NEDA AFLAKI

OPTIMIZATION OF CAROTENOID EXTRACTION IN PEEL AND FLESH OF CANTALOUPE (*CUCUMIS MELO* L.), WITH ETHANOL SOLVENT

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Résumé

Le cantaloup (*Cucumis melo* L.) contient de nombreuses molécules biologiquement actives, telles que les caroténoïdes qui sont des pigments naturels, et qui ont des propriétés souhaitables sur le plan commercial en tant que produits de santé ou nutraceutiques. Les solvants organiques lipophiles tels que l'acétone sont habituellement utilisés pour l'extraction des caroténoïdes, puisque la plupart d'entre eux sont lipophiles et insolubles dans l'eau. Des traces de ces solvants organiques peuvent cependant rester dans l'extrait et être nocifs pour la santé humaine. Dans cette étude, les principaux caroténoïdes qui ont un intérêt nutritionnel ont été extraits, sous conditions optimales, en utilisant de l'éthanol comme solvant car il a une influence limitée sur la santé. Cependant, l'éthanol n'est pas aussi efficace que de nombreux solvants lipophiles pour l'extraction des caroténoïdes.

À cette fin, les caroténoïdes dans la peau et la chair lyophilisées du cantaloup ont été extraits et puis identifiés par chromatographie en phase liquide à haute performance (CLHP). Des conditions optimales d'extraction éthanolique ont été déterminées pour maximiser l'extraction des caroténoïdes totaux (TC). Une durée d'extraction de 2 heures à une température de 50 °C s'est avérée optimale pour la chair alors celle pour la peau était 2 heures à 30 °C. Dans des conditions optimales, les valeurs de TC de la peau et de la chair, mesurées par CLHP, ont été 0,33 mg/g et 0,22 mg/g de poudre sèche, respectivement. Le caroténoïde prédominant dans la chair était le β-carotène, tandis que le caroténoïde majeur de la peau était la lutéine. Ainsi, l'extrait de la peau peut être potentiellement considéré comme une nouvelle source de caroténoïdes naturels pour l'alimentation et des produits nutraceutiques.

Les polyphénols de la peau et de la chair lyophilisées du cantaloup ont également été identifiés en utilisant la chromatographie en phase liquide à ultra performance (CLUP) couplée à la spectrométrie de masse en tandem (SM/SM). Quarante deux composantes de polyphénols ont été identifiées dans la peau et la chair. La valeur de polyphénols totaux de la peau, déterminée par spectrophotométrie, a été plus élevée que celle de la chair. Différents types de flavonoïdes, d'acides hydroxybenzoiques et d'acides hydroxylcinnamiques ainsi que des stylbènes habituellement retrouvés dans le vin rouge

(viniférine) ont été identifiés. De la conidendrin, un lignane, a été également identifiée et pourrait possiblement agir comme des phytoalexines aux propriétés antifongiques. Toutefois, la présence de viniférine et conidendrin doit être confirmée par d'autres méthodes.

Les paramètres optimaux pour l'extraction éthanolique de caroténoïdes ont été utilisés à l'échelle pilote sur le mélange de peau et chair de cantaloup pour valider les résultats de laboratoire et pour produire un produit riche en caroténoïdes. Après extraction et séchage, une poudre et une résine ont été obtenues. Contrairement à la poudre, la résine était riche en caroténoïdes et soluble dans l'huile. Le jus de cantaloup constitue une bonne source de polyphénols.

Résumé court

Les carotenoides et polyphénols de la peau et la chair du cantaloupe ont été identifiés par CLHP et CLUP, respectivement. Des conditions optimales d'extraction éthanolique ont été déterminées pour maximiser d'extraction des caroténoïdes totaux (TC). Une durée d'extraction de 2 heures à une température de 50 °C s'est avérée optimale pour la chair alors celle pour la peau était 2 heures à 30 °C. Les valeurs de TC de la peau et de la chair, mesurées par CLHP, ont été 0,33 mg/g et 0,22 mg/g de poudre sèche, respectivement. Le caroténoïde prédominant dans la chair était le β-carotène, tandis que le caroténoïde majeur de la peau était la lutéine. Différents types de flavonoïdes, acides hydroxybenzoïque et hydroxylcinnamic ont été identifiés. La valeur de polyphénols totaux de la peau a été plus élevée que dans la chair. Les paramètres optimaux de laboratoire pour l'extraction éthanolique de caroténoïdes ont été utilisés dans l'échelle pilote pour valider les résultats de laboratoire et produire un produit riche en caroténoïdes.

Long abstract

Cantaloupe (*Cucumis melo* L.) contains many biologically active molecules such as carotenoids which are natural pigments with desirable health benefits and nutraceutical properties. Organic lipophilic solvents such as acetone and chloroform are usually used for extraction of carotenoids, since most of them are lipophilic and insoluble in water. However, these solvents may remain in the extract and can be harmful for human health. The extract product, therefore, is not safe for addition to food to increase their health value. In this study, the important carotenoids that are found naturally in cantaloupe and are of nutritional and pharmacological interest have been extracted, under optimum condition, using ethanol as solvent which has limited influence on the health. However, ethanol is not as efficient as many lipophilic solvents for extraction of carotenoids.

For this purpose, the carotenoids in freeze dried peel and flesh of cantaloupe, were extracted and identified using high-performance liquid chromatography (HPLC). Optimum ethanolic extraction conditions were determined for maximizing the extraction of total carotenoid (TC). Extraction duration of 2 hours and temperature of 50°C were found to be the optimum extraction conditions for the flesh while it was 2 hours and 30°C for the peel. Under optimal conditions, the amount of TC in peel and flesh, measured by HPLC, were 0.33 mg/g and 0.22 mg/g of dry powder, respectively. The predominant carotenoids identified by HPLC were β-carotene and lutein. β-Carotene was the most prevalent carotenoid in the flesh, while the major carotenoid of peel was lutein. Thus, the extract of peel can be potentially considered as a new source of natural carotenoids for food and nutraceutical products.

Polyphenols in freeze dried peel and flesh of cantaloupe were also identified using ultra performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS). Forty two polyphenol compounds were found in peel and flesh. The amount of total polyphenol in peel, determined by spectrophotometer, was higher than that in the flesh. Many different types of flavonoids, hydroxybenzoic and hydroxylcinnamic acids as well as common stylbene found in red wine (viniferin) were identified in non-negligible quantities. Another stylbene, conidendrin, was identified and probably act as a phytoalexin

with antifungal property. However, the presence of viniferin and conidendrin should be confirmed by other methods.

The optimum laboratory parameters for ethanolic extraction of carotenoids were used in pilot scale on the mixture of peel and flesh of cantaloupe to validate the laboratory results and produce a product rich in carotenoid. After extraction and drying, a powder and a resin were obtained. In contrast to the powder, the resin was rich in carotenoid and soluble in oil. The juice of cantaloupe was a good source of polyphenols.

Short abstract

The carotenoid and polyphenol compositions of the peel and the flesh of cantaloupe were identified using HPLC and UPLC, respectively. Optimum ethanolic extraction conditions were determined for maximizing the extraction of total carotenoid (TC). Extraction duration of 2 hours and the temperature of 50°C were found to be the optimum extraction conditions for the flesh while it was 2 hour at 30°C for the peel. Under optimal conditions, the amount of TC in peel and flesh were 0.33 mg/g and 0.22 mg/g of dry powder, respectively. β-Carotene was the most prevalent carotenoid in the flesh, while the major carotenoid of peel was lutein. Different types of flavonoids, hydroxybenzoic and hydroxylcinnamic acids were identified. The amount of total polyphenol in peel was higher than that in the flesh. The optimum laboratory parameters for ethanolic extraction of carotenoids were used in pilot scale to validate the laboratory results and produce a product rich in carotenoid.

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Dedicated to my parents and my husband
who have been a great source of motivation
and inspiration and have supported me all the way.

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Chapter 1: Introduction

Consumption of fruits and vegetables is considered by many authorities as a public health issue and is the subject of global nutritional recommendations by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). Increased intake of fruits and vegetables is generally associated with a reduced risk of cancer (Mayne, 1996; World Cancer Research Fund/American Institute for Cancer Research, 2007) and cardiovascular disease (Mayne, 1996; Kris-Etherton et al., 2002; Müller, 1997). This association is due to the presence of different phytochemicals, such as carotenoids and phenolic compounds, in fruits and vegetables, which has potential or proved beneficial effects on human health (Mattila and Kumpulainen, 2002). That is why vegetables as well as fruits are widely recommended as healthy foods.

Melons (*Cucumis melo* L.), one of the most widely cultivated and consumed vegetable crops in the world, have significant economic value (Nun˜ez-Palenius et al., 2008). Cantaloupes are commonly grown commodities and popular dietary choices. They are rich sources of vitamin C, vitamin E, polyphenols and carotenoids, which have been suggested as natural sources of antioxidants. They belong to the family of *Cucurbitaceae* and are cultivated in all tropical regions of the world. Consumers are principally interested in the sweetness, texture, flavour/aroma and more recently the health benefit phytonutrients of melons (Lester, 2008).

Carotenoids are one of the valuable constituents of the cantaloupes. Carotenoids, as the main group of natural pigments in nature, are responsible for the red, orange and yellow colors of fruits and vegetables. The restrictions on several certified food colors have stimulated the interest in commercial production and storage stability of these natural carotenoid pigments. Natural colorants in foods can be simply named in ingredient list as colorants and do not need to be certified. Recently, food processors and technologists have shown a great interest in the extraction, identification, and purification of natural pigments, including carotenoids. Such an interest is due to favourable properties of these pigments such as natural origin, nutritional value, high versatility, innocuity and consisting both lipo- and hydro-soluble colorants. In addition to the provitamin A activity of some carotenoids, these pigments have recently been used in the prevention of or protection against serious

human health disorders (Rodriguez-Amaya, 1997; Oliver and Palou, 2000; El-Qudah, 2009).

Phenolic compounds are another group of phytochemicals determined in cantaloupes (Ismail et al., 2010). They have antioxidant capacity, antimutagens, antiproliferative, antimicrobial properties and offer beneficial influences in human health, such as in the treatment and prevention of cancer, cardiovascular diseases, and other pathologies. (Manach et al., 2004; Machlin, 1995; Cacace and Mazza, 2003; Bravo, 1998; Gruz, Novák, and Strnad, 2008).

This project aims to define and introduce a practical way for extraction and analysis of carotenoids in cantaloupe. Besides, polyphenol content in cantaloupe will be determined. The analysis of carotenoids is complicated due to several reasons. These include the presence of *cis–trans* isomeric forms of carotenoids, their characteristic conjugated double bond system and inherent instability (Oliver and Palou, 2000) that will be explained. In addition, organic lipophilic solvents such as acetone, chloroform and ethyl acetate are used for extraction of carotenoids, since most of the carotenoids are lipophilic and insoluble in water (Rodriguez-Amaya, 2001). These solvents, including acetone which is most often used (Wang and Liu, 2009), may remain in the extract and can be harmful for human health. The extract product, therefore, is not safe for addition to food products to increase their health value (Calo et al., 1995). This project, therefore, will focus on the procedures to extract the important carotenoids naturally present in cantaloupe and of nutritional and pharmacological interest, using ethanol as solvent, which has limited influence on the health. However, ethanol is not as efficient as many lipophilic solvents (for extraction of carotenoids).

This thesis contains five chapters. After a brief description of cantaloupes and their constituents, the importance and benefits of the carotenoids and polyphenol will be explained, and the difficulties in the extraction methods of carotenoids will be discussed. Then the methodology and experimental details used for extraction and analysis of the carotenoids and polyphenols will be introduced. Finally, the laboratory and pilot scale results will be discussed and concluded.

Chapter 2: Literature review

2.1. Cantaloupe

Production of melons has been so increased around the world that they are becoming the most highly produced fruits. Different cultivars of melon such as cantaloupes, Persian, honeydews, casaba, Santa Claus, and Christmas melons have been produced. China and Turkey are the world's largest melon producers with 13.7 and 1.7 million tons of yearly production, respectively (Kolayli, et al., 2010). The production of cantaloupe in Canada has increased 60.6% from 19105 tonnes in 2007 to 30681 tons in 2010, as indicated in Figure 2.1.

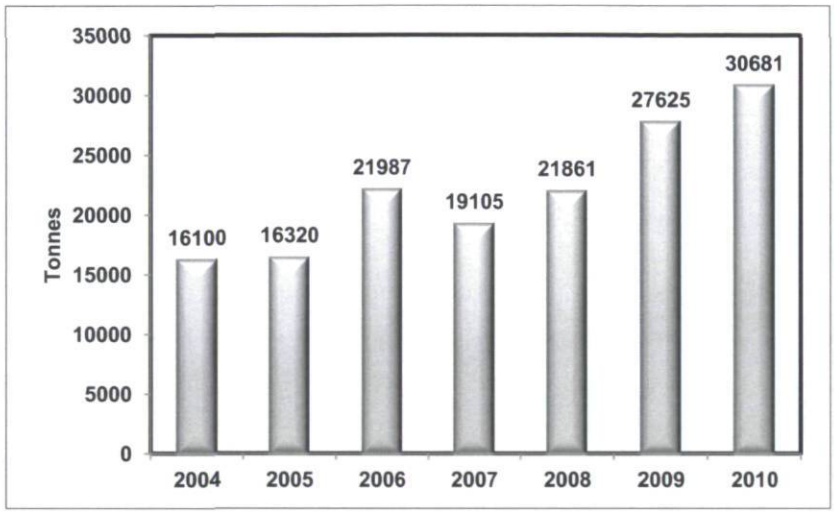


Figure 2.1. Production of cantaloupe in Canada between 2004 and 2010. www.syngenta-vegetables.com/en/announcements/canada-melon-production-on-the-increase.aspx

Cantaloupes contain minerals such as potassium, calcium and iron. They are also qualified as a very good source of vitamin C, vitamin E (tocopherols), polyphenols and carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin) which have been suggested as natural sources of antioxidants (Laur and Tian, 2011; Kolayli et al., 2010; Ismail et al., 2010). It also contains the enzyme superoxide dismutase (SOD) which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Vouldoukis et al., 2004). Cantaloupes contain large quantities of seeds, a rich source of oil and proteins, which are reported to possess medicinal properties. The peel of cantaloupes is commonly discarded because it is indigestible or may be contaminated by sprays or human disease agents. It is reported that most fruits and fruit vegetables have higher carotenoid levels in the peel than in the pulp. Fruit peels, therefore, are a good source of carotenoids (yellow and orange), which are masked by green chlorophyll, and can be treated to make by products

(Rodriguez-Amaya, 1997). It has been reported that the peel of peach contains higher amounts of phenols (Tomas-Barberan et al., 2001), carotenoids and total ascorbic acid than the flesh (Gil et al., 2002). The peel of apple contains a higher amount of phenolic compounds and antioxidant activity (Wolfe, Wu, and Liu, 2003). The level of lycopene in tomato skins is higher than that in the pulp and seeds (Toor and Savage, 2005).

Extraction and separation of carotenoids from natural fruits and vegetables is a method to obtain the supplemental concentrated carotenoids. The intake of supplemental carotenoids improves the health condition and also is a good way to use the waste materials, such as fruit peels and seeds, since waste peels and seeds contain active constituents (Takahashi et al., 2006; de Melo et al., 2000, 2001; Maznah, 2010).

2.2. Carotenoids

2.2.1. Antioxidant activity and carotenoids

Antioxidants are a group of compounds that are produced by the body or that occur naturally in many foods. They may work together in the body to maintain our health by protecting against damages caused by free radicals, which can injure healthy cells and tissues, causing them to lose their structure and function. Oxygen is a highly reactive atom that can become part of potentially damaging molecules called free radicals. Free radicals are unstable and react rapidly and destructively with biomolecules such as protein, lipid, DNA and RNA in the body. Generation of free radicals is associated with peroxidation of lipids and proteins. This peroxidation results in cell structural damage and tissue injury, and finally leads to various health disorders such as Alzheimer's disease, cancer, atherosclerosis, diabetes, mellitus, hypertention and ageing. Antioxidant functions are associated with reduced DNA damage and lipid peroxidation and maintained immune function that are thought to prevent the development of some diseases (Packer and Colman, 2000; Velioglu et al., 1998; Becker, Nissen, and Skibsted, 2004; Valko et al., 2007; Ames, Shigenaga, and Hagen, 1993).

Carotenoids have the ability to act against diseases which has been related to their antioxidant activity, specifically to their ability to quench singlet oxygen and interact with free radicals. The antioxidant property of carotenoids is related to their conjugated double bond system and carotenoids with more than nine double bonds offer the maximum

protection. To ensure a sufficient intake of antioxidants, the human diet should contain 100-500 g/day of carotenoid-rich fruits and vegetables (Müller, 1997).

Carotenoids are one of the most important groups of natural pigments due to their wide distribution, structural diversity and numerous functions. Carotenoids have attracted the interest of researchers of different fields including biochemistry, biology, nutrition and food science, medicine and pharmacy for more than a century. They are responsible for the yellow, orange, and red colors of fruits, roots, flowers, fish, invertebrates and birds. For example, β-carotene and lutein are responsible for the orange and yellow colors, respectively. Carotenoids are synthesized by all plants and many microorganisms (bacteria and fungi), but not by animals. Humans, therefore, rely on dietary intake of carotenoids.

Carotenoid structure provides very special properties which are the basis of their varied functions in all kinds of living organisms. They are essential for photosynthesis and living in an oxygen atmosphere. Carotenoids have two major functions in photosynthetic tissues of plants. They absorb energy for use in photosynthesis and protect the photosystems from photodamage. Up to now, more than 600 carotenoids have been identified in natural sources. However, only about 40 of them are present in a typical human diet and about 20 of them have been identified in human blood and tissues. The carotenoid composition of foods is affected by several factors such as cultivar or variety; stage of maturity; part of the plant consumed; climate or geographic site of production; harvesting and postharvest handling; processing and storage (Rodriguez-Amaya, 2004, 1993). Elevated temperature and longer exposure to sunlight increases the generation of carotenoids in fruits. Tropical climates improve carotenoid biosynthesis, thus, fruits produced in such climates contain higher carotenoid concentrations (Cavalcante and Rodriguez- Amaya, 1992; Kimura et al., 1991; Azevedo-Meleiro and Rodriguez-Amaya, 2004). During ripening, as chloroplasts change to chromoplasts, a decrease in chlorophyll and an increase in β-carotene content will occur (Lester and Eischen, 1996).

The majority of carotenoids are lipophilic and are insoluble in water and soluble in organic solvents. Carotenes are readily soluble in petroleum ether, hexane, and toluene, while xanthophylls exhibit better solubility in methanol and ethanol (Rodriguez-Amaya, 2001).

Lutein and β-carotene have excellent solubility in tetrahydrofuran (THF) (Craft and Soares, 1992). The wavelength maxima and absorptivity of carotenoids depend on the nature of the solvent. For instance, β-carotene dissolved in ethanol has a visible spectrum with little fine structure and absorbance maxima at 453 and 480 nm, while in carbon disulfide exhibits more fine structure and maxima at 484 and 512 nm (Craft and Soares, 1992).

Carotenes are lipophyllic molecules and are expected to be found in a hydrophobic core of thylakoid membranes. Xanthophylls are generally hydrophobic molecules and may have the same location as carotenes. The polar groups of xanthophylls are located at the opposite sides of a long rod-like non-polar skeleton (Gruszecki, 2004). A schematic of an artificial lipid membrane containing β-carotene and zeaxanthin is shown in Figure 2.2. These two carotenoids are oriented in different ways. β-carotene is distributed within the membrane without any preferred orientation. Zeaxanthin is located in the two polar regions of the bilayer while its long axis is almost perpendicular to the membrane. In contrast to β-carotene, zeaxanthin decreases membrane fluidity (Michel, 1998).

