPLC.

HPLC chromatograms were obtained by using following HPLC conditions.

Flow Rate: 1 ml/min

Injection Volume: 25 µl

Auto-sampler Temperature: +4°C

Column Temperature: 20°C

Detector Wavelength: 450 nm

Analyse Time: 85 min

Chromatograms were analysed by ChromQuesst 4.2.34 software system. Lutein and β-carotene contents were determined by comparing the retention times and peak sequence to those of authentic standards.

2.2.6. *In-Vitro* Bioaccessibility

In-vitro bioaccessibility experiments were completed at the Food Engineering Department of Ege University (İzmir, Turkey) according to the method of Minekus et al. (2014). Samples were crashed roughly to simulate chewing action. For each 0.5 g sample 500 µl simulated salivary fluid (Table 2) and human salivary α-amylase (Sigma A 1031) was added and stirred with vortex. The pH was adjusted to 7.0 and then 0.2 ml pepsin solution (Sigma P 7000) and 0.8 ml simulated gastric fluid (Table 2) was added, respectively. Prior to incubation in shaking water bath (Memmert, Type SV 1422, Schwabach, Germany) at 37ºC for 2 hour, pH was adjusted to 3.0. Then, at 5.5 pH dialysis membranes (Spectra/Por 6 8,000 MWCO, Spectrum Lab. Inc.) simulated duodenal fluid (Table 2) containing 0.5N $NaHCO_3$ was placed inside the tubes. pH was elevated to 7.0 and 2.0 ml solution of pancreatin (Sigma P1750;

100 TAME U/ml)-bile (Sigma-Aldrich B8631, USA) mixture was added. Simulated digestion was completed by holding the pH at 7.0 during the incubation period at 37ºC for 2 hour.

Table 2 – Composition of simulated salivary, gastric and duodenal fluids (Minekus et al., 2014).

	Stock Concentration (g/L)	***Volume (ml)***
Simulated Salivary Fluid		
KCl	46.7	10
KH_2PO_4	68	20
$NaHCO_3$	84	4
NaCl	120	1
$MgCl_2(H_2O)_6$	30	1
Simulated Gastric Fluid		
KCl	46.7	28
KH_2PO_4	68	0.9
$NaHCO_3$	168	6.5
NaCl	120	10
$MgCl_2(H_2O)_6$	30	2
Simulated Duedonal Fluid		
KCl	47.7	5.4
KH_2PO_4	68	0.8
$NaHCO_3$	42.5	42.5
NaCl	120	8
$MgCl_2(H_2O)_6$	30	1.1

Digested samples were stored at -20°C, in the dark and at the time of analysis they were defrosted at room temperature. 50 ml extraction solvent composed of acetone:hexane:tetrahydrofuran (50:30:20, v/v/v) was added to the samples taken from outside the dialysis tubes, whereas 25 ml of extraction solvent was added to the samples gained from inside the dialysis tubes. Extraction was completed in 20 minutes on the stirrer (JeioTech-Multichannel Stirrer, MS-52 M) at 250 rpm. Extracted samples were centrifuged (Sigma, 2-16 PK, Germany) at 9500xg at +4°C during 20 minutes and hexane phase separated in the centrifuge was dried under nitrogen evaporator (Biotage, TurboVap-LV, USA) at 38°C. Dried carotenoid was dissolved by 100 µl THF and the nitrogen tubes were washed with known amount of mobile phase. Resulted extract were filtered through 0.45 µm filter (Syringe Filter, PTFE 25 mm) before injection to the HPLC devise. Extraction solvent and the mobile phase used in the bioavailability analysis were same as the ones used in the carotenoid analysis. HPLC conditions were the same as in the carotenoid analysis.

2.2.7. Starch Gelatinization

Samples before and after extrusion were analysed for their starch gelatinization by Differential Scanning Calorimetry (DSC) instrument (Perkin Elmer DSC 4000). Samples were ground and sieved through a 425 µm screen and kept at room temperature until the analysis. Approximately 8 mg sample was weighed on a 30 µL DSC pan (Perkin Elmer) and two fold distilled water was added. Sealed pan was allowed to equilibrate at +4°C during a night. Samples were examined from 0°C to 130°C with a heating rate of 10° C/min. Nitrogen flushing was adjusted to 20 ml/min. Indium and zinc were used for calibration. A sealed empty pan was used as a reference. Gelatinization temperature, peak temperature and the enthalpy were investigated with Pyris software – Version 11.0.0.0449.

2.2.8. Ash Content

To determine the ash content of samples, routine AOAC method (2000) was applied. Crucibles were kept in furnace at 550°C to decontaminate any impurities and then cooled in desiccator prior to weigh approximately 5 g of sample. Crucibles were burned at 550°C until all sample turns into grey. Corresponding ash content was calculated with the following formulas.

$$\%\ \text{Ash Content on Wet Basis} = \frac{\text{Weight of Ash}}{\text{Weight of Sample}} * 100$$

$$\%\ \text{Ash Content on Dry Basis} = \frac{\%\ \text{Ash Content on Wet Basis}}{\text{Dry Matter Content of Sample}} * 100$$

2.2.9. Pectin Content

Pectin content of carrot pulp was determined according to the method by Monsoor (2005). Samples were extracted with 0.05 M HCl by stirring on a stirrer (Heidolph, MR300IK) at 300 rpm at 90°C for an hour. Then extracts were cooled in a water bath (Jeiotech, US-10) to the room temperature prior to centrifugation (Sigma, 2-16 PK, Germany) at 2700xg for 15 min. Supernatants were collected and combined with equal volume of 2-propanol. By adjusting the pH to 3.5 (Crison, Basic pH20), pectin was precipitated. The solution kept at room temperature for 8 hour and centrifuged at 2700xg for 15 min and collected supernatants are mixed with 2-propanol and stirred for an hour. Centrifugation and mixing were repeated twice and at the final centrifugation step, precipitate was collected and dried at 37°C (Bindr, Germany). Dried precipitate was measured to give pectin content.