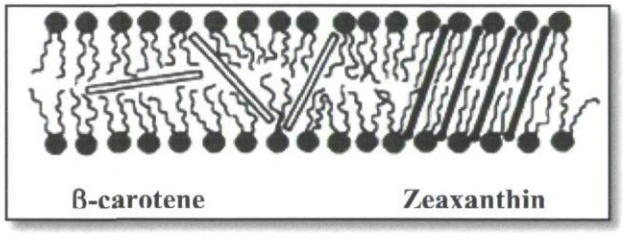


Figure 2.2. Influence of carotenoid orientation on membrane fluidity (Michel, 1998).

2.2.2. Advantages of carotenoids

Carotenoids have recently been used in order to protect human health and to prevent diseases such as various cancers, heart disease, macular degeneration and cataracts (Mayne, 1996). Carotenoids have also been successfully used for many years in the treatment of individuals suffering from photosensitivity disease like erithropoietic protoporphyria. Numerous studies have shown that increased intake of carotenoid-rich foods, fruits and vegetables has a protective effect against several human chronic diseases (Oliver and Palou, 2000). They are also powerful agents for growth inhibition in several human tumor cell lines but not in normal cells. Since many carcinogens inhibit gap junction communications,

protection of this activity by dietary substances could be important for protection against cancer. β-carotene may play an important role in facilitating normal cell to cell communication through gap junction (Acevedo and Bertram, 1995). Lutein is another carotenoid that plays an important role in the human body's antioxidant defence system and is necessary for human health. Intake of lutein-rich fruits and vegetables has been linked to reduced risk of cancers and cardiovascular diseases, most likely due to the antioxidant activity of lutein (Alves- Rodrigues and Shao, 2004; Granado et al., 2003; Laur and Tian, 2011). Lutein is found in dark green leafy vegetables such as spinach and seems to accumulate and act in the macular region of the human retina and is known as a macular pigment (Riso et al., 2004; Johnson, 2000). There is evidence that lutein and zeaxanthin have protective effect on the photo oxidation of eyes (Landrum et al., 1997). In well-nourished populations, β-carotene supplementation is of little or no value in preventing these human health disorders. In smokers, β-carotene supplementation increases rather than decreases lung cancer and cardiovascular disease (Omenn et al., 1996).

Some carotenoids have provitamin A activities. The term provitamin A is used to describe the biological activity that carotenoids exhibit in a human body. β-Carotene, α-carotene and β-cryptoxanthin are the only carotenoid molecules that have provitamin A activities due to the presence of unmodified β-ring(s) (Desobry et al., 1999). The β-ring exist in retinol (vitamin A) structure is essential for its activity and β-carotene has two such rings (Figure 2.3) (Zakaria et al., 1979). In fact, the structure of vitamin A is one-half of the molecule of β-carotene whereas a molecule of water is added at the end of the lateral polyene chain. Theoretically, the vitamin A activity of β-carotene is 100% and provides 80% of vitamin A value of fruit and vegetables while the vitamin A activity of α-carotene is only 52±2% (Desobry et al., 1999). Humans cannot synthesize vitamin A and rely on intake of provitamin A compounds from foods.

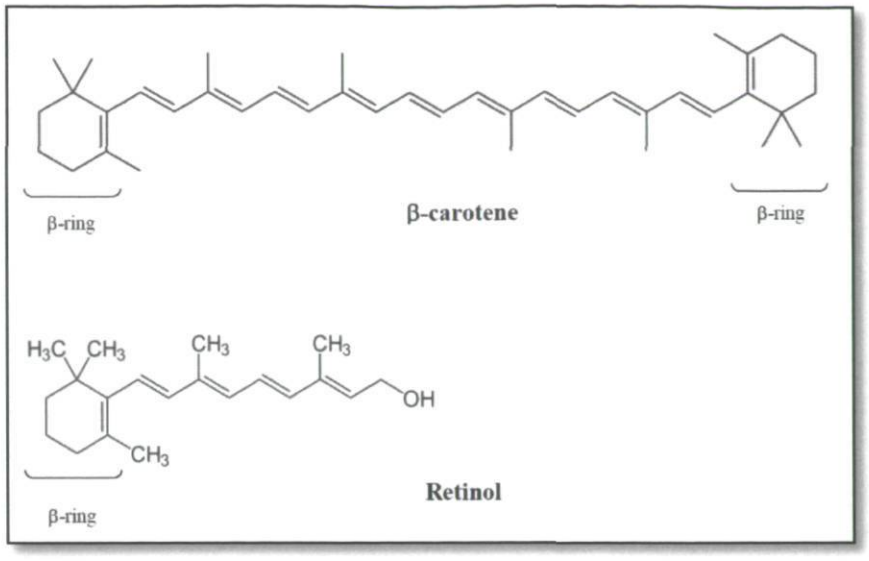


Figure 2.3. Structure of β-carotene and Retinol (vitamin A) (adapted from Zakaria et al., 1979).

2.2.3. Structure of carotenoids

Food carotenoids, with the formula of $C_{40}H_{56}$, are usually C_{40} tetraterpenoids built from eight C_5 isoprenoid units. They are joined in such a way that the sequence is reversed at the center, as shown in Figure 2.4. Modifications, such as cyclization, hydrogenation, dehydrogenation, double-bond migration, chain shortening or extension, rearrangement, isomerization, introduction of oxygen functions, or combinations of these processes result in several structures. Highly conjugated double bond system, consisting alternate double and single C-C bonds, is a unique structural feature of carotenoids. It is usually called as the polyene chain. This portion of the molecule contains delocalized π-electrons and is known as the chromophore. This is responsible for the ability of carotenoids to absorb visible light and is also responsible for the yellow, orange, or red colors of these compounds in many foods (Rodriguez-Amaya, 1997, 2004). In most carotenoids, long conjugated double bond system results in the absorption of visible light, mostly between 400 and 500 nm (Wrolstad et al., 2005). At least seven conjugated double bonds are needed for a carotenoid to impart.

Hydrocarbon carotenoids, made up of only carbon and hydrogen, are called carotenes. Carotenoids containing oxygen, in addition to carbon and hydrogen, are named xanthophylls. Xanthophylls are oxygenated hydrocarbons that contain at least one oxygen

function such as hydroxy (OH) and epoxy (5,6- or 5,8- epoxides), aldehyde (CHO), keto (C=O), carboxy (CO_2H), methoxy (OMe) and carbomethoxy (CO_2Me) groups. In nature, they exist primarily in the more stable all-trans isomeric form.

Carotenoids (carotenes or xanthophylls) can be acyclic, monocyclic, or bicyclic. Cyclization of carotenoids can take place at one or both ends of the molecule, which forms one or two six membered α-rings, so called α-ionone. Therefore, monocyclic α-carotene has one α-ring while bicyclic α-carotene, α-cryptoxanthin and zeaxanthin have two α-rings (Rodriguez-Amaya, 1997).

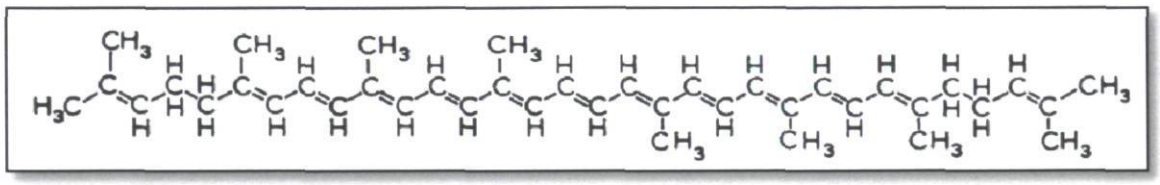


Figure 2.4. Schematic structure of acyclic carotenoids (Nomenclature of Carotenoids, 1972).

Nomenclature of carotenoids: The name of a specific carotenoid hydrocarbon is made by adding two Greek letters as prefixes to the name of stem carotene. These prefixes are characteristics of the two C_9 end groups (Nomenclature of Carotenoids 1972). The prefixes are presented in table 2.1. The prefixes are corresponding to the end-group modifications (Figure 2.5).

Table 2.1. The prefixes used for nomenclature of carotenoids (adapted from Nomenclature of Carotenoids 1972).

Type	*Prefix*	*Formula*	*Structure*
Acyclic	ψ	C_9H_{15}	III
Cyclohexene	β,ε	C_9H_{15}	IV, V
Methylenecylohexane	γ	C_9H_{15}	VI
Cyclopentane	κ	C_9H_{17}	VII
Aryl	ϕ,χ	C_9H_{11}	VIII, IX

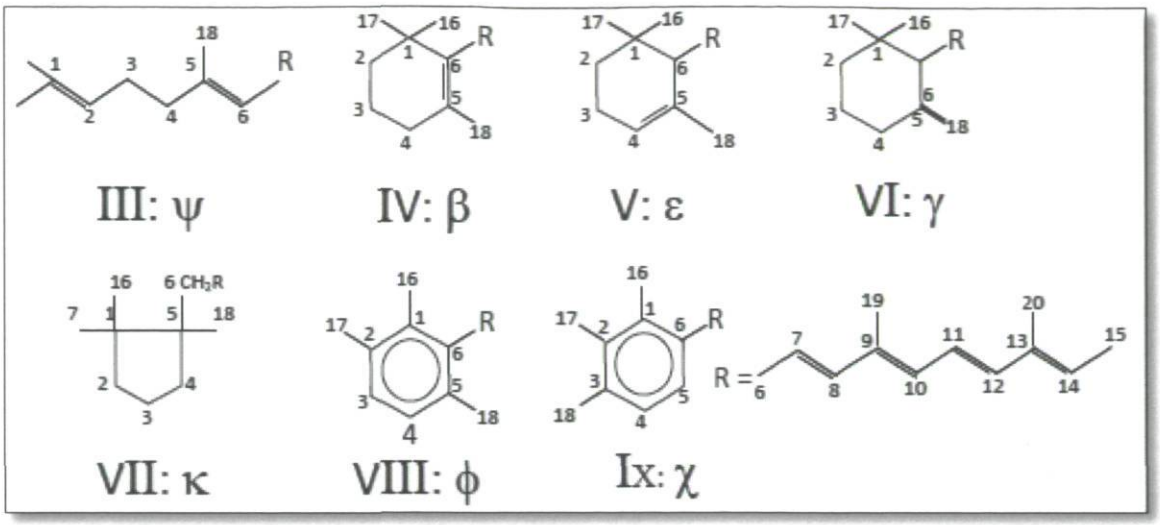


Figure 2.5. The prefixes correspond to the end-group modifications in carotenoid structure (adapted from Nomenclature of Carotenoids, 1972).

Figures 2.6 and 2.7 show the principal carotenoids found in foods and common geometric isomers of β-carotene, respectively. Carotenoids such as β−Carotene, α−carotene, β−cryptoxanthin, lutein, and lycopene are usually found in human plasma (Rodriguez-Amaya and Mieko Kimura, 2004).

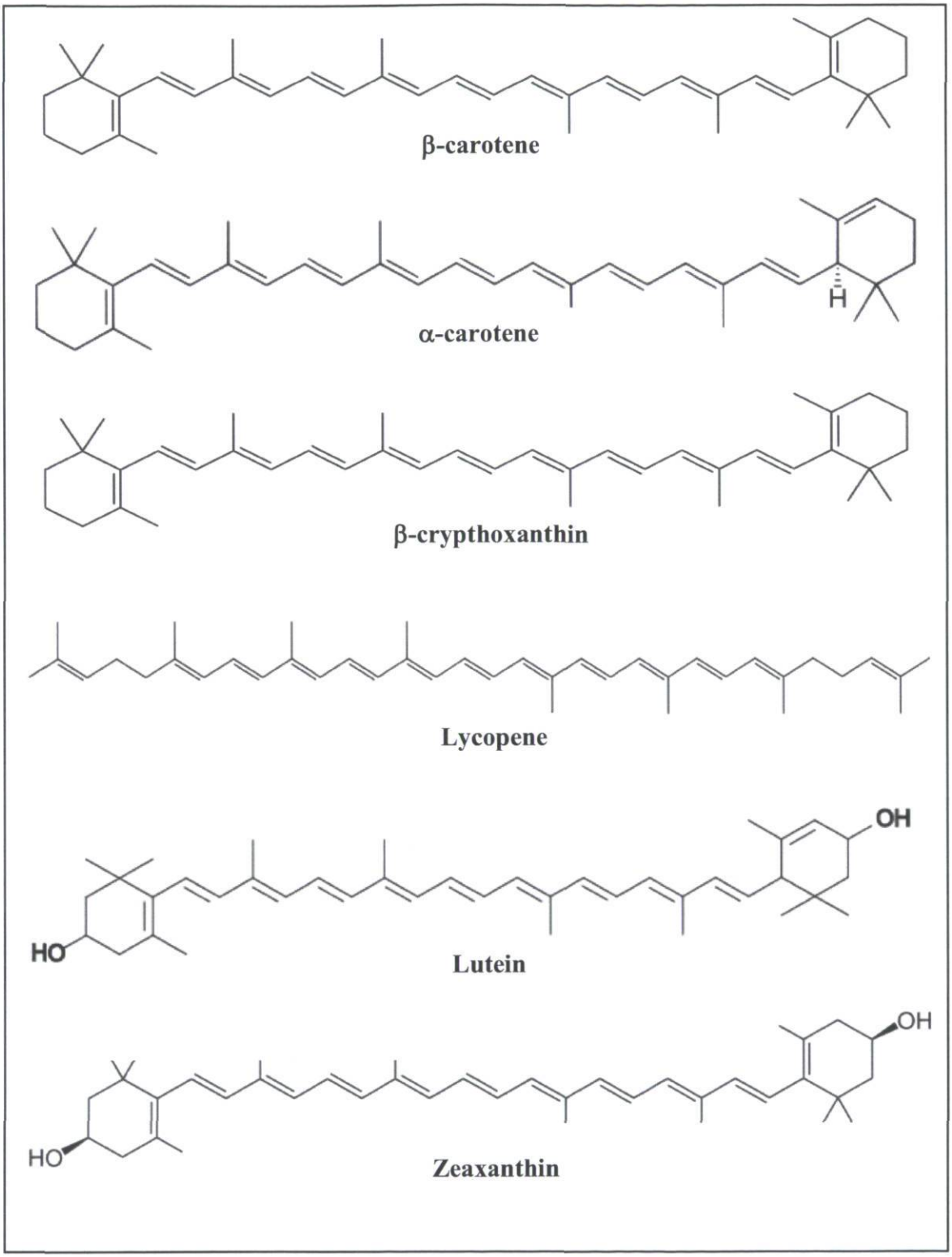


Figure 2.6. Structures of the principal carotenoids in foods and zeaxanthin (adapted from Rodriguez-Amaya and Mieko Kimura, 2004).

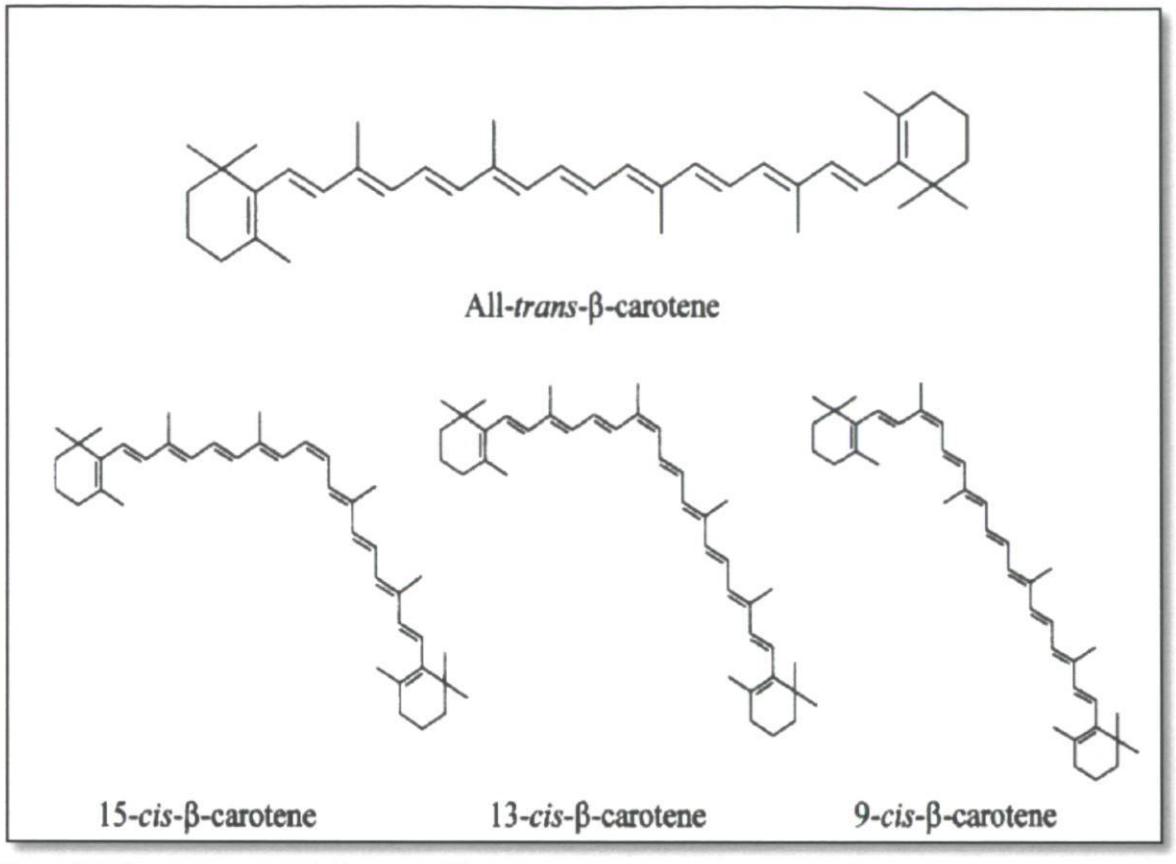


Figure 2.7. Common geometric isomers of β-carotene (Rodriguez-Amaya and Mieko Kimura, 2004).

Figure 2.8 shows the biosynthesis and structures of lutein and zeaxanthin in higher plants.