2.2.10. Protein Content

Kjeldahl method was used to determine the protein content of corn grit and carrot pulp. 5-6 g of Kjeldahl catalyst and 15 ml concentrated H_2SO_4 (0.1 N) were added to 1 g of sample in digestion flask. Samples were burned at 400°C in the digestion unit until the solution become clear. 50 ml distilled water was added to the cooled solution prior to distillation. 25 ml 0.1 N H_2SO_4 was added to the trapping flask. Distillation was completed (Şimşek Labor teknik, DES-1) by adding approximately 75 ml NaOH solution. 2-3 drops of methyl red indicator was added to the trapping flask used in distillation and titrated with 0.1 NaOH until the colour change was observed. Protein content was calculated by the following formula.

$$\%\,\text{Protein Content} = \frac{(\text{Volume of } H_2SO_4 - \text{Volume of titrated NaOH}) * 5{,}75 * 0.0014}{\text{Amount of Sample}} * 100$$

2.2.11. Statistical Analysis

Data were analysed with analysis of variance (ANOVA) and differences between the means were determined by Duncan Multiple Comparison Test. The p values less than 0.05 were considered as significantly different.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Proximate Analysis

Raw material of extrudates, corn grit and carrot pulp, were analysed for their protein, ash and moisture content. Moreover pectin content of carrot pulp was determined. The corresponding results are given in Table 3.

Table 3 - Protein, Ash and Pectin Contents of Carrot Pulp and Corn Grit.

	Carrot Pulp	***Corn Grit***
Protein Content (%)	0.99 ± 0.05	7.34 ± 0.29
Ash Content (%)	0.65 ± 0.13	0.46 ± 0.07
Ash Content on dry basis (%)	5.98 ± 1.50	0.52 ± 0.08
Pectin Content (%)	1.09 ± 0.25	-
Pectin Content (g/100g dry weight)	10.26 ± 2.74	-
Moisture Content (%)	89.93 ± 0.27	11.83 ± 0.32

Values are mean ± SD (n = 3)

According to Holland et al. (as cited in Sharma et al., 2012a) carrot had 88.8 % moisture, 0.9 % protein and 1.1 % total ash. Moreover, USDA National Nutrient Database (2013) stated that fresh carrot had average 88 % moisture, 1 % protein and 1 % ash. Chantaro et al. (2008) analysed the carrot peels in terms of pectin content and found that fresh carrot peel had a pectin content of 9.60 g/100g dry weight. Santosa et al. (2008) analysed the composition of four varieties of corn and found that corn had a protein content of 7.84 – 9.84 %, ash content of 1.03 – 1.55 % and

moisture content of 8.53 – 9.31 %. As chemical composition of vegetables and grains changes according to the several factors such as cultivation, seed or fertilizer quality, area of growth; little differences in studies is ordinary (Santosa et al., 2008).

3.2. Effect of Extrusion and Conventional Heating on the Antioxidant Activity and Total Phenolic Content

As shown in Table 4, antioxidant activity of unprocessed sample was found as 6.80 ± 1.02 μmol TE/ g dry sample. Conventionally heated samples did not differ ($p>0.05$) from feed in terms of antioxidant activity, whereas a significant difference was detected ($p\leq0.05$) between the feed and extrudates. The values changed from 4.91 ± 0.76 to 5.34 ± 0.49 μmol TE/ g dry sample for all extrudates, which means that extrusion process decreased the DPPH scavenging activity by 21.49% - 27.76%. Moreover, shift in temperature from 130 ºC to 160 ºC or screw speed from 125 rpm to 225 rpm did not cause a significant change ($p>0.05$) in antioxidant activity.

Table 5 indicates the differences in the values of total phenolic content of feed, extrudates and conventionally heated samples. Phenolic content of feed (18.15 ± 2.83 mg GAE/g dry sample) was not statistically different ($p>0.05$) from conventionally heated samples. Extrusion caused significant reduction of phenolics. However, extrusion parameters (temperature and screw speed) did not affect the total phenolic content ($p>0.05$) within the range of study. As phenolic compounds show antioxidant property, the correlation ($r= 0.98$) between the changes in the total phenolic content and antioxidant activity was expected.

Effect of high pressure is more dominant than the effect of temperature considering the destruction the cell membranes (Patras et al., 2009). High shear stress in extrusion might destroy the phenolics and antioxidant derivatives; as a result lessens the antioxidant activity of extrudates compared to the conventional heating. Apart from shear stress barrel temperature was also responsible for reduced phenolic

content and antioxidant activity of extrudates as most of the phenolics are heat-labile (Brennan et al., 2011). In the case of exposure to heat over 80°C molecular structure of phenolics might be damaged (Brennan et al., 2011; Sharma et al., 2012b; Zielinska et al., 2007). Moreover, extrusion conditions and mechanical energy supplied by extruder may alter the chemical structure of molecules, which resulted in several reactions (Limsangouan et al., 2010). Polymerization reactions supported by high barrel temperature in extrusion might reduce the extractability of phenolic compounds, which result in the decrease of phenolic content as well as antioxidant activity (Brennan et al., 2011; Sarawong et al., 2014; Sharma et al., 2012b). Additionally, losses of antioxidants by evaporation might be another reason for reduced antioxidant activity of extrudates (Pokorný & Schmidt, 2001).

It is possible to find studies in the literature that investigate the change in antioxidant activity of extrudates. However; even if the feed material or the extrusion parameters were similar, comparison of the results is difficult as numerous methods like DPPH, ABTS, TEAC, ORAC and FRAP assays were used to test (Hodzic et al., 2009). Still; when the literature was searched to have a general idea, there are several studies that confirm our findings. Dlamini et al. (2007) and Anton et al. (2009) found the correlation between antioxidant activity and phenolic content and indicated that extrusion cooking had reduced both the level of these two properties. Abd El-Hady & Habiba (2003) stated that extrusion at 140 ºC and 180 ºC significantly decreased the total phenols of legume seeds. However, there are several other researchers indicating that extrusion process did not affect the antioxidant activity or phenolic content in a negative way. Chiu et al. (2012) stated that extrusion at 105°C die temperature did not cause a decrease on the antioxidant activity of extrudates composed of corn-chinese yam mixtures. Sharma et al. (2012b) investigated that extrusion cooking of barley flour at 150°C and 180°C resulted in the increased level of antioxidant activity.