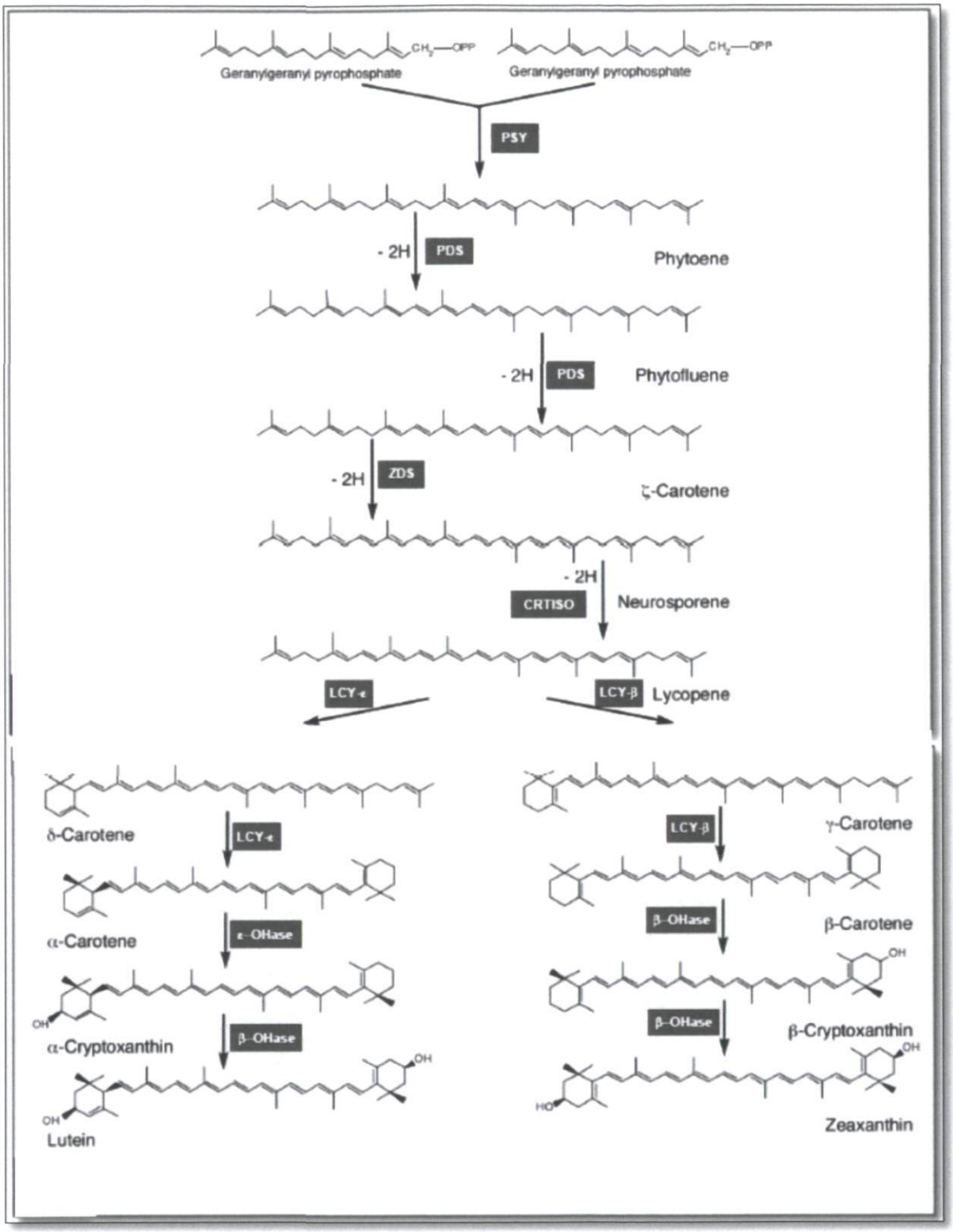


Figure 2.8. Biosynthesis of lutein and zeaxanthin in higher plants. Structures of selected carotenoid molecules are shown (Desjardins, 2008).

2.2.4. Problems with carotenoids extraction

A great variety of food products of plant and animal origin, contain a wide range of carotenoids. In recent years, there has been particular interest on the analysis and extraction of carotenoids in foods for various health and nutrition activities. However, the analysis of carotenoids is complicated due to several reasons. Diversity and the presence of *cis–trans* isomeric forms may affect their biochemistry. Highly unsaturated carotenoids are subjected to isomerisation and oxidation which produces problems associated with work and manipulation on carotenoids. Isomerisation of usual configuration of carotenoids (*trans* forms) to the *cis* forms can occur due to heat, light, acids, and adsorption on an active surface. This isomerisation leads to some loss of color and provitamin A activity. Oxidative degradation is the main reason for loss of carotenoids. This degradation depends on the presence of oxygen and is enhanced by light, metals and enzymes. Carotenoids have different susceptibilities to oxidation. The first step of oxidation is formation of epoxides and apocarotenoids. Subsequent fragmentations produce a series of low-molecular-weight compounds, as shown in Figure 2.9. The last consequences are total loss of color and of vitamin A and other biological activities. Conditions necessary for isomerization and oxidation of carotenoids exist during preparation, processing, storage and analysis of food (Rodriguez-Amaya, 2001). For this reason, several precautions are necessary when handling carotenoids.

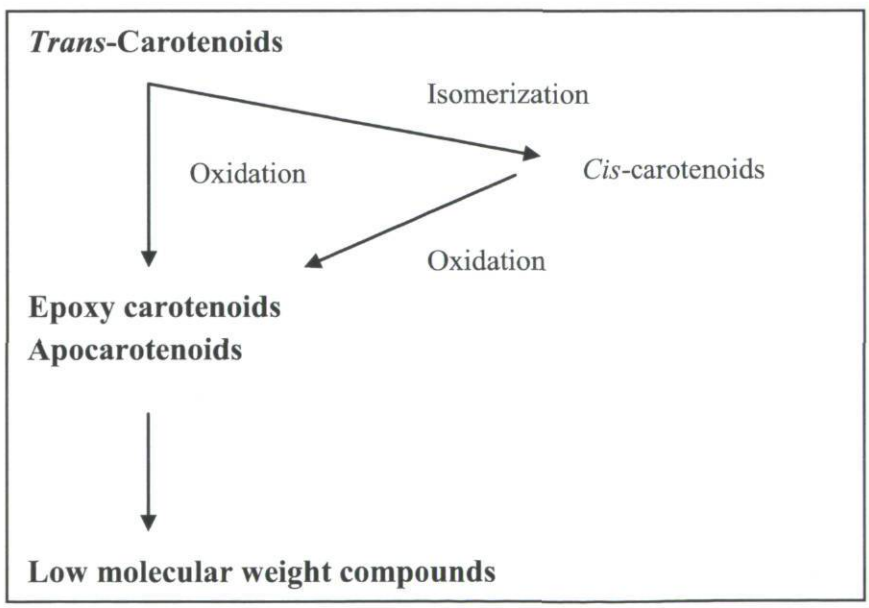


Figure 2.9. Isomerization and oxidation of carotenoids (adapted from Rodriguez-Amaya, 2001).

Structural diversity of carotenoids and difficulty in obtaining standard compound are other problems related to analysis of carotenoids. Composition of carotenoids changes qualitatively and quantitatively in foods. Thus, the analytical procedure, specifically the chromatographic step, should be adapted to the carotenoid composition. In a given food, carotenoid concentrations changes in a wide range. One to four principal carotenoids are typically identified while a series of carotenoids with low or trace concentrations are present. The separation, identification, and quantification of trace carotenoids are a challenge in food analysis (Rodriguez-Amaya and Mieko Kimura, 2004).

Other common sources of error in carotenoid analysis are:

- Non representative samples for food lots;
- Incomplete extraction and chromatographic separation;
- Physical losses of carotenoids during different steps, such as transfer of carotenoids from one solvent to the other during partitioning, loss of carotenoids in the washing water, sticking on the container walls when solutions are evaporated;
- Chemical losses due to oxidation;
- Incorrect identification, quantification and calculation.

2.2.5. Precautions

Precautionary measures should be considered for all analytic methods to prevent artifact formation and quantitative losses. These measures include the shortest analysis time, elimination of oxygen, protection from light and high temperature, avoiding contact with acids, and using high purity solvents. Oxygen, particularly in combination with light and heat, is extremely destructive. The presence of traces of oxygen in stored samples (even at deepfreeze temperatures) and of peroxides in solvents as THF can quickly lead to bleaching and the formation of artifacts, such as epoxy carotenoids (Britton, 1991). Helium bubbling as well as nitrogen or argon atmosphere can be used during analysis and storage to eliminate oxygen. Antioxidants such as butylated hydroxytoluene, pyrogallol, and ascorbyl palmitate can also be used, particularly when the analysis time is long. They can be used through saponification or can be added to solvents, standard solutions, and isolates.

Exposure to light, particularly UV or direct sunlight leads to *trans-cis* photoisomerization and photodestruction of carotenoids. Therefore, open columns and vessels containing carotenoids should be kept in dark or wrapped with aluminum foil. Polycarbonate shields can be used for fluorescent lights. For extracts containing chlorophylls or other potential sensitizers, rapid manipulation and covering from light are very important. The presence of these sensitizers leads to rapid photodegradation and isomerization, even with brief exposure to light. Extraction should be performed immediately after sample preparation, since tissue disruption releases enzymes (e.g., lipoxygenase), which catalyze oxidation of carotenoid, and liberates acids that accelerate *trans–cis* isomerisation (Rodriguez-Amaya and Mieko Kimura, 2004).

Heating should be performed only when is really required. Concentrating of carotenoid extracts or solution should be done in a rotary evaporator at low pressure and a temperature below 40°C. Complete dryness of extract in the rotary evaporator should be prevented since it may lead to carotenoids degradation (especially lycopene) (Tonucci et al. 1995). In addition, the more polar part of the carotenoids may stick strongly to the glass walls.

The majority of carotenoids are stable against alkali. Calcium carbonate, magnesium carbonate and sodium bicarbonate can be used as neutralizing agents during extraction to neutralize acids released from the food sample itself. Acidic reagents and strong acids should not be used in the same place where carotenoids are handled.

2.2.6. Extraction solid-liquid of carotenoids

In the extraction process of active molecules such as carotenoids from a solid material, diffusion takes place from solid to liquid or solvent. Extraction is a solid-liquid interaction in which one or more solid, crystals and liquids components (solute) are dissolved in a solvent to produce a solution or extract (solvent+solute). A concentration gradient exists between solvent and solute due to which solute transfers from solid (concentrated) to liquid phase. Towards the end of the process, conditions tend to equilibrium and diffusion is almost terminated. If fresh liquid phase is supplied, diffusion will continue until the entire solid is consumed (Brianceau, 2010). Diffusion controlled solid-liquid interactions have five consequent steps, as shown in Figure 2.10:

1- Transfer of the solvent to the solid surface
2- Diffusion of the solvent into the solid phase
3- Dissolution of the solute in the solvent
4- Diffusion of the dissolved solute to the solid surface
5- Transfer of the solute in the solution by diffusion or convection

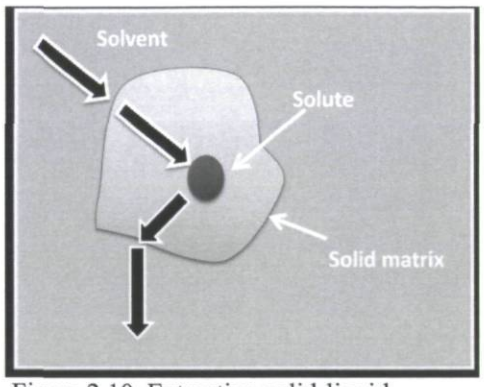


Figure 2.10. Extraction solid-liquid
(adapted from Brianceau, 2010).

The performance of extraction process is influenced by several factors such as extraction time and temperature, particle size, stirring, nature of solvent and pH. The most important factors affecting solvent extraction for the determination of total carotenoids (TC) include extraction duration, repeated extraction cycles, solvent-solid ratio and extraction temperature (Wang and Liu, 2009).

Following extraction, an efficient way of initiating the isolation of carotenoids is to saponify the extract. Carotenoids in fruits and vegetables are predominantly esterified by fatty acids. Saponification removes many of the unwanted lipids and chlorophyll present in the sample. This method is done in a KOH solution: aqueous, methanolic or ethanolic solution (Wrolstad et al., 2005; Rodriguez-Amaya et al., 2004). However, saponification increases the analysis time and enhances the formation of artifacts and degradation of carotenoids. Despite the resistance of provitamin A carotenoids (β−carotene, α−carotene, β−cryptoxanthin and γ-carotene) to saponification (Rodriguez-Amaya et al., 1988), significant amount of lutein, violaxanthin, dihydroxy, trihydroxy and epoxycarotenoids can be lost through saponification and washing step (Rodriguez-Amaya and Mieko Kimura, 1988; Khachik et al., 1986).

2.2.6.1. Samples preparation

The sample that is brought to the laboratory is usually too large, in bulk or particle size, for direct analysis. It must be transformed into a homogeneous, small sample for analysis while maintaining its representativeness. Inedible portions (i.e., peel, seed, shell, etc.) may be removed and prepared for separate analysis. Physical operations, such as chopping, cutting into pieces, mixing, milling, blending, and sieving, are carried out, along with bulk reduction, for example, by quartering and riffling. The process can be done manually or through commercially available mills, blenders, grinders, riffle cutters, etc.

Several problems may occur in sample preparation including difficulty in taking small representative samples, loss of material, difficulty in elimination of inappropriate material without removal of plant constituents (analyte), and changes in enzymes and unstable components during preparation and analysis. Nature of the food and analyte, analyte distribution in the food, and analytic method are determining factors to select the sample preparation procedure (Rodriguez-Amaya, 2001).

Water content is considered an important factor for extraction of carotenoids. It has been found that working with low-moisture samples simplifies the extraction process. Industrial extraction normally is done with dry material, which reduces complications arising from the solvents used for processing and their recovery. The plant material may contain some water (<10%), which will not affect extraction. Therefore, complete dehydration is not recommended before extraction. A small quantity of water can be advantageous when a solvent with low polarity is used. On the other hand, excess water may reduce extraction effectivness (Wrolstad et al., 2005).

Freeze-drying is a widely used method for dehydrating a vast range of materials, including foodstuffs. Compared to conventional drying and many other processing methods, freeze-drying offers a number of advantages such as maintaining food quality and weight reduction. It is an appropriate way to preserve samples that have to be stored before carotenoid analysis. By freeze-drying, the moisture content is reduced to such a low level appropriate for an efficient extraction. However, degradation of carotenoids takes place during lyophilisation. In addition, this processing technique increases sample porosity and exposure of carotenoids to oxygen during storage (Park, 1987; Craft et al. 1993).

In addition to the moisture content of samples, particle size may influence the performance and efficiency of extraction procedure. The grinding of solid material can intensify the transfer of solvent through increasing the exchange interface between the solvent and the solid and also reducing the distance of penetration of the solvent in the solid. For example, the extraction yield of phenolic compounds is increased by decreasing particle size.

2.2.6.2. Solvents

The selection of the solvent to promote the extraction is a very important issue since it determines the degree of affinity to the chemical composition of the substances to be extracted (Henriques et al., 2007). A solvent, by definition, is a substance that has the power to form a homogeneous solution with other substances. The solvent should be selective, with a high dissolving capacity, a low boiling point and low viscosity. The pigment polarity is a determining factor to choose the extraction solvent. If this factor is unknown, an acetone/hexane (1:1, v/v) mixture is usually used. When the carotenoids are nonpolar or in the ester form, hexane is an efficient solvent. Ethanol is able to extract polar carotenoids, while a nonpolar solvent such as hexane lead to crystallization (Wrolstad et al., 2005).

In addition to the dissolution ability, the solvent has also an important effect on cell lysis. In cells with strong walls, the extraction yield is increased using more aggressive solvents. Methanol was the first solvent used for extraction of chlorophylls, however, because of its toxicity it has been replaced by other solvents. Till 1995, acetone was used for evaluation of the chlorophyll content. Since then, ethanol has been recommended as the extraction solvent (Henriques et al., 2007).

Methanol (MeOH) or a mixture of MeOH and other more apolar solvents are usually used for extraction procedures. A MeOH–tetrahydrofuran (1:1, v/v) solution was used by Hart and Scott (1995) for carotenoid analysis of raw and cooked vegetables and fruits. MeOH and acetone–hexane (Gregory et al., 1987), MeOH and hexane (Schmitz et al., 1989), MeOH and diethyl ether (Weissenberg et al., 1997), and MeOH and chloroform (Oliver et al., 1998), have also been reported. Acetone alone or in combination with light petroleum (Mouly, Gaydou, and Corsetti, 1999) has also been applied to food products. Chen and co-workers (1991) reported a more complex extracting solution consisting of hexane–acetone–

MeOH–toluene (10:7:6:7, v/v), for analysis of carotenoids in carrot and water convolvulvus (*Ipomoea aquatica*). Craft and Wise used methanol and nine mixtures of methanol and other solvents to extract seven different carotenoids. They measured the absorbance of the extract by chromatography, as shown in Figure 2.11, and reported that 95% methanol/5% THF was the best solvent for carotenoids (Craft and Wise, 1992). Table 2.2 shows valuable information on the selection of solvents for carotenoid extraction as well as the absorption coefficient $A^{1\%}_{1cm}$ of the carotenoids (absorbance at a given wavelength of a 1% solution in spectrophotometer cuvette with a 1-cm light path) which is used for the calculation of the carotenoid concentration.

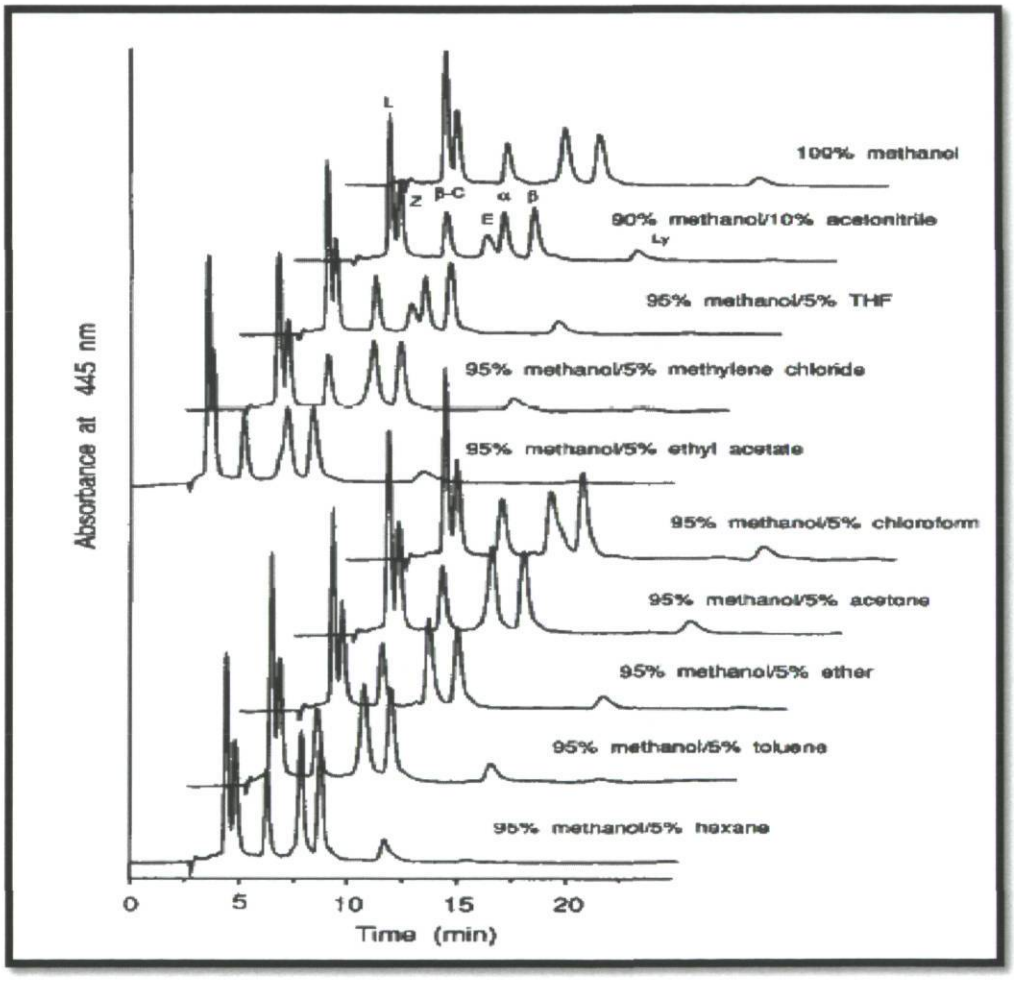


Figure 2.11. The effect of nine solvent modifiers on the separation of seven carotenoids (L=Lutein; Z= Zeaxanthin; β-C= β-cryptoxanthin; E= echinenone; α= α-carotene; β= β-carotene; Ly= lycopene) (Craft and Wise, 1992).

Table 2.2. Relative solubility, stability, and absorptivity of Lutein and β-Carotene in organic solvents (adapted from Craft and Soares, 1992).

Solvent	*Lutein*			*β-carotene*		
	Solubility (mg/L)	***λ_{max} nm***	***Absorptivity $E^{1\%}$, cm^{-1}***	***Solubility (mg/L)***	***λ_{max} nm***	***Absorptivity $E^{1\%}$, cm^{-1}***
Acetone	800	446	2540	200	452 (452)	2559
Acetonitrile	100	446	2559	10	452	2540
Benzene	600	456 (458)	2350	4000	462 (462)	2304
Chloroform	6000	454 (458)	2369	2000	462 (461)	2330
Cyclohexane	50	448	2520	2000	454 (457)	2508
Cyclohexanone	4000	454	2359	2000	462	2359
dichloromethane	800	452	2320	6000	460	2369
DMF	1000	454	2390	200	460	2389
DMSO	1000	460	2369	30	466	2259
Ethanol	300	444 (445)	2550	30	450 (449)	2529
ethyl acetate	800	446	2529	500	452	2520
ethyl ether	2000	444	2629	1000	448	2659
Hexane	20	444 (445)	2589	600	448 (453, 450)	2592
2-propanol	400	444	2599	40	450	2508
Methanol	200	442 (444)	2629	10	450	2540
MTBE	2000	444	2589	1000	450	2588
THF	8000	450	2469	10000	456	2399
Toluene	500	456	2290	4000	462 (463)	2270

2.2.6.2.1. Ethanol for carotenoid extraction

Carotenoids are usually extracted with organic solvents such as chloroform, hexane, acetone, petroleum ether and etc., since carotenoids are liposoluble. In the case of samples with large amounts of water, water-miscible organic solvents such as ethanol, acetone, etc. are also used. A mixture of various solvents is usually used in carotenoid extraction. Carotenoid extraction methods are generally time consuming procedures with errors due to oxidation and losses during extraction. The obtained carotenoid-rich extract is usually used in food additives, health foods, medicines and cosmetics. For these applications, the residual solvents should be eliminated to obtain a safe extract. This problem can be avoided using food grade solvents such as ethanol and ethyl acetate. Carotenoids are enclosed within cells and the cell walls have a complex composition. It is also known that ethanol can break cell walls. Therefore, good accessibility of ethanol to the carotenoids may lead to a good extraction yield. On the other hand, higher polarity of ethanol than ethyl acetate can influence the higher concentration obtained with ethanol. Calo et al. (1995) mentioned that using ethanol as solvent for the extraction of pigments has several advantages including; lower toxicity as compared to other solvents used for carotenoid extraction; and the

prevention of toxic residues that would be derived from the use of acetone. Calvo et al. (2007) evaluated the extraction yield of food grade solvents (ethanol and ethyl acetate) by extracting lycopene, β-carotene, phytoene and phytofluene from tomato peel powder. In the extractions performed with ethanol, the concentration of lypidic extract and the yield of each carotene were higher than those obtained with ethyl acetate. Taungbodhitham et al. (1998) evaluated a carotenoid extraction method for a wide range of fruit and vegetable samples. Their results showed that the extractions with ethanol and hexane (solvents with low biological hazard) have good recovery and precision and can be successfully used for the analysis of carotenoids in fruits and vegetables.

Takahashi et al. (2006) extracted the carotenoids from Japanese persimmon peels by supercritical fluid extraction (SFE), using CO_2 as solvent. 5 to 20 mol% of ethanol was used to increase the selectivity and extraction yield. According to their results, increasing ethanol concentration resulted in a nonlinear increase of β-carotene extraction yield. The β-carotene yield was considerably enhanced in the range of 10 to 15 v/v% ethanol. This may be due to the fact that solvent polarity changes with addition of ethanol. They also indicated that water is not efficient for β-carotene extraction. Sun and Temelli (2006) studied the effect of water on the extraction of carotenoids from carrot. They observed that extraction yield of lutein was increased, using water as solvent, while the extraction yields of α- and β-carotene were reduced. They reported that interaction between the OH groups of lutein and water molecules, through hydrogen bonding, was the reason for improving the lutein extraction. Lutein has two OH groups, while α- and β-carotene does not have any oxygen in their structures.

Canals et al. (2005) studied the influence of ethanol concentration and ripeness on the extraction of color and phenolic compounds from grape skins and seeds during maceration/fermentation process. According to their results, ripeness and ethanol concentration can significantly influence the extraction of color and phenolic compounds. However, the effect of ripeness on the extractability was more than that of ethanol content. The presence of ethanol in the solvent improves the extraction of anthocyanin and especially proanthocyanidin, but it decreases co-pigmentation phenomena, which can decrease the color intensity. The extraction of anthocyanin and the astringency of

proanthocyanidins increased in all stages of ripening with increasing the ethanol concentration in the maceration media. Long maceration will lead to wines with a high concentration of proanthocyanidin and high astringency. Because skins and especially seeds have been in contact with a medium rich in ethanol for a long time.

2.2.6.3. Factors influencing carotenoid extraction

In nature, carotenoids are protected by cellular structure. The susceptibility of carotenoids to degradation increases with destruction of the cell structure. On the other hand, this natural protection limits the bioavailability of carotenoids. Food processing causes denaturating of proteins and breaking of cell walls, therefore, facilitating the release of carotenoids from the food matrix during digestion. It has been shown that by processing of spinach and carrots, the bioavailability of β−carotene in humans increased (Castenmiller et al. 1999). It is also reported that the bioavailability of lycopene was increased in heat-processed tomatoes compared with unprocessed tomatoes (Gärtner et al. 1997; Stahl and Sies 1992; Hof et al. 2000). Processing conditions should be optimized to minimize losses of carotenoids while enhancing their bioavailability (Rodriguez-Amaya and Mieko Kimura, 2004).

Extraction conditions such as temperature and stirring can affect the extraction results. Mechanical stirring results in continued suspension of particles in the solvent and homogenization of the medium, and has a positive effect on extraction yield. In the case of aqueous extraction, stirring can reduce resistance to the transfer of solutes at the solid-liquid interface (boundary layer) and can increase the transfer coefficient.

Although carotenoids are susceptible to light oxidation, they are more stable than chlorophylls during thermal processing (Bergquist et al., 2006). It is also reported that carotenoids of cooked or processed foods have a better bioavailability than those of raw products (Gartner, Stahl, and Sies, 1997; Hedren, Diaz and Svanberg, 2002; Stahl and Sies, 1992). The stability of these nutrients is dependent on their location and distribution in plant tissues.

The heat, below the degradation temperature, facilitates the extraction of solute by permeabilization of cell walls, increasing the solubility of solutes, increasing of diffusion

coefficients and decreasing the viscosity of the solvent extraction. Nguyen et al. (2001) reported that heat treatment changes the physical structure of the tomato tissue and the bioavailability of carotenoids. Hart and Scott (1995) found an increase in the concentration of carotenoid in heated vegetables which could be due to changes in the cell structure and therefore the availability of the carotenoid. Calvo et al. (2007) used different heating intensities and solvents to study the influence of these factors on carotenoids isomerization and degradation during extraction. They extracted lycopene, β-carotene, phytoene and phytofluene from tomato peel powder to investigate the extraction yield of ethanol and ethyl acetate. The heat treatment temperatures of 25, 35, 50 and 60 °C were applied for 5, 10, 20, 30 and 40 minutes. Their results showed that carotenoid concentrations increased with increasing the temperature; however using ethanol at 60 °C resulted in lower yield of (all-*E*)-lycopene and their (*Z*)-isomers, comparing to that obtained at 50 °C. This can be an indication for great isomerisation occurs when extraction is performed at high temperature with ethanol; however, the oxidative degradation is the principal reaction. In contrast, they found that the isomerization is the main reaction when ethyl acetate is used. Barth et al. (1995) reported that there is an optimum temperature of 50°C for extraction of α-carotene and β-carotene from freeze-dried carrots.

2.2.6.4. Characterization

2.2.6.4.1. Measurement of total carotenoid concentration by spectrophotometer

Spectrophotometer is a quantitative measurement method which uses the reflection or transmission properties of a material as a function of wavelength. Spectrophotometer is much less expensive and much faster than HPLC, however it is not able to estimate the individual content of carotenoids (Henriques et al., 2007).

Carotenoids in solution follow the Beer-Lambert law in which absorbance *(A) is* equal to concentration multiplied by extinction coefficient $(A^{1\%})$. The extinction coefficient $(A^{1\%})$ is the absorbance of a 1% (10 g/liter) solution of carotenoid, in a defined solvent, in a 1-cm path-length cuvette, at a specific wavelength (λ). This equation can be used to determine the concentration of carotenoid in a standard sample or in a mixture or extract of carotenoids. The extinction coefficient can also be expressed in molarity (Wrolstad et al., 2005). The following equation is used for determination of the carotenoids concentration:

$$C = \left(\frac{A}{E^{1\%} \times 1\ cm}\right) \times \left(\frac{V}{M}\right) \times \left(\frac{10^6\ \mu g}{1\ g}\right) \times D \qquad (2.1)$$

C: concentration (μg/g)

A: absorption at 450 nm

V: volume of solvent (dl)

$E^{1\%}$: absorptivity of lutein and β-carotene in organic solvents (cm^{-1})

M: mass of sample (g)

D: dilution factor

2.2.6.4.2. High- Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a chromatographic technique to separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify and quantify the individual components of the mixture. HPLC is used for analysis of carotenoids levels due to its ability to distinguish between similar conformational structures of carotenoids. The rapidity, non-destructiveness and ease in automating as well as small amount of required sample make the new HPLC methods a suitable technique for analysis of carotenoids (Oliver and Palou, 2000).

2.2.6.4.2.1. Determination of carotenoids by HPLC

Some researchers have worked on the extraction of carotenoids of cantaloupe. Bureau and Bushway (1986) determined alpha- and beta-carotene and beta-cryptoxanthin in twenty-two fruits and vegetables such as cantaloupe, carrots and grapefruit. They used the method that Bushway and Wilson employed in 1982. They used a Partisil 5 ODS column 25 cm × 4.6 mm ID and a solvent mixture of acetonitrile-tetrahydrofuran-water (85:12.5:2.5) pumped at a flow rate of 2.0 mL/min. Absorbance was determined at 470 nm.

A carotenoid analysis of carrot, spinach, tomatoes, corn (canned) and tangerines using a stainless steel (250×4.6 mm I.D.) column packed with Vydac 201 TP, 5 mm particle size, and a mobile phase consisting of MeOH–THF (95:5, v/v) have been performed (Konings and Roomans, 1997). The flow-rate was at 1 ml /min, the chromatogram was recorded at 450 nm and ethyl-β-apo-8'-carotenoate was used as internal standard. Lutein, zeaxanthin, α-carotene, β-carotene and lycopene were separated and quantified with this method.

Craft and Wise (1992) optimized the isocratic HPLC separation of carotenoids using a polymeric C_{18} column and MeOH-based mobile phase with nine solvent modifiers at several column temperatures. They found that 3 to 5% THF in MeOH at a column temperature of 20°C results in a good separation of seven carotenoids including lutein, zeaxanthin, β-cryptoxanthin, echinenone, α-carotene, β-carotene and lycopene.

El-Qudah (2009) quantified carotenoids using a high-performance liquid chromatography equipped with C_{30} column (3 μm, 150×4.6 mm). Absorbance of carotenoids was determined at 455 nm using a Waters 2996 photodiode array detector. The HPLC mobile phases were methanol: Methyl-tert-Butyl Ether (MTBE): water (85:12:3 by volume, with 1.5% ammonium acetate in water) and methanol: MTBE: water (8:90:2 by volume, with 1.0% ammonium acetate in water) for solvent A and B, respectively. The flow rate of 0.4 $ml.min^{-1}$ and the injection volume of 20 μl were employed. HPLC was calibrated by standard solutions. Peak areas under the curve were determined to quantify the carotenoids. The peaks were confirmed by the retention time and characteristic spectra of the standards.

Cis-isomers have different biological potencies than their *trans* counterpart and it is, therefore, necessary to separate and quantify *cis*-isomers when they exist in considerable amounts. Detailed analysis, however, makes the analysis more complicated. The polymeric C_{30} column has been developed specifically for this purpose (Sander et al. 1994). YMC_{30} column is much more hydrophobic than classical C_{18} stationary phases. Using YMC_{30} column, many sample solutes are retained even when pure organic eluents are used. The stationary phase of YMC_{30} provides sufficient phase thickness to increase interaction with long chained molecules (Figure 2.12). Therefore, YMC_{30} phase is capable to recognize and resolve geometric and positional isomers of conjugated double bonding systems.

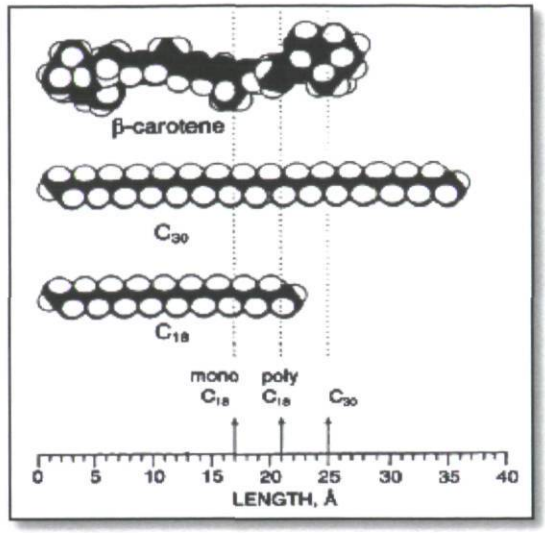


Figure 2.12. Comparison of the film thickness of C_{18} and C_{30} stationary phases with the molecular length of β-carotene (Sander et al., 1994).

This column, with an isocratic solvent system consisting of methanol:methyl-tert-butyl ether (89:11), has been used for the quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables (Lessin et al. 1997).

2.3. Polyphenols

Phenolic compounds or polyphenols are one of the most numerous and universal groups of plant metabolites and are an intrinsic part of both human and animal diets. Polyphenol structure consists of several hydroxyl groups on aromatic rings. Several thousand of molecules with a polyphenol structure have been identified in higher plants, while in edible plants several hundred have been found. These molecules are secondary metabolites of plants and are involved in defense against ultraviolet radiation or aggression by pathogens. Phenolic compounds may be classified based on the number of phenol rings and the structural elements that bind these rings together. Using this classification method, polyphenols are classified into the phenolic acids, flavonoids, stilbenes, and lignans. The flavonoids can be divided into 6 subclasses as a function of the type of heterocycle involved. The flavonoids subclasses are flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (Manach et al., 2004; Bravo, 1998; Kondratyuk and Pezzuto, 2004). The subclasses of polyphenols are presented in Figure 2.13.

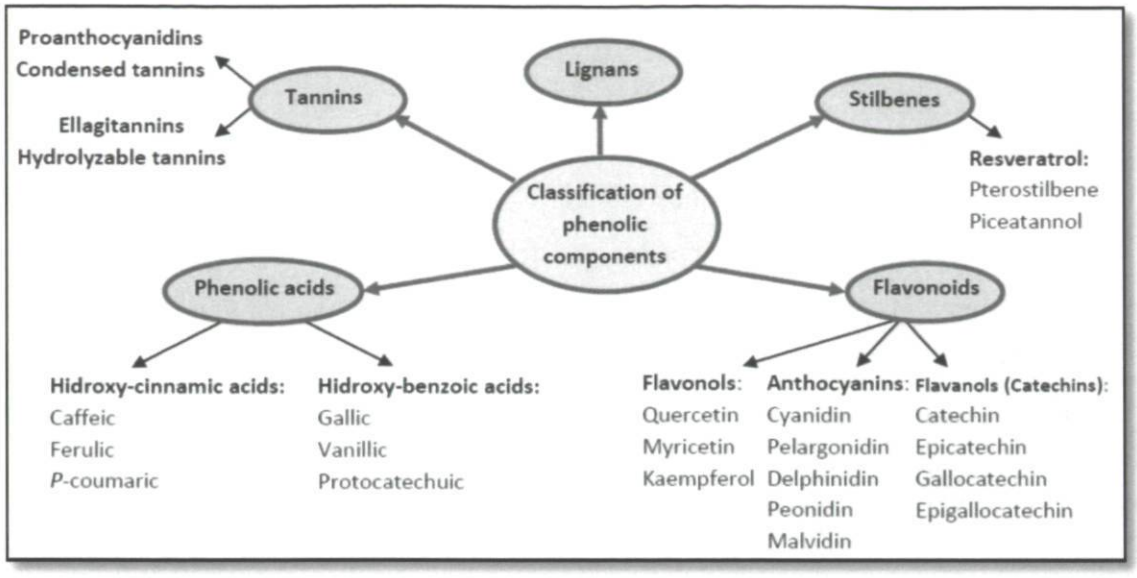
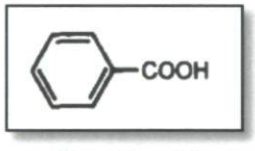


Figure 2.13. The subclasses of polyphenols (adapted from Paredes-López, 2010).

Phenolic acids can be classified into two groups: derivatives of benzoic acid and cinnamic acid. Hydroxybenzoic acids are components of complex structures such as hydrolyzable tannins (gallotannins and ellagitannins). Edible plants have generally very low content of hydroxybenzoic acids. The hydroxycinnamic acids are more common than the hydroxybenzoic acids and include *p*-coumaric, caffeic, ferulic, and sinapic acids. These acids are rarely found in the free form, except in processed food that has been freezed, sterilized, or fermented (Manach et al., 2004). Chemical structures of phenolic acids, benzoic acids and hydroxycinnamic acids are presented in Figure 2.14.



Phenolic acids

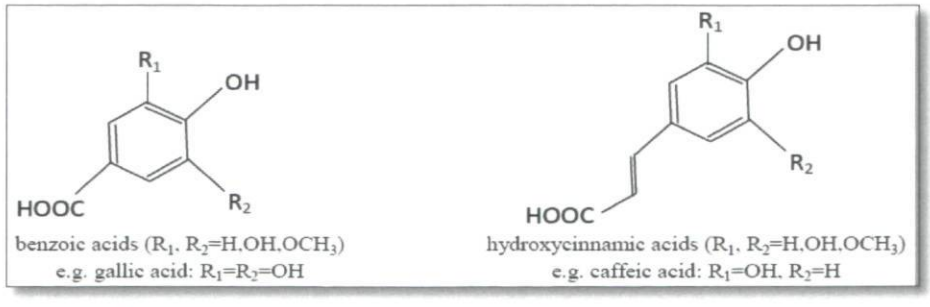


Figure 2.14. Chemical structures of phenolic acids, benzoic acids and hydroxycinnamic acids (adapted from Bravo, 1998; Cheynier, 2005).