Table 4 – Effect of extrusion and conventional heating process on antioxidant activity

	Trolox Equivalent Antioxidant Activity (µmol/ g dry sample weight)	***% Loss***
Extrusion Process		
Feed	6.80 ± 1.02 [a]	
Extrudate (130ºC & 125 rpm)	5.34 ± 0.49 [b]	21.49
Extrudate (130ºC & 225 rpm)	4.91 ± 0.76 [b]	27.76
Extrudate (160ºC & 125 rpm)	5.20 ± 0.55 [b]	23.55
Extrudate (160ºC & 225 rpm)	5.09 ± 0.68 [b]	25.18
Conventional Heating Process		
Feed heated at 160ºC	6.32 ± 0.51 [a]	7.06
Feed heated at 200ºC	6.37 ± 0.34 [a]	6.32

Values are mean ± SD (n = 3)
Values with different letters are significantly different ($P \leq 0.05$)

Relatively higher residence time and temperature in conventional heating might cause to the physical disruption of the cell wall and release the free phenolics (Sarawong et al., 2014). Another approach for the same phenolic content and antioxidant level of conventionally heated sample and feed might be explained by Maillard reactions (Sharma et al., 2012b). Maillard reactions start at 100°C - 120°C and reach the maximum rate of formation at 150°C (Pokorný & Schmidt, 2001). As a result of short residence time of extrusion, high temperature might be inadequate for the formation of the phenolic compounds and Maillard reaction products compared to conventional heating. However, in conventional heating combined effect of relatively long residence time (10 min) and high temperatures (160°C - 200°C) contributes to transformation to Maillard reaction products, which show strong antioxidant property (Chiu et al., 2012; Rufián-Henares & Delgado-Andrade 2009; Pokorný & Schmidt, 2001).

Table 5 – Effect of extrusion and conventional heating process on total phenolic content

	Gallic Acid Equivalent Phenolic Content (mg/ g dry sample weight)	***% Loss***
Extrusion Process		
Feed	18.15 ± 2.83 [a]	
Extrudate (130ºC & 125 rpm)	10.26 ± 2.27 [b]	43.50
Extrudate (130ºC & 225 rpm)	9.45 ± 0.94 [b]	47.96
Extrudate (160ºC & 125 rpm)	9.60 ± 1.49 [b]	47.16
Extrudate (160ºC & 225 rpm)	10.01 ± 0.36 [b]	44.86
Conventional Heating Process		
Feed heated at 160ºC	17.49 ± 0.32 [a]	3.64
Feed heated at 200ºC	17.23 ± 0.18 [a]	5.07

Values are mean ± SD (n = 3)
Values with different letters are significantly different ($P \leq 0.05$)

In the literature, both positive and negative effect of thermal treatment on antioxidant activity and phenolic content were stated. Huber et al. (2014) studied the change in the antioxidant activity of beans by two different method (DPPH and ABTS) with regard to cooking at 121ºC for 10 min and found that cooked beans had higher DPPH antioxidant activity than raw beans, however; ABTS test gave no significant difference between the antioxidant activity of cooked and raw beans. Dewanto et al. (2002) found that thermal treatment at 115ºC for 25 min increased both the antioxidant activity and total phenolic content of sweet corn by 44% and 54%, respectively. Sensoy et al. (2006) roasted dark buckwheat flour at 200ºC for 10 min and found that antioxidant activity was increased after roasting, while no change was observed in total phenolic content.

3.3. Effect of Extrusion on β-Carotene and Lutein Content

Effect of extrusion on the carotenoid content was investigated by analysing the samples before and after extrusion process. Results indicating the change of β-carotene and lutein content after extrusion process were given in Table 6.

Extrusion process at 130°C and 160°C last treatment zone temperatures with a screw speed of 225 rpm degraded the lutein content to some extent ($p \leq 0.05$). Extrudates were not statistically different from each other with regard to their lutein content within the studied range ($p > 0.05$). In the case of β-carotene, extrusion at 130°C last treatment zone temperature reduced the β-carotene content from 11.13 ± 2.43 to 4.23 ± 0.98 (μg/g dry weight), whereas extrudates formed at 160°C last treatment zone temperature was not significantly different from feed in terms of its β-carotene content ($p > 0.05$).

During extrusion food material was subjected to both thermal and mechanical stresses (Emin et al., 2012). Heat and shear occurred during extrusion promoted the loss of β-carotene and lutein due to their unsaturated structure. Heat induces the oxidation and isomerization, which were the major factors responsible for the extensive loss of carotenoids (Rodriguez-Amaya, 2001; Carbonell-Capella et al., 2014; Hill & Johnson, 2012).

In carrots, β-carotene usually exits in trans-forms, however; due to heat application isomerization from trans to cis configuration was seen (Rodriguez-Amaya, 2001; Castenmiller & West, 1998). Mild heat treatment at 130°C might be insufficient to promote isomerization of β-carotene, whereas extrusion at 160°C induced the conversion of trans-cis form. Compared to its trans-form, cis-β-carotene is more polar; thus more soluble, which might compensate the lost β-carotene due to extrusion (Faulks & Southon, 2005; Van den Berg et al., 2000; Sharma et al., 2012b; Carbonell-Capella et al., 2014). Therefore; no statistical change was observed with respect to total β-carotene content between feed and extrudates processed at 160°C.