Flavonols such as quercetin and kaempferol are the most universal flavonoids in foods, however their concentration is relatively low. Fruits often contain 5 to 10 different flavonol glycosides. These flavonols accumulate in the outer and aerial tissues (skin and leaves) where the biosynthesis is enhanced by light (Manach et al., 2004). Flavanols are a group of plant polyphenols responsible for many bitter and astringent flavors in food products which exist in two forms: monomer (catechins) and polymer (proanthocyanidins). The most abundant and widely distributed flavonoids consumed in the diet are proanthocyanidins. These compounds have health-promoting properties toward chronic diseases. However the isolation and quantification of proanthocyanidins are difficult, since they have large degrees of chemical variation and stereochemistry. Although there is no current industry standard, the 4-dimethylaminocinnamaldehyde (DMAC) spectrophotometric assay is a rapid and popular technique for quantification of the amount of proanthocyanidins exists in beverages and foods (Wallace and Giusti, 2010). Chemical structures of flavonols and proanthocyanidins are presented in Figure 2.15.

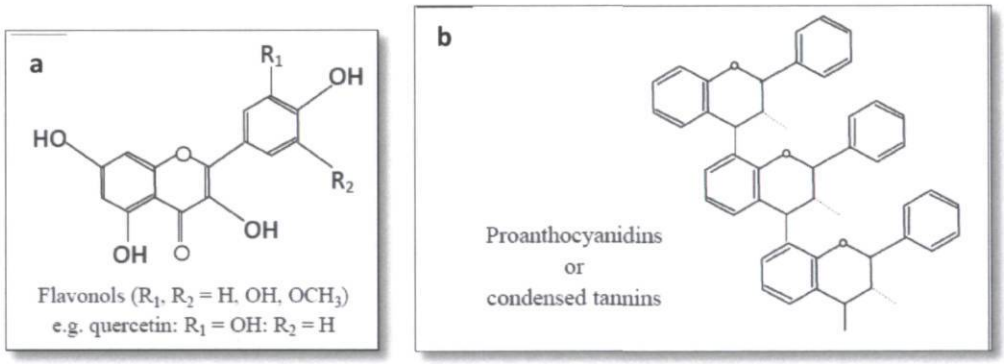


Figure 2.15. Chemical structures of a) flavonols and b) proanthocyanidins (adapted from Cheynier, 2005; Bravo, 1998).

Stilbenes are another class of polyphenols. Most stilbenes in plants are synthesized only in response to infection or injury and act as antifungal phytoalexins. Resveratrol (trans-3, 5, 40-trihydroxystilbene) is the most extensively studied stilbene and is a phytoalexin or a class of antibiotics of plant origin and are produced mainly in grapes and peanuts. Grape skin contains the highest concentration (50–100 μg/g) of resveratrol. It has been used in oriental medicine for the treatment of lipid, inflammatory and heart disorders (Kondratyuk and Pezzuto, 2004). It has been reported that resveratrol has a wide range of pharmacological properties, such as effects on cell signaling pathways, cell proliferation,

tumor growth, and apoptosis (Stewart et al., 1999), anti-inflammatory, estrogen receptor agonist (Gehm et al., 1997), anticancer (chemopreventive), antifungal and antimicrobial properties (Kondratyuk and Pezzuto, 2004). However, since resveratrol is found in small quantities in the diet, the protective effects of this molecule cannot be achieved by nutritional intakes (Manach et al., 2004). Figure 2.16 shows the chemical structures of stilbenes.

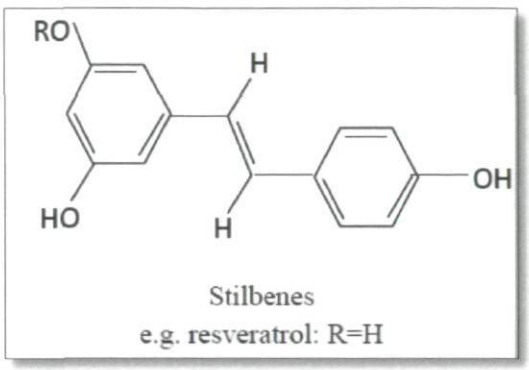


Figure 2.16. Chemical structures of stilbenes (adapted from Cheynier, 2005).

Phenolics are not uniformly distributed in tissue, cellular and subcellular levels of plants. Soluble phenolics are components of plant cell vacuoles while insoluble phenolics are found in cell walls. The outer layers of plants contain higher concentrations of phenolics than the tissues in their inner parts (Bengoechea et al., 1997).

2.3.1. Beneficial health effects of polyphenols

Scientists have been interested in plant polyphenols because they are essential to plant physiology. Polyphenols contribute to plant pigmentation, growth and reproduction and protection of crops from plague and preharvest seed germination. They also act as phytoalexins, or increase food astringency to provide resistance against pathogens and predators. Recent interest in food phenolics has increased greatly, due to their antioxidant capacity, antimutagens, antiproliferative, and antimicrobial properties as well as their beneficial applications in treatment and prevention of cancer, cardiovascular disease and other pathologies. Health beneficial effects of polyphenols are presented in Figure 2.17. Phenolic compounds also protect other natural antioxidants such as β-carotene and α-tocopherol and result in the reduction of risk of disease. The health effects of polyphenols

depend on two factors: the amount consumed and their bioavailability. Polyphenols are used in the food industry as additives (natural colorants and preservatives). In addition, some phenolic compounds such as flavonoids, are used as antibiotics, antidiarrheal, antiulcer, and anti-inflammatory agents, as well as in the treatment of hypertension, vascular fragility, allergies and hypercholesterolemia (Manach et al., 2004; Machlin, 1995; Cacace and Mazza, 2003; Bravo, 1998; Gruz, Novák, and Strnad, 2008).

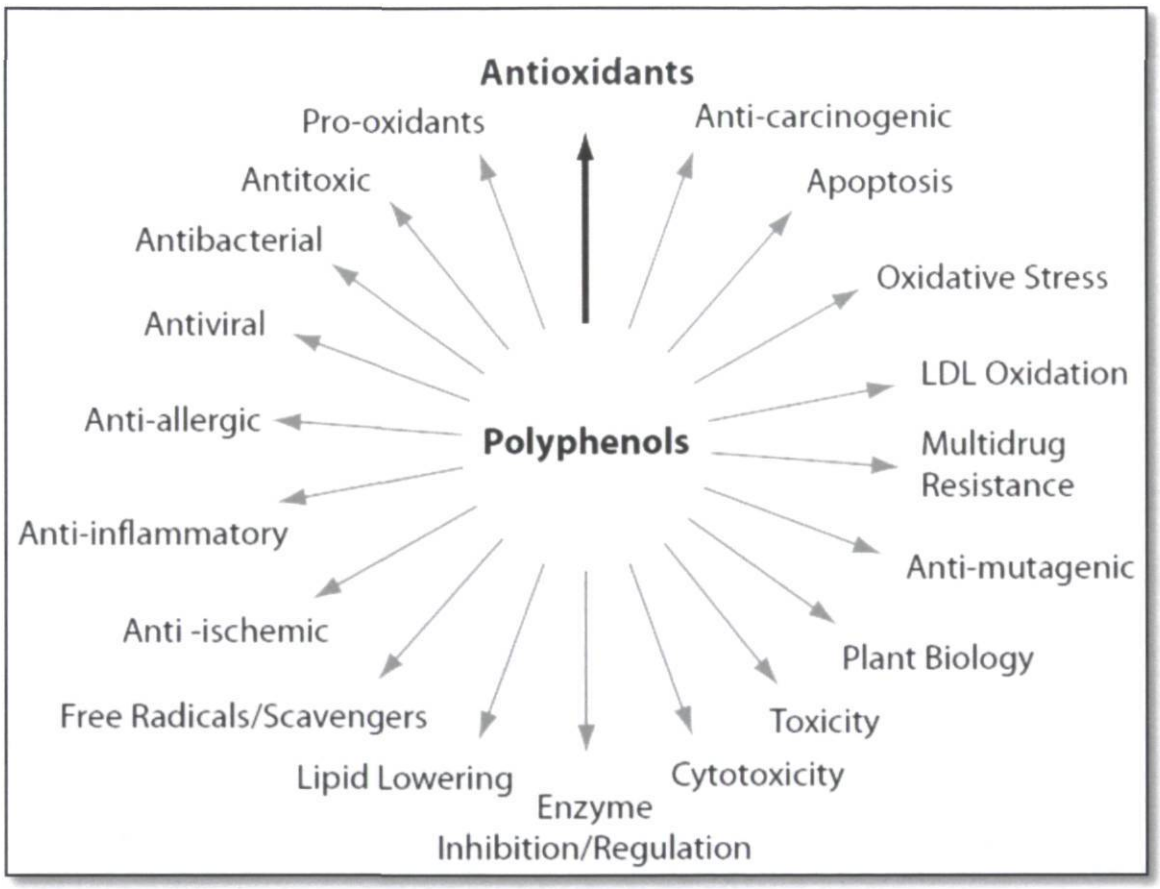


Figure 2.17. Health beneficial effects of polyphenols (http://www.scribd.com/doc/61911692/polyphenols).

2.3.2. Factors influencing polyphenol content

The polyphenol content is mostly influenced by environmental factors. These factors may be divided into pedoclimatic and agronomic factors. Pedoclimatic factors include soil type, sun exposure and rainfall while agronomic factors are culture in greenhouses or fields, biological culture, hydroponic culture, fruit yield per tree and etc. Exposure to light has a significant effect on most flavonoids. The degree of ripeness also changes the concentrations and proportions of the polyphenols. Generally, anthocyanin concentration

increases during ripening, whereas phenolic acid concentration decreases. Storage is another factor which may affect the content of polyphenols that are easily oxidized. Oxidation of polyphenols may result in the formation of polymerized substances, which changes the quality of foods, specially the color and organoleptic properties. Grinding of plant tissues may also results in oxidative degradation of polyphenols. Because cellular decompartmentation and contact between cytoplasmic polyphenol oxidase and phenolic substrates present in the vacuoles may occur. It leads to transformation of polyphenols into brown pigments that are polymerized to different levels. Peeling of fruits and vegetables can remove a considerable amount of polyphenols because polyphenols are more concentrated in the outer parts than in the inner parts (Manach et al., 2004).

There are many parameters influencing the extraction of phenolic compounds in plant materials. The most important parameters are solvent, temperature, solid-liquid ratio, flow rate, extraction time, particle size, extraction method, sample conditions, storage time and condition, presence of interfering substances and etc. In addition, plant polyphenols have various structures which make it practically impossible to develop an extraction method suitable for all plant phenolic compounds (Naczk and Shahidi, 2004). Solubility of polyphenolic compounds as well as their diffusion into the solvent depends on their chemical structure and changes greatly from simple compounds to highly polymerised compounds. Therefore, the choice of solvent is one of the most important steps in extraction process. Solubility of phenolic compounds depends on several parameters including the degree of polymerization of phenolics, polarity of solvent, interaction between phenolics and other food constituents, and formation of insoluble complexes. According to the literatures, the most frequently used solvents for extraction of plant phenolics are methanol, ethanol, acetone, water and ethyl acetate however, their efficiency is not the same. The other solvents used to a lesser extent are propanol, dimethylformamide, and their combinations. Water and different aqueous solution of ethanol are not toxic for human health and have the environmental friendly effect. Compared to water and ethanol, some other organic solvents such as methanol, acetone and ethyl acetate have better efficiency in polyphenol extraction, however, they are not preferable in view of food applications of the extracts (Escribano- Bailon and Santos-Buelga, 2003; Naczk and Shahidi, 2006). It should be noted that the solvent used for the

extraction may not be suitable for all phenolics. In the other words, polyphenols may exist in complex compositions with plant components such as carbohydrates and proteins. Phenolics and their complexes with high molecular weight may be completely insoluble. On the other hand, phenolic extracts of plants are composed of different classes of phenolics dissolved in the solvent. Unwanted phenolics and non-phenolic substances such as chlorophylls, fats, waxes and terpenes may be removed by additional steps (Naczk and Shahidi, 2004; Bucić-Kojić et al., 2009).

2.3.3. Quantification of phenolics

A number of spectrophotometric methods have been developed for quantification of phenolic compounds in plant materials. The most widely used method for quantification of total phenolics in plant materials is the Folin–Denis assay. In this method, in the presence of phenolic compounds and in an alkaline solution, phosphomolybdic–phosphotungstic acid (Folin–Denis) reagent is reduced to a blue colored complex. Swain and Hills (1959) modified the Folin–Denis method for routine analysis of a large number of samples. The Folin–Ciocalteu assay is also used to determine the total phenolics content of plant foods. Folin–Denis and Folin–Ciocalteu reagents are not specific and detect all phenolic classes found in extracts. The interference of reducing substances such as ascorbic acid may be another disadvantage of this assay. The 4-dimethylaminocinnamaldehyde (DMAC) assay has also been proposed for determination of proanthocyanidins. Thies and Fischer (1971), for the first time, reported the formation of a green chromophore between catechin and DMAC.

2.3.4. Ultra high performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS)

Ultra high performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS) is a rapid method which is developed for qualitative and quantitative analysis of phenolic acids (PHA). For determination of PHAs, analytical methods based on reverse-phase high-performance liquid chromatography (HPLC) are used. Another separation technique used for PHAs, is gas chromatography or capillary electrophoresis, followed by ultraviolet (UV), electrochemical (EC), fluorescence (F) or mass spectrometric (MS) detection. Most of these methods require complex extraction, pre-

concentration and hydrolysis before quantification. Unfortunately, these steps often result in the oxidation/degradation of analysed PHAs. UPLC is an advanced form of liquid chromatography (LC) in which narrow-bore columns packed with very small particles and mobile phase delivery systems operate at high back-pressures. Compared to conventional HPLC, UPLC has major advantages such as improved resolution, shorter retention times and higher sensitivity (Yu et al., 2006). Coupling UPLC with electrospray ionisation (ESI) tandem mass spectrometry (MS/MS) offers more advantages than conventional HPLC–MS/MS (Gruz, Novák, and Strnad, 2008). Gruz et al. used this method for qualitative and quantitative analysis of phenolic acids in different beverages (Gruz, Novák, and Strnad, 2008).

2.4. Large scale extraction of carotenoids

Carotenoids can be extracted in large scale to produce carotenoid rich supplements. In this scale, the equipment may be different from those used in laboratory scale and the extraction conditions are not easy to control. For example, filtration and drying processes of the extraction solution in large scale are different from lab scale methods. Residual pectins in fruit juice lead to formation of colloids which may result in blockage during filtration. A possible solution in fruit juice processing can be enzymatic degradation of the colloids (Will et al., 1994).

Another possible problem is degradation of sensible carotenoids during drying and storage. Using additives to encapsulate the product may protect it against heat and moisture to solve the problem.

2.4.1. Enzymatic treatment

Raw press juice is viscous due to persistent clouds formed from cell wall and middle-lamina pectin of the fruit with cytoplasmic protein. The clouds are degraded by pectinase treatment. Pectinase leads to an increase in press capacity and the yield of juice. It has also a positive effect to increase carotene and dry matter content of the product (Demir et al., 2001). In addition, pectinase reduces the viscosity of the juice and agglomerates the cloud particles to larger units which sediment and can be easily removed by centrifuge or filtration (Pilink and Voragen, 1989).

Pectinases, cellulases and hemicellulases are three enzymes used for fruit juice treatment. Grohmann and Baldwin (1992) studied the effect of commercial cellulose and pectinase enzymes on the hydrolysis of polysaccharides in comminuted orange peel. Pectinase enzyme resulted in high levels of conversion to monomeric sugars but cellulose enzyme lead to only limited solubilisation. Treatment of comminuted orange peel with a combination of cellulose and pectinase enzymes was the most effective method for enzymatic hydrolysis of polysaccharides.

Viscozyme is a multi-enzyme prepared from *Aspergillus aculeatus*, and contains a wide range of carbohydrases, such as arabanase, cellulase, hemicellulase, and xylanase (NCBE, 2011). Viscozyme is commercially used in food industry to process cereals and vegetables. Viscozyme is able to degrade the non-starch polysaccharides and branched pectins in plant cell wall, which reduces the viscosity of plant extract and increases the yield of juice (Sun, 2007).

2.4.2. Additives

β-carotene is sensitive to oxidation during storage. Wagner and Warthesen (1995) reported a half-life of 2–3 days at 23°C in spray dried carrot juice. Encapsulation isolates the sensitive ingredient, like β-carotene, in a wall of coating material and provides a barrier to oxygen and water vapour. Emulsification of the sensitive ingredient in a solution which contains the wall material is the easiest encapsulation method. The solution is so dried that the sensitive ingredient is coated by or entrapped in the wall material. Figure 2.18 shows the schematic of encapsulation.

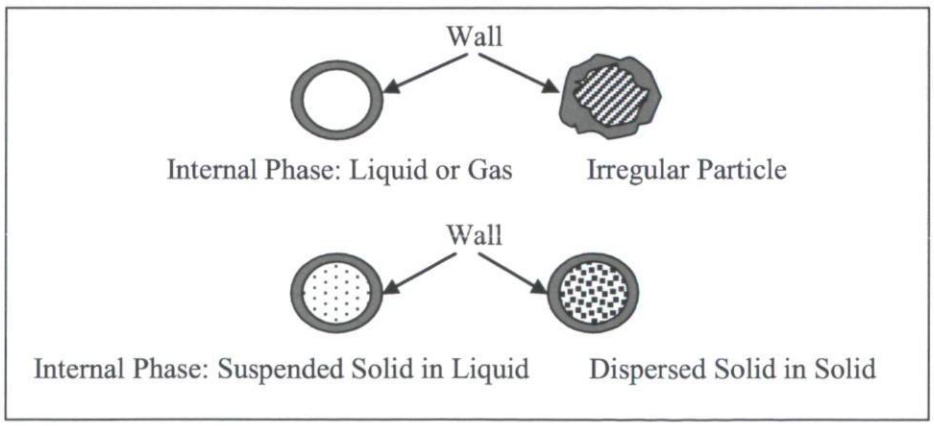


Figure 2.18. Encapsulation of gas and solid particles in single capsule (adapted from Balassa et al., 1971).

Application of this technique has widely used in food industry to enhance their stability and maintaining viability. Encapsulation is also used to mask odours or tastes, and to make the application of the powder easier and more widespread. Several techniques are performed to form the capsules, including spray drying, spray chilling or spray cooling, extrusion coating, fluidized bed coating. Encapsulation is used for a wide variety of foods including flavouring agents, artificial sweeteners, colorants, antioxidants and agents with undesirable flavours and odours (Gibbs et al., 1999).

Large surface area in spray dryer may increase oxidation. Thus, the wall material should be thick enough to provide a barrier against oxygen. In general, 80% of the total weight must be the wall material which reduces delivery efficiency of the product. Maltodextrins are efficient and cost effective and have mild flavour. They exhibit low viscosity at high solid ratios and are accessible in several average molecular weights. Maltodextrines have dextrose equivalent (DE) values of 4, 10, 15, 20, 25, 30 and 42 while the average molecular weight decreases with increasing DE. This feature allows for blending to produce different wall densities which provides the protection of the encapsulated ingredient against oxidation. Using maltodextrins with higher DE values provide a denser matrix which is more impermeable to oxygen and results in longer shelf life (Desobry et al., 1997).

In addition to maltodextrin, other agents are used industrially such as acacia gum, sucrose and starch. However, maltodextrins are the most frequently used agent because of their good efficiency and low price. Desobry et al. (1999) revealed that addition of glucose to maltodextrin 4-DE to reach 25 DE extended the half-life of the encapsulated β-carotene to 17 weeks as compared to 6 weeks with a commercial maltodextrin 25 DE. It can be used to extend the shelf-life of provitamin A in foods.