Table 6 - Effect of Extrusion on the β-Carotene and Lutein Content

	Prior to in-vitro digestion	***After in-vitro digestion***		***Relative in-vitro Bioaccessibility(%)***
		Dialyzed Portion	*Not Dialyzed Portion*	
Lutein Content (µg lutein / g dry weight)				
Feed	2.66 ± 0.47 [a]	0.20 ± 0.04 [b]	ND	7.50 ± 0.77 [b]
Extrudate (130°C & 225 rpm)	1.91 ± 0.28 [b]	0.18 ± 0.04 [b]	ND	9.54 ± 2.37 [b]
Extrudate (160°C & 225 rpm)	1.38 ± 0.30 [b]	0.34 ± 0.05 [a]	ND	24.91 ± 0.89 [a]
β-carotene *Content* *(µg β-carotene / g dry weight)*				
Feed	11.13 ± 2.43 [a]	2.47 ± 0.32 [a]	TR	22.55 ± 2.33 [b]
Extrudate (130°C & 225 rpm)	4.23 ± 0.98 [b]	1.69 ± 0.44 [b]	TR	39.84 ± 5.93 [a]
Extrudate (160°C & 225 rpm)	9.26 ± 2.02 [a]	3.16 ± 0.33 [a]	TR	34.72 ± 4.36[a]

Values are mean ± SD (n = 3)
Values in the same column followed by different letters are significantly different ($P < 0.05$)
ND means 'Not Detectable Level', TR means 'Trace Level'

3.4. Effect of Extrusion on *in-vitro* bioaccessibility of β-Carotene and Lutein

Relative *in-vitro* bioaccessibility (%) of lutein was found as 7.50 ± 0.77, 9.54 ± 2.37 and 24.91 ± 0.89 for feed, extrudate processed at 130°C last zone temperature and extrudate processed at 160°C last zone temperature, respectively (Table 6). Extrusion process at 130°C last zone temperature did not affect the *in-vitro* bioaccessibility, however; extrusion at 160°C last zone temperature caused a significant increase ($p \leq 0.05$). In the case of β-carotene, feed had a relative *in-vitro* bioaccessibility of 22.55 ± 2.33 % and after extrusion at 130°C and 160°C last zone temperatures, it was increased to 39.84 ± 5.93 % and 34.72 ± 4.36 %, respectively; which were not statistically different from each other ($p > 0.05$).

In-vitro bioaccessibility of β-carotene increased by heat treatment regardless of the studied temperature range. One reason for increased bioaccessibility is more release of β-carotene due to softened structure of cell wall, which was linked to thermal stress during extrusion (Hornero-Méndez & Mínguez-Mosquera, 2007; Southon & Faulks, 2003). Moreover, increase in extrusion temperature disrupted the protein-carotenoid complexes by the denaturation of proteins, which increased the amount of free carotenoid molecules ready to release (Parker, 1996; Mulokozi et al., 2004). Instead of thermal stress, mechanical stress also played role in improved bioaccessibility as crushing between extruder screws reduced the particle size, which created more surface area for digestive enzymes to come into contact (Erdman et al., 1993; Hedren et al., 2002; Donhowe & Kong, 2014; Southon & Faulks, 2003).

Thermal and mechanical stresses formed during extrusion process at 130°C last zone temperature and 225 rpm was not be severe enough to destruct the protective matrix of lutein, thus did not effect to the *in-vitro* bioaccessibility ($p > 0.05$). However, shift in temperature to 160°C accelerated the release of lutein by breaking of the cell wall, thus increased its bioaccessibility (Hornero-Méndez & Mínguez-Mosquera, 2007).

In the literature there are several studies in agreement with our findings, which stated the negative effect of thermal or mechanical stress on carotenoid content, in the meantime their positive effect on *in-vitro* bioaccessibility. Hornero-Méndez & Mínguez-Mosquera (2007) searched the effect of cooking (100°C for 15 min) on carotenoid content of carrot and observed both the degradation of carotenoids and increment of *in-vitro* bioaccessibility with respect to heat. Veda et al. (2010) studied the effect of different cooking methods on carotenoids and examined the loss in β-carotene content of vegetables which were subjected to the pressure-cooking, open-pan boiling or stir-frying. Furthermore, improvement in *in-vitro* bioaccessibility was recorded for each type of cooking. Courraud et al. (2013) investigated the bioaccessibility of lutein and β-carotene in raw and cooked spinach (hydrothermal cooking at 98ºC for 20 min). It was stated that hydrothermal cooking destroyed 33.8 % of β-carotene (from 57.9 ± 5.2 to 38.3 ± 1.6 mg/100 g dry matter) and 57.6 % of lutein content (from 58.9 ± 8.3 to 25.0 ± 5.9 mg/100 g dry matter). Moreover, bioaccessibility of both carotenoids increased after cooking, which was explained by the disruption of food matrix by heat or pressure. Pugliese et al. (2014a) investigated the bioaccessibility of carotenoids in 20 varieties of sun dried chilli peppers (for 1 month at 15-30ºC) in pursuit of *in-vitro* digestion procedure. It was stated that β-carotene had lower bioaccessibility then capsanthin, zeaxanthin and lutein.

As well as processed food samples, *in-vitro* bioaccessibility of lutein and β-carotene for raw materials has also been studied several times. Granado-Lorencio et al. (2007) investigated *in-vitro* bioaccessibility of several food samples such as green vegetables (broccoli, spinach, lettuce), non-green vegetables (tomato, carrot, red pepper, fresh tomato) and fruits (orange, kiwi, pineapple, loquat). Green vegetables showed lowest rate of incorporation for β-carotene, lutein and zeaxanthin compared to non-green vegetables and fruits, which was associated to cellular localization. Schweiggert et al. (2012) compared the bioaccessibility of selected carotenoids (β-cryptoxanthin, β-carotene, lycopene and lutein) in carrot, papaya, mango and tomato by using *in-vitro* digestion model. *In-vitro* bioaccessibility (%) of β-carotene was found as 0.5, 5.3, 10.1 and 3.1 for raw carrot, papaya, mango and tomato, respectively. The results indicated that carotenoids could be ranked as lycopene, β-

carotene, β-cryptoxanthin and lutein according to relative bioaccessibility from less to more. The difference in the bioaccessibilities was explained by different physical form and different hydrophobicity of each carotenoid.

In addition to studies related to *in-vitro* digestion and bioaccessibility, there are few *in-vivo* bioavailability researches that examined the mutual effects of β-carotene and lutein. Although its mechanism was not clarified yet, it was stated that interaction of β-carotene with lutein decreases the absorption of lutein by measuring the plasma area under curve (Southon & Faulks, 2001). Kostic et al. (1995) investigated that β-carotene scaled down the lutein response in plasma by 40 %, whereas lutein did not have an effect on the plasma β-carotene response in humans. In several studies less response of lutein was taken as long as β-carotene supplementation was executed, which implied the antagonistic effect of β-carotene on lutein absorption (Hughes et al., 1997; Micozzi et al., 1992; Faulks et al., 1998).

Each carotenoid has different sensitivity to oxidation. Lutein together with ζ-carotene and violaxanthin were grouped as the most susceptible carotenoids to oxidation (Rodriguez-Amaya, 2001), which may result in the higher destruction. Moreover, interaction between carotenoids could complicate to make certain comment on carotenoids' behaviour. For example, interaction between β-carotene and lutein is reported as a reason for much better β-carotene absorption (Southon & Faulks, 2001). In addition, interaction of lycopene with β-carotene reported to have no effect on β-carotene absorption while improving the lycopene absorption (Johnson et al., 1997). Moreover; nature of food matrix such as presence of fat and fiber or physical form of carotenoids might affect the carotenoids' digestion or absorption thereby resulted in various outcomes in the literature (Southon & Faulks, 2001). Additionally, it should be also noted that differences in procedures and analysing methods limits making general implications about carotenoids' behaviour.

3.5. Effect of Extrusion on Starch Gelatinization

Selected DSC diagrams for each feed and extrudates are given in Figures 1-3. Although gelatinization peak was observed for the samples before extrusion; none of endothermic transition was seen in DSC diagrams of extrudates, which means complete gelatinization was observed for the samples extruded at both 130ºC and 160ºC, with a screw speed of 225 rpm. Extrusion resulted in total or partial destruction of starch granules (Gray & Chinnaswamy, 1995). Bouvier (2001) stated that loss of crystalline-ordered structure of starch molecules took place in shorter time when sufficient shear was applied instead of performing heat alone. This could be explained by disruption of amylopectin molecules between screws of extruder, which caused the physical breakdown of starch granules. Hence, molecular weight of granules was reduced and starch became more easily accessible (Camire, 2001; Hagenimana, Ding, & Fang, 2006). Consequently, processing parameters of extrusion like temperature, shear and pressure might accelerate the rate of starch gelatinization (Camire, 2001; Altan et al., 2009).

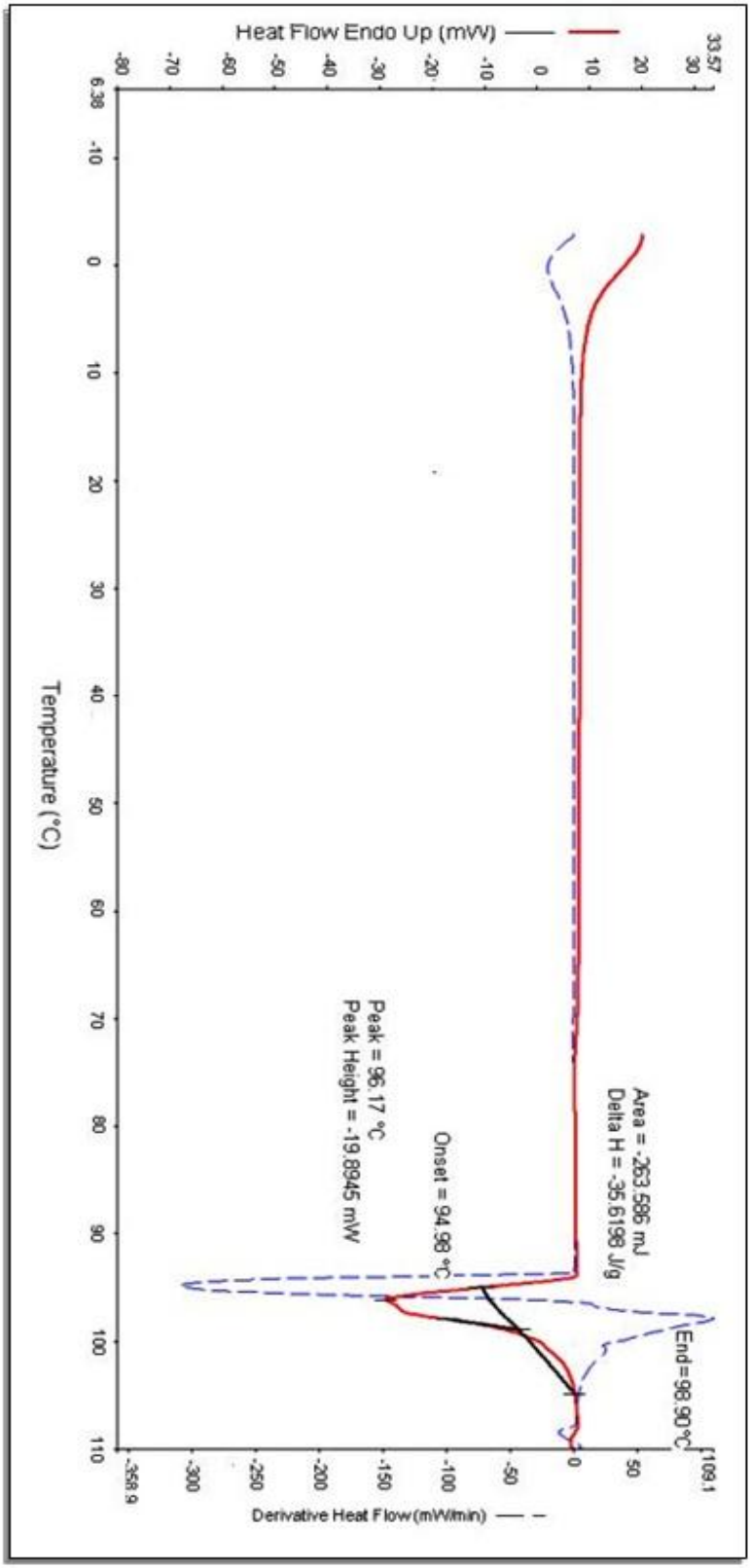

Figure 1 – DSC chromatogram of feed.