2.4.3. Spray drying

Atomization or spray drying is the most widely used industrial process for formation of solid powders, granulates and agglomerate particles from solutions, emulsions and pumpable suspensions. It is also the most common method of encapsulation of the extract due to its short drying time and low cost (Robert et al., 2003).

The quality of food powders is based on a variety of properties depending on the specific application. Final moisture content, solubility index, rheological properties and bulk density are among the important powder properties (Straatsma et al., 1999). Several parameters such as solid content of feed, inlet and outlet air temperature have to be carefully controlled to obtain a powder with desired properties (Bhandari et al., 1992). For example, the low solid content can increase the moisture content, while the high solid content can increase the viscosity of the feed (Chen and Tang, 1998).

Figure 2.19 shows the schematic of a typical spray dryer. Most spray dryers consist of a vertical cylindrical chamber (A). Material is sprayed into this chamber in the form of small droplets (B) and at the same time a large volume of heated gas (C), usually air, is fed into the chamber. The volume and temperature of the heated gas must be sufficient to completely evaporate the solvent from the droplets. Drying should be completed before separating the particles from the gas. This separation is partially done in the drying chamber due to the gravity of the larger dried particles (D). The fine particles are separated from the gas in external cyclones (E) and secondary bag collectors (F) (Balassa et al., 1971).

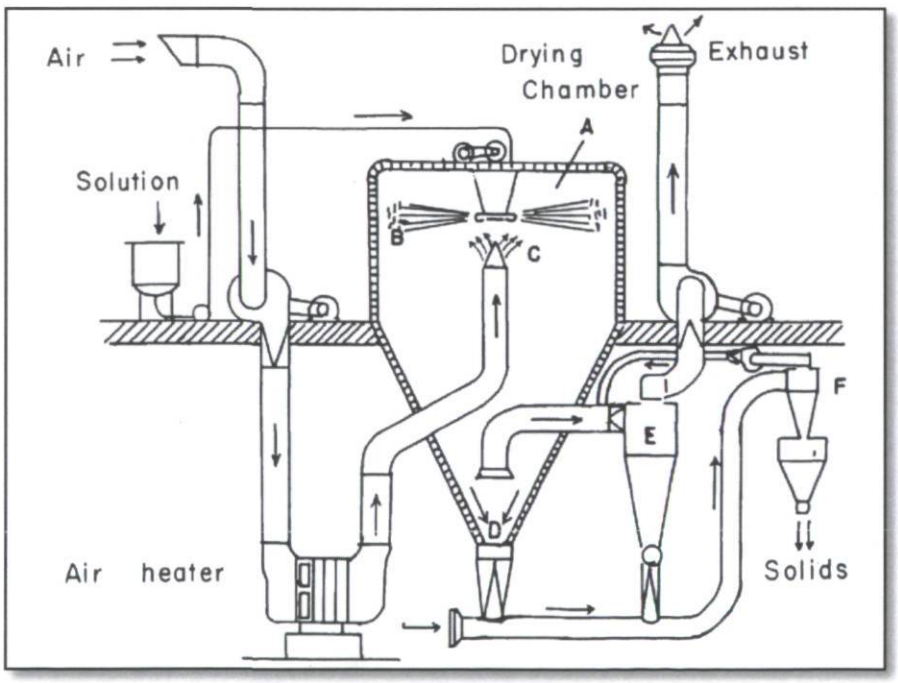


Figure 2.19. Diagram of a typical spray dryer (Balassa et al., 1971).

Hypothesis and objectives

Hypothesis:

A powder extract from cantaloupe rich in carotenoids and phenolic compounds and with high quality, can be obtained by using ethanol as extraction solvent.

Objectives:

1. To study the effect of time and temperature of extraction by ethanol on the carotenoid content.
2. To characterize (qualification and quantification) each type of carotenoid and phenolic compounds in the skin, seed and flesh of cantaloupe.
3. To produce a dry extract rich in the carotenoids at pilot scale through using additives to enhance the quality of the final powder extract.

Chapter 3: Methodology

The proposed approach involves five sections:

3.1. Material preparation

Cantaloupes were purchased and separated into three parts; flesh, peel and seed, which were freeze dried. The powder obtained after freeze drying was used for extraction and characterization in the next steps.

3.2. Extraction and characterization of carotenoids and polyphenols

3.2.1. Extraction and characterization of carotenoids using tetrahydrofuran (THF) as solvent

The carotenoid content of each part of cantaloupe (flesh, peel and seed) was extracted at room temperature, using tetrahydrofuran. The extraction procedure was done in triplicate, as shown in table 3.1. The total carotenoid of extracts was determined by spectrophotometer. High performance liquid chromatography (HPLC) was used for qualitative and quantitative determination of carotenoids present in the extracts. The results of this step provided a reference point to compare the results of sections 3.3 and 3.4.

Table 3.1. Experimental parameters for extraction and characterization of carotenoids with tetrahydrofuran.

Dependent variable	***Sample***	***Measurement method***	***Repeats***	***Number of measurements***	***Number of samples***
Total carotenoid content	Flesh	Spectrophotometer	3	12	24
	Peel				
	Seed				
Individual carotenoid content	Flesh	HPLC	3	12	
	Peel				
	Seed				

3.2.2. Extraction and characterization of polyphenols

The polyphenol content of each part of cantaloupe (flesh, peel and seed) was extracted, using methanol as solvent. The extraction procedure was done in triplicate, as shown in table 3.2. The total and individual polyphenol of extracts were determined by spectrophotometer and UPLC, respectively. The results of this step provided a reference point for the results of section 3.3.2.

Table 3.2. Experimental parameters for extraction and characterization of polyphenols with methanol

Dependent variable	*Sample*	*Measurement method*	*Repeats*	*Number of measurements*	*Number of samples*
Total polyphenols content	Flesh	Spectrophotometer	3	9	9
	Peel				
	Seed				
Individual polyphenols content	Flesh	UPLC	3	9	
	Peel				
	Seed				

3.3. Optimization of carotenoid extraction parameters

3.3.1. Optimization of time and temperature for extraction of carotenoids, using ethanol as solvent

Ethanol was used as extraction solvent and optimum extraction parameters including time and temperature were determined. Six extraction time and four extraction temperature were considered and carotenoid content of the extracts was characterized by spectrophotometer. The comparison of the results obtained in sections 3.2.1 and 3.3.1 revealed the efficiency of extraction carried out with different parameters. Table 3.3 shows the experimental plan for this step.

Table 3.3. Experimental parameters for optimization of extraction parameters.

Dependent variable	*Independent variable*	*Levels*	*Sample*	*Measurement method*	*Repeats*	*Number of measurements*	*Number of samples*
Total carotenoid content and extraction yield	Time (minute)	0	Flesh	Spectrophotometer and drying	3	4*6*2*3=144	4*2*3=24
		30					
		60					
		120	Peel				
		180					
		360					
	Temperature (°C)	25	Flesh		3		
		30					
		40	Peel				
		50					

3.3.2. Optimization of ethanol/water ratio for extraction of carotenoids and polyphenols

Five ethanol/water ratios were used as extraction solvent, using the optimum extraction time and temperature obtained in section 3.3.1. The carotenoid and polyphenol content of the extracts were characterized by spectrophotometer. Individual carotenoid and polyphenol contents of the extracts were characterized by HPLC and UPLC, respectively. The comparison of the results obtained in sections 3.2.1, 3.2.2 and 3.3.2 revealed the efficiency of extraction carried out with different solvent ratios. Table 3.4 shows the experimental plan for this step.

Table 3.4. Experimental parameters for optimization of solvent ratio

Dependent variable	***Independent variable***	***Levels***	***sample***	***Measurement method***	***Repeats***	***Number of measurements***	***Number of samples***
Total and individual polyphenol contents	Ethanol/water ratio	Ethanol 95%, 70%, 50%, 30% and 0%	Peel and flesh	Spectrophotometer and UPLC	3	150	30
Total and individual carotenoid contents				Spectrophotometer and HPLC			
Extraction yield				Drying			

3.4. Ethanolic extraction of carotenoids

The optimum extraction time, temperature and solvent ratio obtained in sections 3.3.1 and 3.3.2 were used for ethanolic extraction of carotenoids in the flesh and peel (table 3.5). Spectrophotometer was used to measure the total carotenoid and polyphenol content while HPLC was used to distinguish the type of carotenoid and determine the content of each type of carotenoid. THF was used as a solvent for extraction of the remained carotenoids in the extraction residue.

Table 3.5. Experimental parameters for ethanolic extraction.

Dependent variable	*Independent variable*	*Sample*	*Measurement method*	*Repeats*	*Number of measurements*	*Number of samples*
Total carotenoid and polyphenol content in extract	Optimum extraction time and temperature and solvent ratio	Flesh Peel	Spectrophotometer	3	18	12
Total carotenoid content in residue						
Individual carotenoid (type & content) in extract	Optimum extraction time and temperature and solvent ratio	Flesh Peel	HPLC	3	12	
Individual carotenoid (type & content) in residue						

3.5. Semi-industrial procedure for ethanolic extraction of carotenoids

The results obtained in section 3.4 revealed the individual carotenoids which can be extracted with ethanol from each part of cantaloupe. The aim of this step was to validate the laboratory scale results through a semi-industrial procedure. Based on the carotenoid content and extraction efficiency obtained, ethanolic extraction procedure was applied to selected parts in a semi-industrial scale and a carotenoid rich powder was produced and characterized. In this step, centrifuge filtration and spray dryer were used to filter the extract and produce a powder from peel and flesh of cantaloupe. Maltodextrine was used as additive to protect the solution during spray-drying.

3.6. Statistics

Analysis of variance was performed using the GLM procedure of Statistical Analysis System (SAS). In the case of optimisation of the extraction parameters, MIXED procedure of SAS 9.2 was used. The results were presented as mean values and standard deviations (mean ± SD).

3.7. Impact of the research

The current research is supposed to extend our knowledge of carotenoids in cantaloupe and lead to a protocol for ethanolic extraction of these carotenoids. Using this protocol, we will be able to produce a powder extract from cantaloupe rich in carotenoids and with high quality. This powder can be used as nutritional additive to potentially reduce the risk of serious human health disorders such as cancer, heart disease, macular degeneration and cataracts. The use of ethanol as extraction solvent will provide such an extract and prevent the harmful effects of other solvents.

Chapter 4: Experimental

4.1. Material preparation

Cantaloupes with a total weight of 6 kg were purchased at a local grocery store (Figure 4.1). The cantaloupes were separated into three parts, flesh, peel and seed. From each cantaloupe, three samples of flesh, peel and seed were kept at -80°C. Three grams of each sample was dried at 105°C during 3 hours and humidity, dry matter and their ratio were determined using the following equations. Then the flesh of all cantaloupes was blended together at high speed. The peel and seeds of all cantaloupes were also mixed separately. One sample was kept at -80°C and the remained mixture of the flesh, peel and seed were freeze dried separately. Their humidity and dry matter were determined. All samples were protected against heat and were covered with aluminum sheet to prevent the exposure to light.

Figure 4.1. Cantaloupe used in the experiments.

The powder obtained after freeze drying was used for extraction and characterization in the next steps.

$$\%Dry\ matter = \frac{Weight\ of\ dry\ matter}{weight\ of\ fresh\ material} \times 100 \quad (4.1)$$

$$\%Humidity = \frac{weight\ of\ fresh\ material - weight\ of\ dry\ matter}{weight\ of\ fresh\ material} \times 100 \quad (4.2)$$

4.2. Extraction and characterization of carotenoids with tetrahydrofuran (THF)

4.2.1. Extraction of carotenoids with tetrahydrofuran (THF)

Total carotenoid extraction was carried out by employing the method of Bureau and Bushway (1986). These extracts will be used as a reference for the ethanolic extraction experiments. Two grams of freeze dried powder samples of each part of cantaloupe (flesh, peel and seed) were separately added to 12.5 ml THF as solvent. They were mixed using a

ULTRA-TURRAX at high speed (9500 rpm) for 5 minutes at room temperature. The extract was vacuum-filtered through a Buchner funnel fitted with Whatman No. 42 filter paper. The filter cake was re-extracted two more times to remove all the carotenoids. The filtrates were combined and brought to a 25 ml volume with THF.

4.2.2. Determination of total carotenoids by spectrophotometer

Three milliliters of supernatant of the extract was transferred to a 50 ml round bottom flask and evaporated to dryness under vacuum, using a Buchi rotary evaporator at a temperature of 30°C. The evaporated sample was redissolved in 3 ml of hexane and then transferred to a 3 mL cuvette. The absorbance of UV at the wavelength of 450 nm was measured by spectrophotometer.

The total concentration of carotenoids was calculated using the following equation:

$$C = \left(\frac{A}{E^{1\%} \times 1\ cm}\right) \times \left(\frac{V}{M}\right) \times \left(\frac{10^6\ \mu g}{1\ g}\right) \times D \qquad (4.3)$$

C: concentration (μg/g)

A: absorption at 450 nm for lutein and 448 nm for β-carotene

V: volume of solvent (dl)

$E^{1\%}$= absorptivity in organic solvents (cm^{-1}), 2592 for lutein and 2589 for β-carotene

M: mass of sample (g)

D: dilution factor

4.2.3. Determination of carotenoids by High-performance liquid chromatography (HPLC)

HPLC was used in this study because it is quick, accurate and reproducible for carotenoid analysis. Ten milliliters of supernatant of the extract was transferred to a 50 ml round bottom flask and evaporated to dryness under vacuum, using a Bushi rotary evaporator at a temperature of 30°C. The evaporated sample was redissolved in 1 ml of methanol/MTBE (9:91) and transferred to a 1 ml vial and placed in -80°C until injection into the HPLC. During the analysis, the sample was wrapped in aluminum foil to protect against light.

4.2.4. Preparation of β-carotene standard

Solution of β-carotene was prepared by weighing 0.001 g of β-carotene into a 100 ml volumetric flask. The flask was brought to volume with stabilized hexane. Aliquots of 500 µl and 2.0 ml were taken and poured into two 10 ml volumetric flasks. This working standard was brought to volume of 10 ml with stabilized hexane then 3 ml of each transferred to a cuvette. The absorbance of UV at the wavelength of 450 nm was measured by spectrophotometer. The total concentration of carotenoids was calculated using the following equation:

$$C(ppm) = \left(\frac{Abs}{E\ dl\ cm^{-1}g^{-1}\times 1\ cm}\right) \times \left(\frac{10mg/ml}{1}\right) \times \left(\frac{1000}{1}\right) \quad (4.4)$$

E dl cm^{-1} g^{-1}= 2592 dl g^{-1}=absorptivity of β-carotene in hexane

The concentration of β-carotene in 2 ml and 500 µl standard solutions were used to perform calibration curve. Ten microliters of standard was injected, and quantification was performed using peak area. Each peak was confirmed by the retention time and characteristic spectra of the standards. Three milliliters of solvent B (methanol/MTBE) was taken and poured into a vial as a blank solution and then 10 µl of that was injected into HPLC.

4.2.5. High-performance liquid chromatography (HPLC)

Carotenoids were quantified using a high-performance liquid chromatographic system equipped with column YMC C_{30} 250 mm × 4.6 mm. The column was maintained at 30°C using a heated column. The wavelength of UV detector photodiode (Waters 996) was 450 nm. A 10 µl sample was injected using a Waters 717 plus. A standard solution was prepared (β-carotene) over a concentration range of 1 to 5 mg/l diluted in solvent B (methanol/MTBE, 9:91). All pigments were quantified by the β-Carotene. A Waters pump 600 was used for making the gradient. The mobile phase flow rate was 1.0 ml/min. The mobile phases were A: methanol/MTBE/water (81:15:4) and B: methanol/MTBE (9:91). The mobile phase gradient used was as follow: starting at 100% A to 50% A/50% B in 45 minutes followed by 50% A/50% B for 24:58 minutes. The column was re-equilibrated between samples for 25 minutes. The rebalancing was necessary to remove residual effects of solvent B.

Table 4.1. The mobile phase gradient table

Time (min)	% A	% B
-	100.0	0.0
45	50.0	50.0
69:58	50.0	50.0
69:59	100.0	0.0

4. 3. Extraction and characterization of polyphenols

In order to quantify the extractable polyphenols, two successive extractions were performed on 1 g powder of flesh, peel and seed. In these extractions, methanol:water with the volume ratio of 80:20 and acetone:acide acetic 2% with the volume ration of 70:30 were used as solvents extraction, respectively.

4.3.1. Double extraction of polyphenols

One gram of the powder of each part of cantaloupe was weighed accurately in a 50 ml tube. Five milliliters of water was added and stirred. Twenty milliliters of methanol was then added and mixed well by the vortex at high speed. Samples were mixed for 30 seconds, sonificated for 30 minutes and then centrifuged at 3500 rpm for 5 minutes. The supernatant (No. 1) was collected for analysis. One hundred microliters of the supernatant was used for analysis of total polyphenol and 2 ml was used for determination of dry matter and yield. The residue was used for a second extraction. Sixteen milliliters of acetone and then 4 ml of acetic acid solution 2% were added to the residue. Samples were mixed for 30 seconds, sonificated for 30 minutes and then centrifuged at 3500 rpm for 5 minutes. The supernatant (No. 2) was collected for analysis. One hundred microliters of the supernatant was used for analysis of total polyphenol and 2 ml was used for determination of dry matter and yield. First and second supernatant were mixed together and centrifuged at 3500 rpm for 5 minutes. They were transferred into a 250 ml round bottom flask and were evaporated completely at temperature of 45°C. Twenty five milliliters of water was added and mixed well. The Brix of each sample was measured. Then, the extract went through the column chromatography to purify the extract. The passage of extract through the chromatography column allowed to separate polyphenols from other soluble materials present in the extract, ie, sugars, polysaccharides and organic acids mainly. Twenty five milliliters of aqueous extract was passed through 50 ml of resin. One hundred and fifty milliliters of ethanol was

used to wash the column and detach the polyphenols from the resin. The volume of the output of chromatography was measured and then transferred into a 500 ml round bottom flask and were evaporated at 45°C completely. One milliliter of ethanol 95% was added and mixed well. Then the solution was transferred into a UPLC vial and kept at -80°C. All experiments were performed in triplicate.

In the case of purification of extract by column chromatography, the adsorption of polyphenols on the resin is much more effective when the chromatography step is performed directly after the evaporation step, without refrigeration and waiting time. In fact, the resins are more efficient when heated (Schieber et al., 2003). Cooling has an irreversible negative impact on the adsorption properties of polyphenols, since reheating of the solution cannot provide the initial effectiveness of the resin (Brianceau, 2010).

4.3.2. Analysis of total polyphenol with Folin-Ciocalteu

One hundred microliters of sample, as well as the standard and blank solutions were transferred into a 3 ml cuvette. Two milliliters of distilled water and 200 µL of Folin-Ciocalteu were added, respectively. The samples were mixed well for about 1 minute and were left for 5 minutes. Nine hundred microliters of sodium carbonate solution (200 g sodium carbonate in 1000 ml of distilled water) was added and mixed well. The reading was done by spectrophotometer at wavelength of 765 nm, two hours after addition of sodium carbonate. The samples were kept in dark. The total polyphenol was calculated using equations (4.5) and (4.6):

$$\text{Concentration of polyphenol (mg/g)} = (\text{C(mg/l)} \times \text{V(ml)})/(1000 \times \text{M(g)}) \quad (4.5)$$

$$\%\ \text{Polyphenol} = C(\tfrac{\text{mg}}{\text{g}}) \times \left(\tfrac{\text{V(ml)}}{1000}\right) \times \left(\tfrac{1}{\text{M(g)}}\right) \times (\tfrac{1}{1000}) \quad (4.6)$$

C: concentration (mg/g)

M: mass of sample (g)

V: volume of solvent (ml)

4.3.3. Preparation of standard solutions

Gallic acid in methanol, with the concentrations of 100 ppb, 1000 ppb, 50 ppm and 250 ppm was used as a standard solution.

4.3.4. UPLC–MS/MS instrumentation and conditions

UPLC–MS/MS analyses were carried out using an ACQUITY H-Class Ultra Performance LC™ system (Waters, Milford, MA, USA) linked simultaneously to both a PDA detector and TQD triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating in negative mode.

MassLynx™ software (version 4.1, Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing. Sample solutions were injected into a reversed phase column (Acquity UPLC ® HSS T_3, 1.8 µm, 2.1 x 150 mm (Waters, Milford, MA), which was maintained at 30°C. Formic acid 0.1% and acetonitrile were used as a solvent A and B respectively at a flow rate of 200 µL min^{-1}. At the end of this sequence the column was equilibrated under initial conditions for 1.40 minutes. The pressure ranged from 4000 to 7000 psi during the chromatographic run. The effluent was introduced into a PDA detector (scanning range 210–600 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 120°C, desolvation temperature 350°C, capillary voltage 3.05 kV, cone voltage 39 V). Nitrogen was used as desolvation gas (650 L/h).

Table 4.2. The mobile phase gradient table

Time (min)	*% Solvent A*	*% Solvent B*
-	95.0	5.0
6.00	88.0	12.0
12.00	85.0	15.0
24.00	75.0	25.0
33.00	70.0	30.0
39.00	65.0	35.0
51.00	40.0	60.0
60.00	5.0	95.0
61.80	5.0	95.0
62.40	95.0	5.0
69.00	95.0	5.0

4.4. Extraction of proanthocyanidin from peel, flesh and seed of cantaloupe

4.4.1. Solutions to prepare

1. Acidified ethanol: 12.5 ml of hydrochloric acid (36%) was added to 12.5 ml of distilled water and 75 ml of ethanol (95%). They were mixed and kept at 18-25°C.
2. Dilution solution: 8 ml of ethanol (95%) was added to 19.5 ml of deionised water and mixed well.
3. DMAC reagent: 0.1% mg of DMAC was weighted and added to 50 ml of acidified ethanol.

4.4.2. Extraction

Five hundred milligrams of the powder was weighed in a 50 ml tube. Five milliliters of acetic acid solution 2% was added and mixed well. Fifteen milliliters of acetone was then added and mixed for 30 seconds. The extract was sonificated for 30 minutes and centrifuged at 3500 rpm for 5 minutes, respectively. The supernatant was collected for the analysis. All experiments were performed in triplicate.

4.4.3. Analysis of proanthocyanidin

Seven hundred microliters of the sample as well as the standard and blank (dilution solution) were transferred into three 3 ml cuvettes. Then, 2100 µl of DMAC was added to each cuvette and mixed well. The reading was done by spectrophotometer at wavelength of 640 nm from 15 to 20 minutes to select the highest absorption.

4.5. Optimization of parameters for carotenoids extraction using ethanol as solvent

4.5.1. Optimization of extraction time and temperature

The extraction of carotenoids was carried out by maceration of the powder in the ethanol. The solid: liquid ratio of 1:20 (w:v) and ethanol 95% are most suitable in terms of industrial production and were used for this step. To optimize the extraction parameters at the laboratory scale, 1 g of the ground powder was mixed with 20 ml of ethanol using the ULTRA-TURRAX at high speed (9500 rpm). Water bath was used for heating the extraction solvent to 25°C, 30°C, 40°C and 50°C. After soaking times of 0 h, 0.5 h, 1 h, 2 h, 3 h and 6 h a sample was taken and analysis of total carotenoid was performed. The impacts of extraction time and temperature on the extraction yield were investigated. After 6 h at different extraction temperatures a sample of 2 ml of extract was taken and

transferred into a small glass tube. The sample was dried in the oven at 45°C and %yield and %dry matter were calculated. All experiments were performed in triplicate.

The total carotenoid (TC) was determined by UV-visible spectrophotometer (Unico 1200). The concentration of total carotenoids such as lutein and β-carotene was calculated based on the absorbance of the wavelength of 444 nm and 450 nm, respectively, and using the following formula:

$$C = \left(\frac{A}{E^{1\%} \times 1\ cm}\right) \times \left(\frac{V}{M}\right) \times \left(\frac{10^6\ \mu g}{1\ g}\right) \times D \qquad (4.7)$$

C: Concentration (μg/g)

A: Absorption at 444 nm for lutein and 450 nm for β-carotene

V: Volume of solvent (dl)

$E^{1\%}$= Absorptivity in organic solvents (cm^{-1}), 2550 for lutein and 2529 for β-carotene

M: Mass of sample (g)

D: Dilution factor

4.6. Optimization of the ethanol/water ratio for extraction of carotenoids and polyphenols

4.6.1. Optimization of the ethanol/water ratio

One gram of freeze dried powder samples of flesh and peel were extracted using 20 ml of ethanol 95%, 70%, 50% and 30%, and Ultra pure water as solvent to study the extraction yield. The impacts of different percentages of ethanol were investigated. ULTRA-TURRAX at 9500 rpm was used to mix the powder and solvent for 2 minutes at room temperature. Water bath was used for keeping the solvent at 50°C. The soaking time was 2 hours for flesh and peel. The extracts were vacuum-filtered with Whatman 42 filter paper. The supernatant was separated, centrifuged at 3500 rpm for 2 minutes and kept at -80°C for the analysis. For calculation of dry matter, 1 ml of each supernatant was transferred into a glass tube and dried in the oven at 45°C. All experiments were performed in triplicate.

4.6.2. Analysis of total polyphenol

One hundred microliters of supernatant was used for analysis of total polyphenol by spectrophotometer using Folin-Ciocalteu, as explained in section 4.3.2.

4.6.3. Analysis of polyphenols by UPLC

Three milliliters of supernatant was transferred into a glass tube. Ethanol was evaporated under nitrogen, at a temperature of 50°C. After evaporation, the volume was adjusted to 3 ml by water. Five milliliters of methanol, 5 ml of HCl 0.01 M and 3 ml of supernatant were passed through the SEP-PAK (solide phase extraction, C_{18}), respectively. Then the SEP-PAK was washed with 5 ml of HCl 0.01 M. The polyphenols were recovered with 3 ml of methanol and transferred into a vial of UPLC and kept at -80°C for analysis with UPLC, as explained in section 4.3.4.

4.6.4. Determination of total carotenoids in the extract by spectrophotometer

Three milliliters of supernatant of the sample was transferred to a 50 ml round bottom flask and evaporated to dryness under vacuum, using a Buchi rotary evaporator (30°C). Evaporated sample was redissolved in 500 µl of water. Then, 2.5 ml of acetone was added and centrifuged for 3 minutes. The supernatant was transferred to 3 mL cuvette. The concentration of total carotenoids such as lutein and β-carotene was calculated based on the absorbance of the wavelength of 446 nm and 452 nm, respectively, and using the following formula:

$$C = \left(\frac{A}{E^{1\%} \times 1\ cm}\right) \times \left(\frac{V}{M}\right) \times \left(\frac{10^6\ \mu g}{1\ g}\right) \times D \qquad (4.8)$$

C: Concentration (µg/g)

A: Absorption at 446 nm for lutein and 452 nm for β-carotene

V: Volume of solvent (dl)

$E^{1\%}$: Absorptivity in organic solvents (cm^{-1}), 2540 for lutein and 2559 for β-carotene

M: Mass of sample (g)

D: Dilution factor

This small volume of water was used to dissolve the dried extract, since it was not soluble in acetone and other solvents. The absorbance of water was unknown; therefore acetone was used, after dissolving, for determination of total carotenoids.

4.6.5. Determination of carotenoids in the extract by HPLC

Three milliliters of supernatant of extract was transferred to a 50 ml round bottom flask and evaporated to dryness under vacuum, using a Bushi rotary evaporator (30°C). The

evaporated sample was redissolved in 1 ml of methanol/MTBE with the volume ratio of 80:20 which was found as the optimum ratio. The dried extract was less soluble in other ratios of methanol/MTBE. The supernatant was transferred to 1 ml vial and kept at -80°C until injection into the HPLC. HPLC was performed as explained in section 4.2.5.

4.7. Ethanolic extraction of carotenoids

4.7.1. Extraction of carotenoids with ethanol

One gram of freeze dried powder samples of flesh and peel were used for extraction of carotenoids by ethanol 95%, as explained in section 4.6.1. The extract was centrifuged at 3500 rpm for 3 minutes and vacuum-filtered through a Buchner funnel fitted with a 0.7 μm Whatman GF/F filter paper. The filter cake was separated for the analysis and the supernatant was filtrated by filter paper of HPLC (0.45 μm) and was kept at -80°C for the analysis. One milliliter of the supernatant was dried in the oven at 45°C for calculation of dry matter.

4.7.2. Analysis of total polyphenol

One hundred microliters of supernatant was used for analysis of total polyphenol by spectrophotometer using Folin-Ciocalteu, as explained in section 4.3.2.

4.7.3. Determination of total carotenoid in the extract by spectrophotometer

Three milliliters of supernatant was transferred to a 3 ml cuvette and the absorbance of UV at wavelength of 444 nm and 450 nm was measured by spectrophotometer. The total concentration of carotenoids was calculated using the equation (4.7).

4.7.4. Determination of carotenoids in the extract by HPLC

The carotenoids in the extraction residue were determined by HPLC, as explained in section 4.2.3. Evaporated sample was redissolved in 3 ml of methanol/MTBE (9:91). The extracts were transferred to a 1 ml vial and kept in -80°C until injection into the HPLC.

4.7.5. Extraction of carotenoids in the extraction residue, using tetrahydrofuran (THF)

The carotenoids in the extraction residue were extracted by THF, as explained in section 4.2.1. The filter cake was re-extracted one more time to remove all the carotenoids. The filtrates were combined and brought to a 20 ml volume with THF and was used for determination of total and individual carotenoids remained in the residue.

4.7.6. Determination of total carotenoids in the extraction residue by spectrophotometer

The concentration of total carotenoids in the extraction residue was measured using the method explained in section 4.2.2. The total concentration of carotenoids was calculated using the equation (4.3).

4.7.7. Determination of carotenoids in the extraction residue by HPLC

HPLC was used to determine the carotenoids in the extraction residue. The methods are explained in sections 4.2.3 and 4.2.5.

4.8. Semi-industrial procedure for ethanolic extraction of carotenoids

Before starting the semi-industrial procedure, the amount of polyphenol and carotenoids were measured in the presence and absence of enzymes (Kleryme and Viscozyme) in the lab-scale experiments and the best enzyme was chosen for using in pilot scale.

For this purpose, 1 g of the powder of flesh of cantaloupe was mixed with 6 ml of water and heated to 45°C for 1 hour. Then, 200 µl of Viscozyme and 3 µl of Kleryme was added separately to three samples and kept at 45°C for 1 hour. The samples were centrifuged for 30 minutes at 5000 rpm. The water was separated and the amounts of polyphenol and extraction yield were measured in the water. Twenty milliliters of ethanol 95% was added to the residues. After mixing well, the samples were put in the water bath at 50°C for 2 hours. After centrifugation, the amounts of polyphenol and carotenoid as well as extraction yield were measured for the extract.

Figure 4.2 shows the schematic of different steps of semi-industrial procedure for ethanolic extraction of carotenoids.

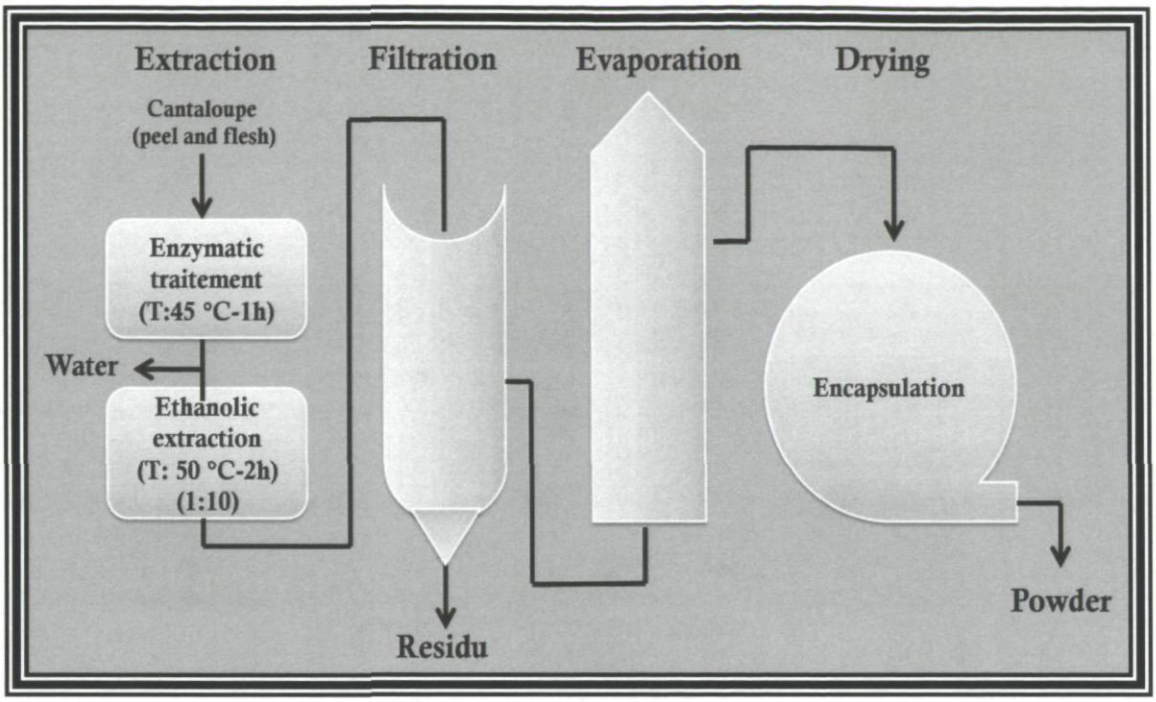


Figure 4.2. Semi-industrial procedure for ethanolic extraction of carotenoids.

4.8.1. Material preparation

Cantaloupes with a total weight of 10 kg were purchased from a local grocery store. The cantaloupes were separated into two parts, flesh-peel and seed (Figure 4.3). Each part was measured separately. Then the flesh-peel of all cantaloupes was blended at a high speed (Figure 4.3.d). One sample of flesh-peel was kept at -80°C. The humidity and dry matter were determined. All samples were protected against heat and will be covered with aluminum sheet to prevent the exposure to the light.

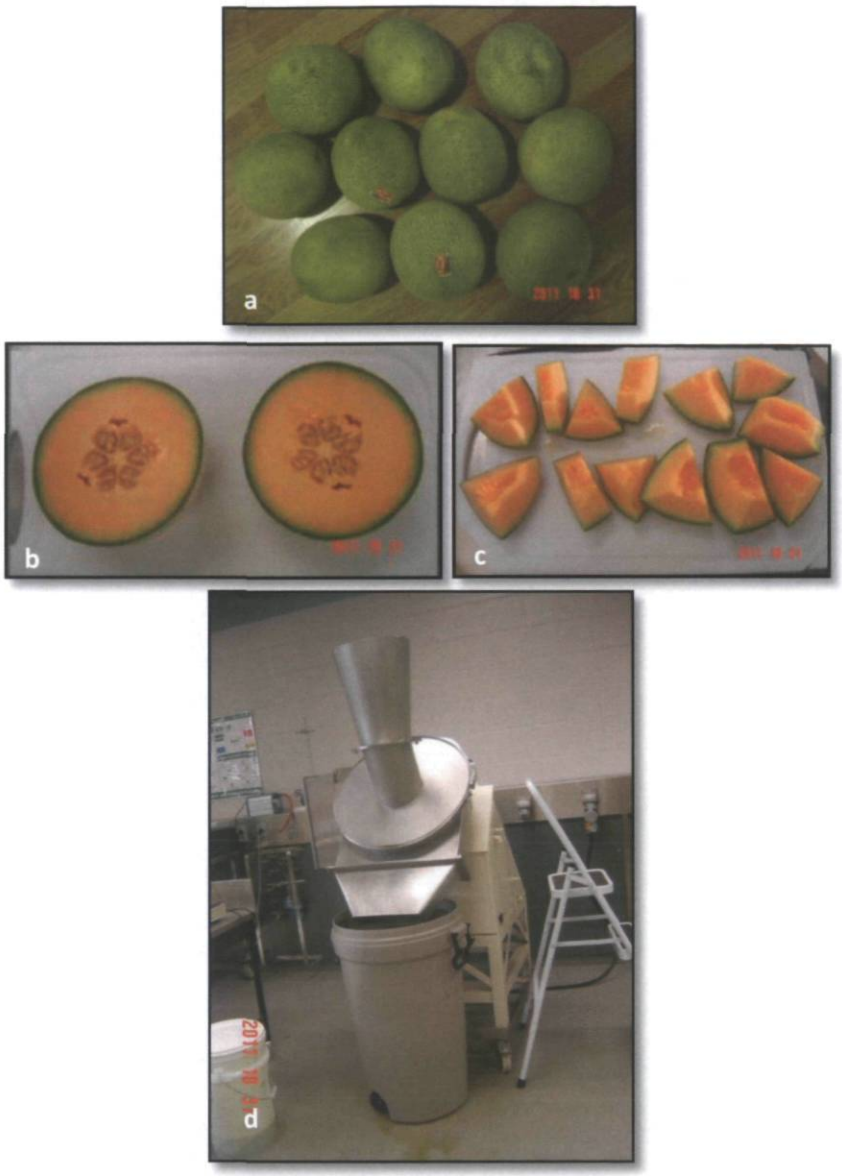


Figure 4.3. a,b and c) Cantaloupe preparation, d) Blender.

4.8.2. Enzyme treatment

Viscozyme (200 µl/g dry powder or 200 ml/kg dry powder) was used as an enzyme for separating the pectin and facilitating the filtration. For this purpose, the remained mixture of the flesh-peel was weighed and transferred into a mixer and the temperature was set at 45 °C. The mixture was heated for 1 hour at this temperature.

4.8.3. Centrifuge-filtration

After enzyme treatment, the mixture was transferred into a centrifuge to separate the residue from the juice. After centrifuge-filtration, the mass of residue, the volume and the dry matter of liquid were measured. The juice was centrifuged again to separate the particles. Then the juice was freeze dried and 588 g of dry powder was obtained. The powder obtained after freeze drying was too hydroscopic. The quantity of total polyphenols in the separated juice, after freeze drying, was measured by spectrophotometer.

4.8.3.1. Analysis of total polyphenol with Folin-Ciocalteu

Five hundred milligrams of the powder (after freeze drying of juice) was weighed accurately in a 50 ml tube. Five milliliters of water was added and stirred. Twenty milliliters of methanol was then added and mixed well by the vortex at high speed. Samples were mixed for 30 seconds, sonificated for 30 minutes and then centrifuged at 3500 rpm for 5 minutes. One hundred microliters of sample, as well as the standard and blank solutions were used for analysis of total polyphenol by spectrophotometer using Folin-Ciocalteu, as explained in section 4.3.2.

4.8.4. Ethanolic extraction

To obtain the ratio 1:10, 6.5 litres of ethanol 95% was added to 650 g of the residue and was mixed well for 2 hours at 50°C (Figure 4.4). Samples were taken at 30 min, 1 h and 2 h for measuring the carotenoid content.