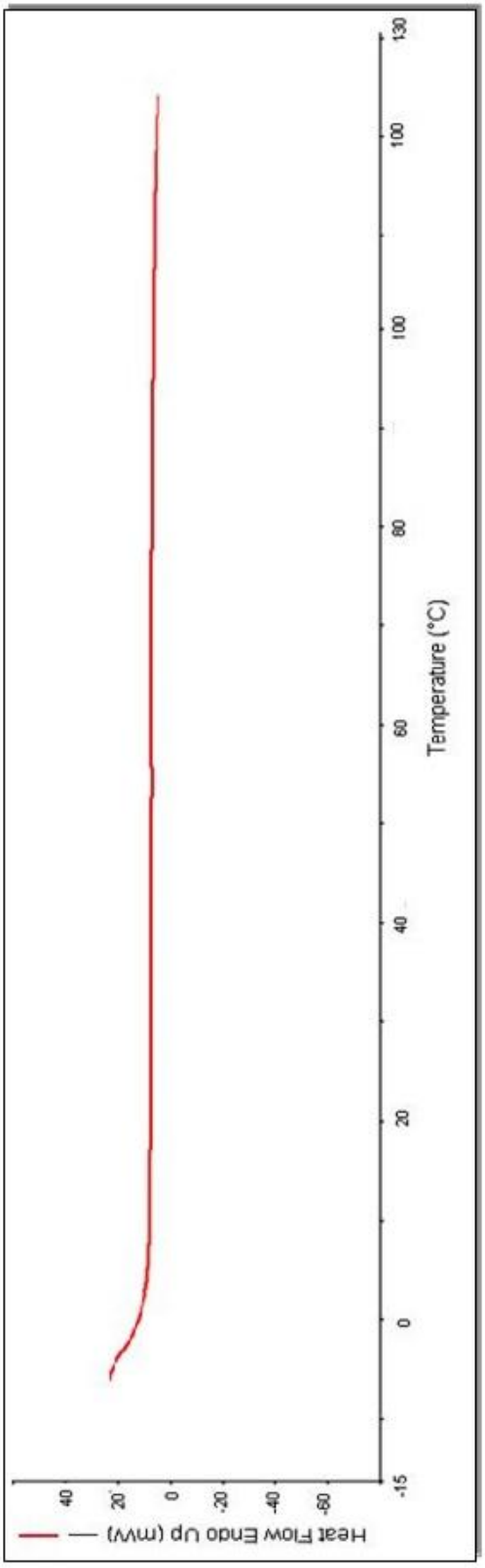

Figure 2 – DSC chromatogram of extrudates produced at 130 °C & 225 rpm.

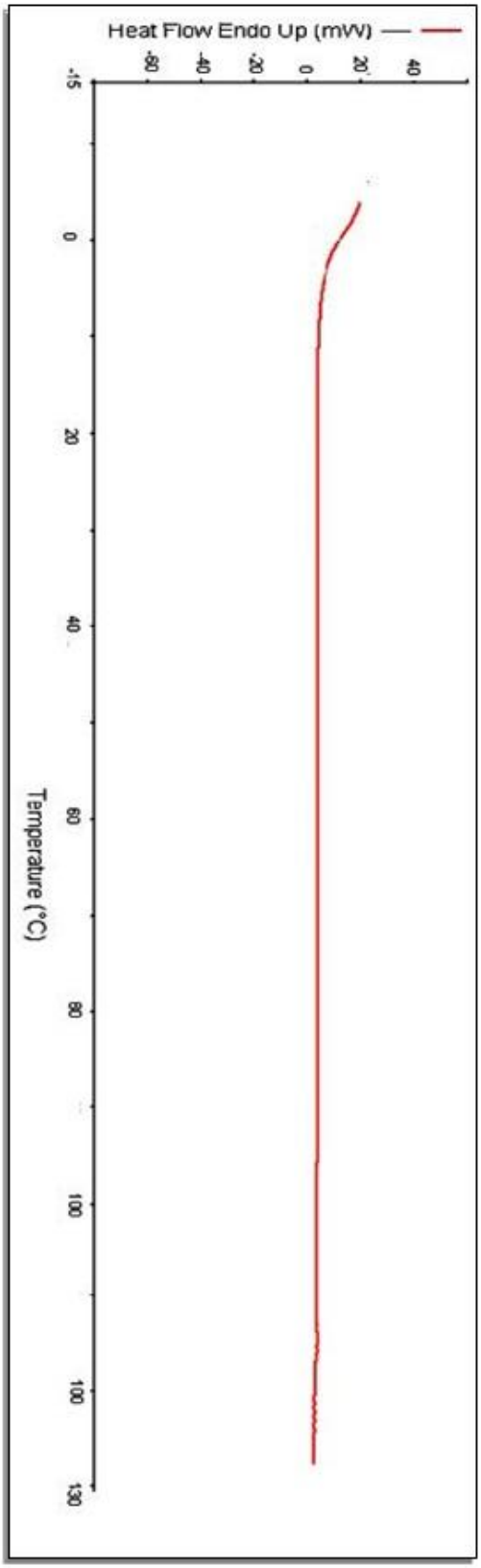

Figure 3 – DSC chromatogram of extrudates produced at 160 °C & 225 rpm.

CHAPTER 4

CONCLUSION

It was found that extrusion process reduced the antioxidant activity and total phenolic content ($p \leq 0.05$) whereas conventional heating process did not promote a change in antioxidant activity and phenolic content ($p > 0.05$). Moreover, extrusion decreased the lutein content both at 130°C and 160°C last zone treatment temperatures at 225 rpm screw speed, while increasing its *in-vitro* bioaccessibility only at 160°C last zone temperature ($p \leq 0.05$). Extrusion also reduced β-carotene content at 130°C last zone temperature, while showed no effect at 160°C last zone temperature ($p > 0.05$). In the case of *in-vitro* bioaccessibility of β-carotene, extrusion process significantly increased the bioaccessibility within the ranges studied ($p \leq 0.05$). Amount of absorbed carotenoid is more important than their consumed amount. Therefore; even though some favourable effect of extrusion was stated in present study on the *in-vitro* bioaccessibility, further research or *in-vivo* studies are needed to understand the behaviour of carotenoids in human digestive system.

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APPENDIX A

SCREW CONFIGURATIONS OF THE EXTRUDER

Table A1 - Screw configurations of the extruder

8 D	Twin lead feed screws
7 x 30°	Forward kneading elements
4 D	Twin lead feed screws
4 x 60°	Forward kneading elements
4 x 30°	Reverse kneading elements
2 D	Twin lead feed screws
6 x 60°	Forward kneading elements
4 x 30°	Reverse kneading elements
1 D	Single lead feed screws
7 x 90°	Kneading elements
2 D	Single lead feed screws
	Die

Screw diameter (D) = 25 mm.

One kneading element = 0.25 D.

APPENDIX B

EXTRUDATE PRODUCTION

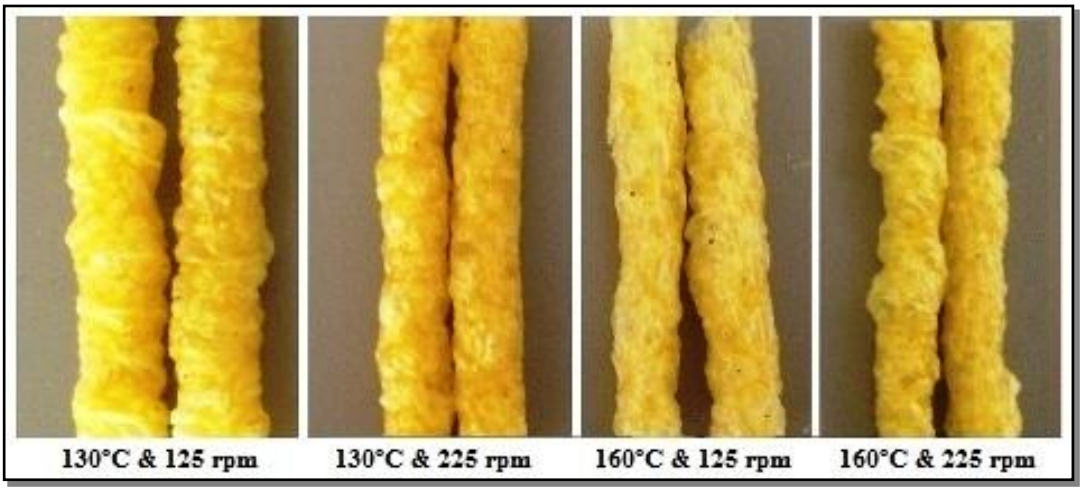

Figure B1 – Extrudates processed at different parameters.

APPENDIX C

β-CAROTENE & LUTEIN STANDARD CURVES

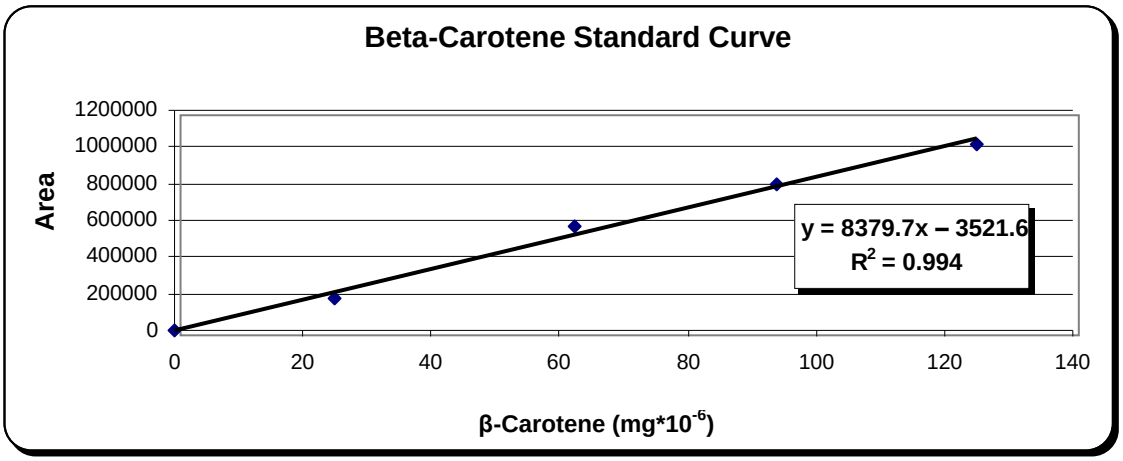

Figure C1 - β-Carotene Standard Curve in the range of 200000 - 10000000 Area.

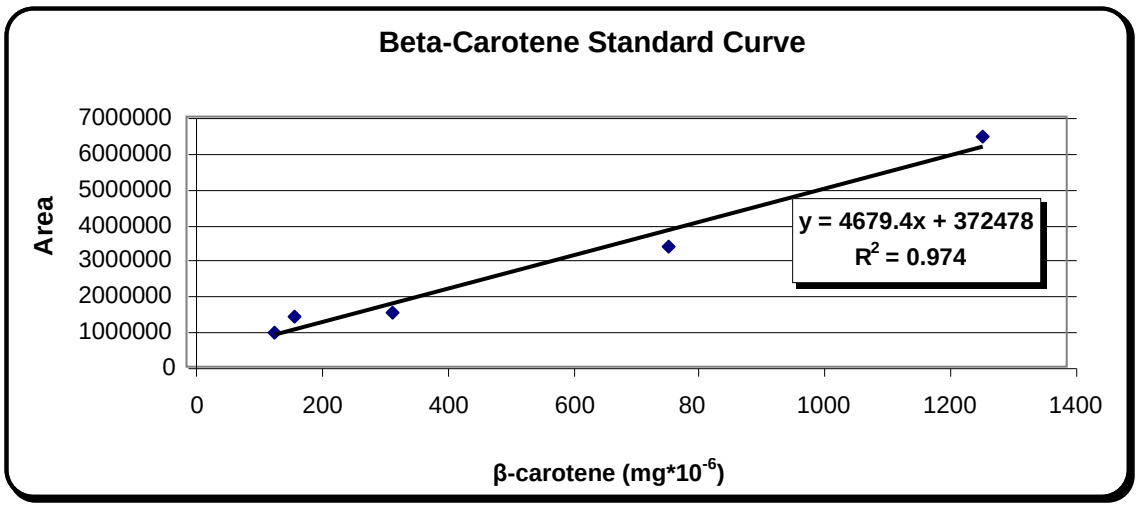

Figure C2 - β-Carotene Standard Curve in the range of 1000000 - 6500000 Area.

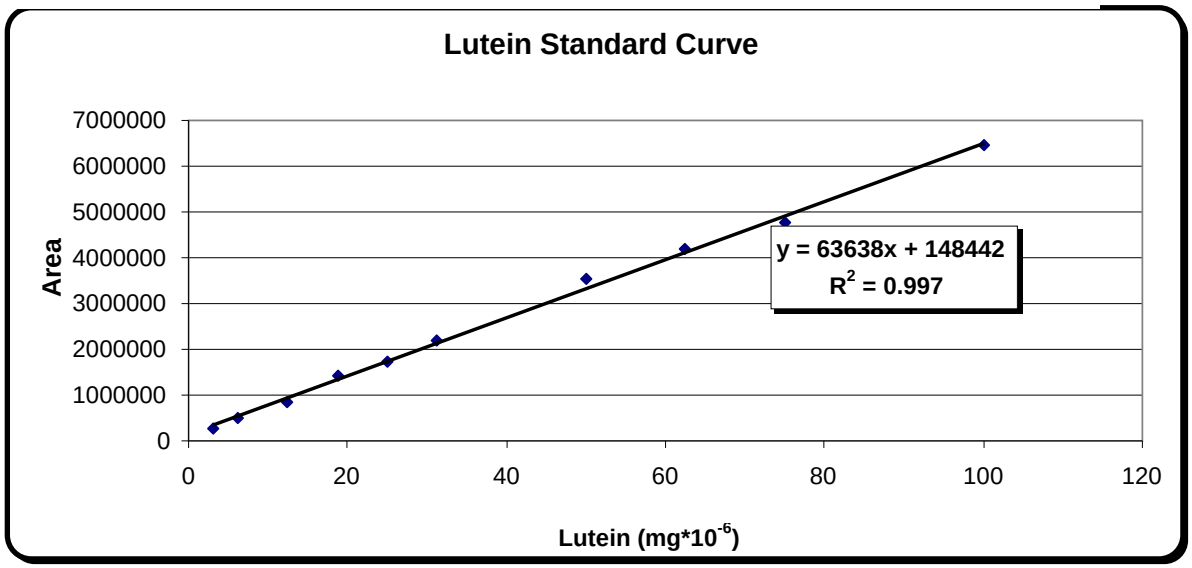

Figure C3 - Lutein Standard Curve.

APPENDIX D

HPLC CHROMATOGRAM

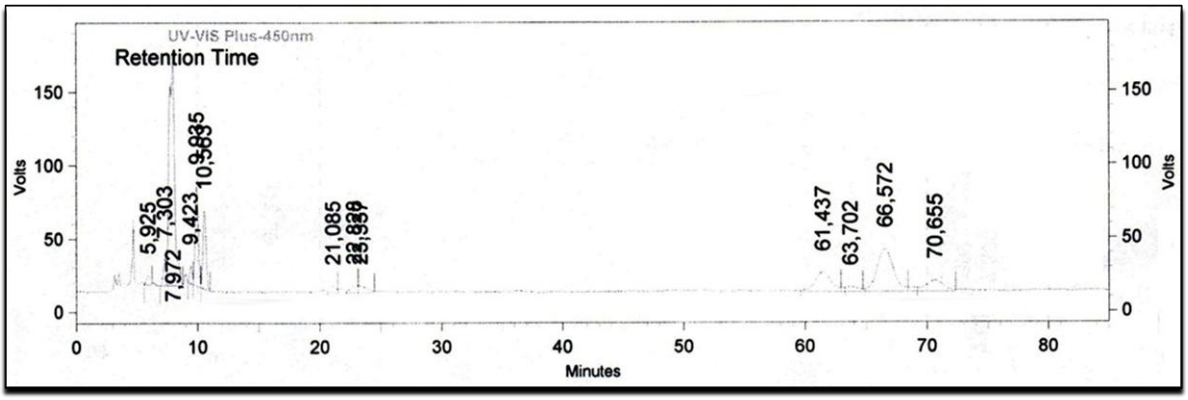

Figure D1 – Sample HPLC chromatogram for lutein and β-carotene whose retention times are 7.30 and 66.57 seconds, respectively.

APPENDIX E

SCHEMATIC DIAGRAM OF EXTRUDER

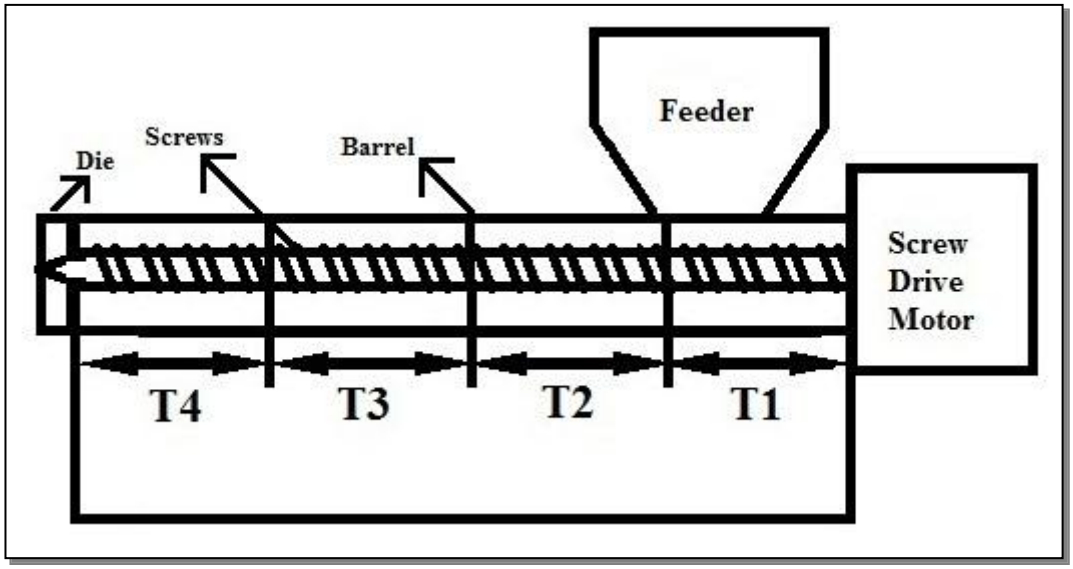

Figure E1 – A schematic diagram of an extruder where T1, T2, T3 and T4 are temperature zones.