Figure 4.4. Ethanolic extraction of carotenoids

4.8.4.1. Analyse of total carotenoids by spectrophotometer

After centrifuge, 3 ml of solvent was transferred to a 3 ml cuvette and the absorbance of UV at wavelength of 444 nm and 450 nm was measured by spectrophotometer.

4.8.5. Centrifuge-filtration

After extraction, the mixture was transferred into a centrifuge to separate the residue from ethanol. A white residue was obtained after centrifuge (Figure 4.5). After centrifuge-filtration, the mass of residue, volume, Brix and dry matter of the extract were measured. The quantity of total polyphenols in the extract was measured by spectrophotometer.

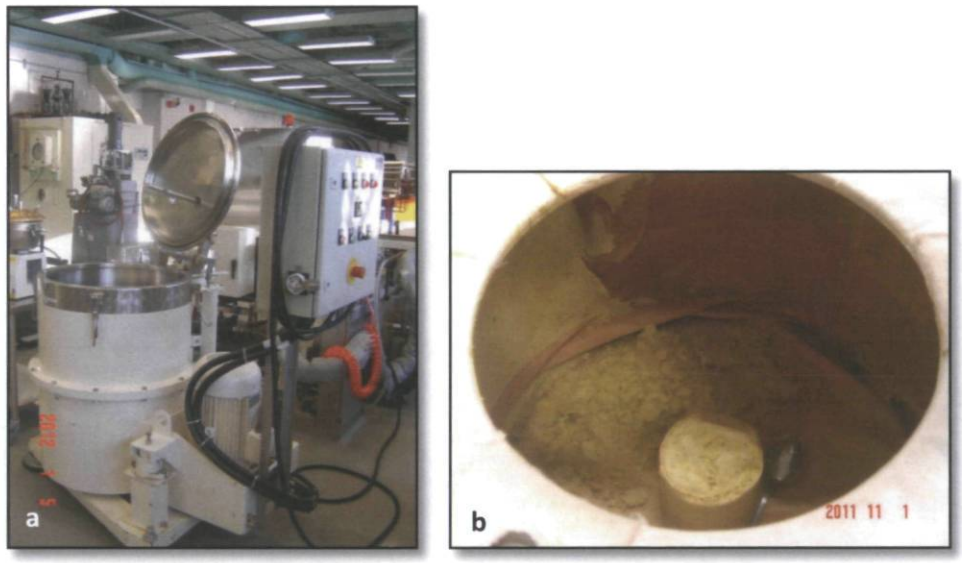


Figure 4.5. a) Centrifuge-filtration, b) Residue after Centrifuge-filtration

4.8.5.1. Analysis of total polyphenol with Folin-Ciocalteu

One hundred microliters of extract was used for analysis of total polyphenol by spectrophotometer using Folin-Ciocalteu, as explained in section 4.3.2.

4.8.6. Evaporation of ethanol

Ethanol was evaporated from 5400 ml of the solvent at temperature of 35°C and vacuum pressure of 750 mm/Hg (Figure 4.6). As a result, the extract was concentrated about 27 times and 200 ml of aqueous fraction with dry matter of 20% was obtained.

Figure 4.6. Evaporation of ethanol

After evaporation, a resin (Figure 4.6) was obtained which was the residue of carotenoids and chlorophyll, sticked around the vessel. This resin was gathered and its carotenoid content was determined after drying through two different ways; drying in oven at 45°C and freeze drying. The results were compared with those obtained without drying (direct analysis) to verify the effect of drying method on the degradation of carotenoids.

The volume of solvent (water), Brix, dry matter, total carotenoid and total polyphenol were measured in the solution. Solution was protected against heat and was covered with aluminum sheet to prevent the exposure to light.

4.8.7. Additives

Sixty grams of maltodextrine was mixed with 100 ml of water and added as additive to 200 ml of final extract (with the Brix of 20%) to obtain the extract to maltodextrine ratio of 1:1.5. One gram of silica was also mixed with 50 ml of water and added to this solution. These additives were used to protect the solution during spray-drying.

4.8.8. Spray dryer

In this step, spray dryer (Figure 4.7.a) was used to produce a powder of the extract. The inlet and outlet temperature of air were 170°C and 70°C, respectively. Fifty two grams of

powder (Figure 4.7.b) with the humidity of 2.8% was obtained. The quantity of total polyphenols and carotenoids in the final powder was measured by spectrophotometer.

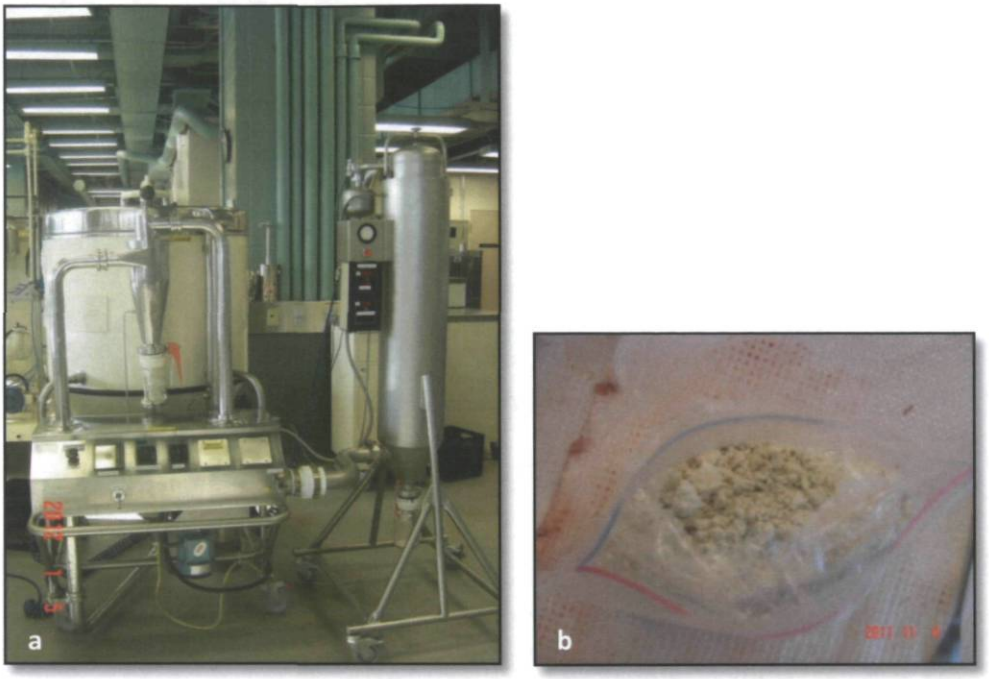


Figure 4.7. a) Spray dryer, b) Dried powder obtained after spray drying.

4.8.8.1. Analysis of total polyphenol with Folin-Ciocalteu

The concentration of total polyphenols in the powder was measured using the method explained in section 4.8.3.1.

4.8.8.2. Determination of total carotenoids by spectrophotometer

Five hundred milligrams of the powder was weighed accurately in a 50 ml tube. Ten milliliters of acetone was added and mixed well by the vortex at high speed. Sample was centrifuged at 3500 rpm for 20 minutes and then was filtered with filter paper No. 0.45. Three milliliters of supernatant of the extract was transferred to a 3 ml cuvette. The absorbance of UV at the wavelength of 446 and 452 nm was measured by spectrophotometer.

Chapter 5: Results and discussion

5.1. Material preparation

The percentage of dry matter and humidity of three parts of cantaloupe (flesh, peel and seed) are presented in the table 5.1. The results were obtained by taking the average of humidity and dry matter of seven cantaloupes. According to table 5.1, the moisture content of flesh is more than that of peel and seed. The dry matter of seed is higher than that of peel and flesh of cantaloupe.

Table 5.1. % Dry matter and humidity in peel, flesh and seed of cantaloupes.

	% Dry matter	*% Humidity*
Peel (Average of seven cantaloupes)	10.1±1.8	89.9±1.8
Flesh (Average of seven cantaloupes)	9.9±2.1	90.1±2.1
Seed (Average of seven cantaloupes)	26.7±2.6	73.3±2.6

Percentage of humidity and dry matter of mixture of peel, flesh and seed, which were determined after freeze drying are presented in table 5.2. It was used to calculate the amounts of carotenoid and polyphenol in the fresh peel and flesh.

Table 5.2. % Dry matter and humidity in peel, flesh and seed of cantaloupes.

	% Dry matter	*% Humidity*
Peel (mixture)	10.2	89.8
Flesh (mixture)	9.8	90.2
Seed (mixture)	27.0	73.1

Ratio of dry matter and humidity in peel, flesh and seed are presented in table 5.3. These ratios can be used to calculate the amounts of carotenoid and polyphenol in the fresh matter of whole cantaloupe.

Table 5.3. Ratio of dry matter and humidity in peel, flesh and seed of cantaloupes.

Ratio between different parts of cantaloupe	*Dry matter*	*Humidity*
Peel : Flesh (Average of seven cantaloupe)	1.0	1.0
Seed : Flesh (Average of seven cantaloupe)	3.1	0.8
Peel : Seed (Average of seven cantaloupe)	0.3	1.3
Peel : Flesh (mixture)	1.1	1.0
Seed : Flesh (mixture)	2.8	0.8
Peel : Seed (mixture)	0.4	1.2

Percentage of peel, seed and flesh in the whole fresh cantaloupe is 21.3%, 8.9% and 69.8% respectively (Figure 5.1.a). As shown in Figure 5.1.b, peel, seed and flesh constitute 18.7%, 24.7% and 56.6% of an entire dry cantaloupe, respectively. The flesh constitutes the largest part of the cantaloupe.

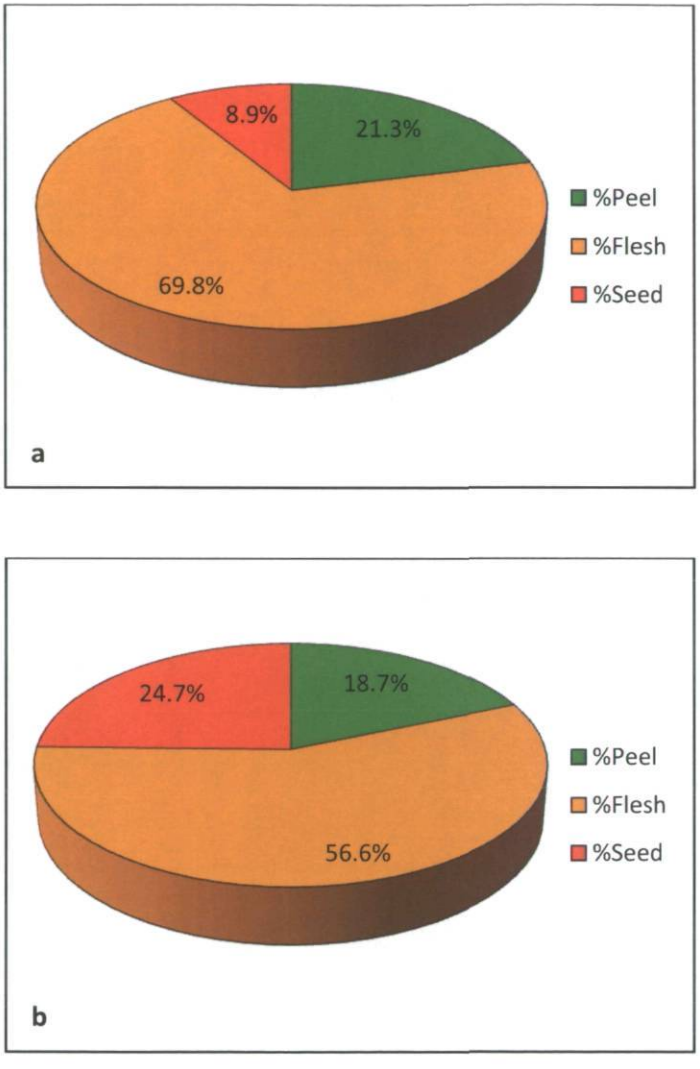


Figure 5.1. Average percentage of flesh, seed and peel in the entire cantaloupe a) based on fresh matter, b) based on dry powder.

5.2. Extraction and characterization of carotenoids using tetrahydrofuran (THF) as solvent

Carotenoid extraction was carried out by employing the method of Bureau and Bushway (1986) and using tetrahydrofuran (THF) as solvent. Figure 5.2 shows the extracts. The concentration of extracted carotenoids was measured using spectrophotometer. As shown in table 5.4, the outer part (peel) of cantaloupe was the richest part and, therefore, a good source for carotenoids. This is in agreement with the findings of Gross et al. (1973) and Remorini et al. (2008) who have determined the total carotenoid content in the peel and flesh of avocado and peach, respectively. Their results indicated that the peels of avocado and peach had a higher content of carotenoids than the flesh. After the peel, the flesh and seed had the highest amount of carotenoids, respectively. The results of statistical analysis (LSD test) in table 5.4 show that the difference between the amount of total carotenoids in the peel and flesh of cantaloupe is significant.

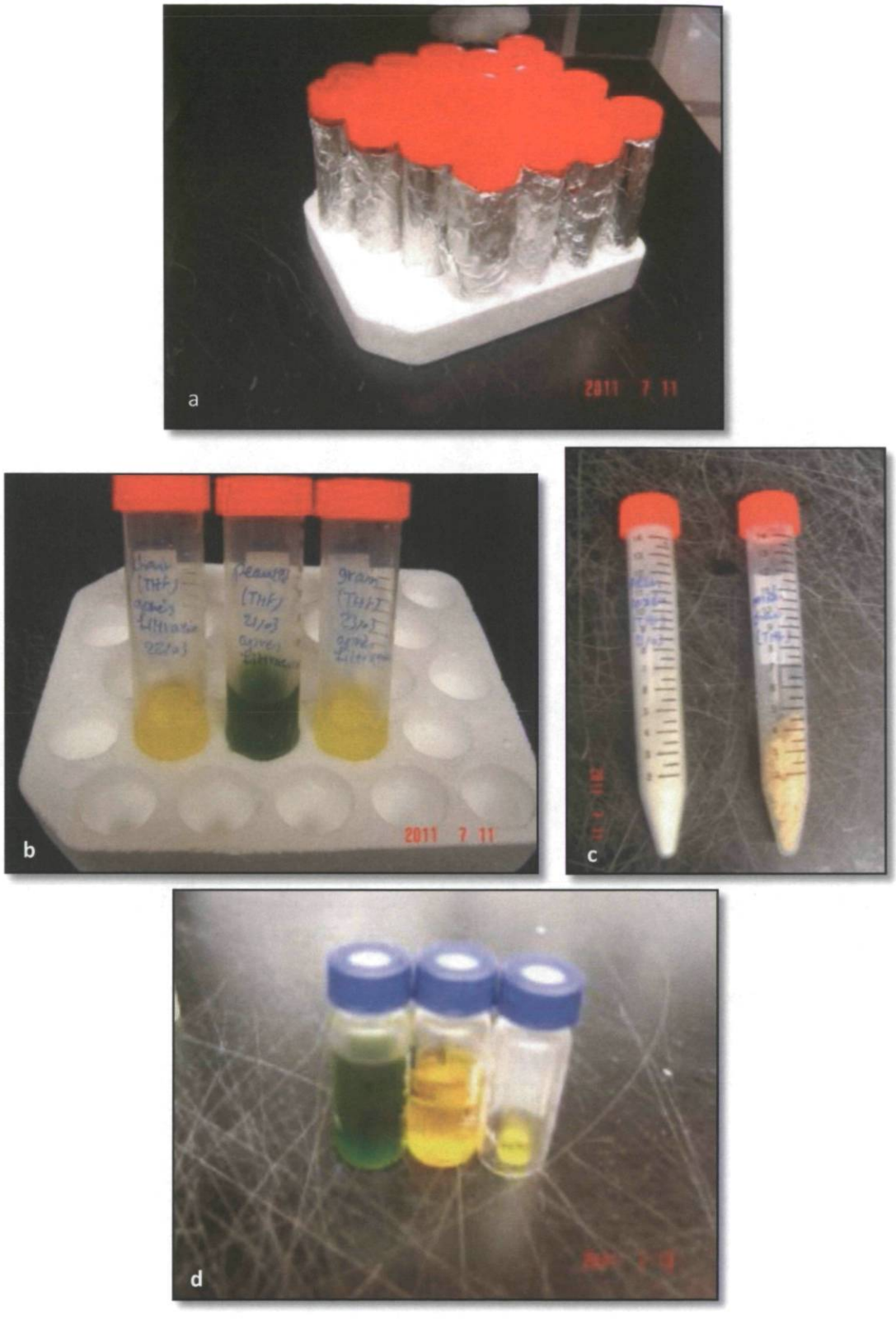


Figure 5.2. a) Protection of samples against light, b) Samples of peel, flesh and seed, after extraction by THF, c) Residue of peel and flesh after extraction, d) Samples of peel, flesh and seed in vial, prepared for HPLC.

Table 5.4. Total concentration of carotenoids in different parts of cantaloupe measured by spectrophotometer.

	Total carotenoid in dry powder (fresh matter) (mg/g)
Peel	0.49±0.08 [a] (0.05)
Flesh	0.28±0.06 [b] (0.03)
Seed	0.19±0.09 [b] (0.05)

Different letters within columns represent significant difference at $p < 0.05$, using LSD test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

The carotenoid constituents separated from extracts of different parts of cantaloupe and their quantitative amount determined by HPLC and using a C_{30} column are shown in table 5.5. Seven components were present which were separated and identified by comparing retention times and UV/visible absorbtion spectra with commercial standard (β-carotene). The carotenoids were β-carotene, lutein, *cis* isomers of β-carotene (9-*Cis*-β-carotene and 13-*Cis*-β-carotene), xanthophils, α-carotene and β-cryptoxanthin. In addition to known carotenoids, there were unidentified carotenoids in the extracts (table 5.5). The major hydrocarbon carotenoids in this fruit were identified as β-carotene and lutein. The *cis* isomers of β-carotene, xanthophils, α-carotene and β-cryptoxanthin were identified as minor components. The comparisons between the HPLC results of the carotenoid extracts from peel, flesh and seed indicate that the highest level of β-carotene was detected in the flesh (0.36 mg/g dry powder), compared with 0.14 mg/g dry powder in the peel (table 5.5). On the contrary, the highest level of lutein was detected in the peel (0.21 mg/g dry powder), compared with 0.005 mg/g dry powder in the flesh and 0.002 mg/g dry powder in the seed.

Table 5.5. Concentration of β-carotene, α-carotene, β-cryptoxanthin, Lutein, Xanthophilles, 9-*Cis*-β-carotene and 13-*Cis*-β-carotene in different parts of cantaloupe, determined by HPLC.

Carotenoid	*Carotenoid in dry powder (fresh matter) of peel (mg/g)*	*Carotenoid in dry powder (fresh matter) of flesh (mg/g)*	*Carotenoid in dry powder (fresh matter) of seed (mg/g)*	*P-value*
β-Carotene	0.14 ± 0.01^{b} (0.01)	0.36 ± 0.03^{a} (0.04)	0.20 ± 0.02^{b} (0.05)	
β-Cryptoxanthin	ND	0.0013±0.0004 (0.0001)	0.0013±0.0002 (0.0003)	0.3739
α-Carotene	0.009 ± 0.001^{a} (0.001)	0.004 ± 0.001^{b} (0.0004)	0.00578 ± 0.00004^{ab} (0.00155)	
Lutein	0.21 ± 0.03^{a} (0.02)	0.005 ± 0.002^{b} (0.001)	0.002 ± 0.001^{b} (0.001)	
Xanthophylles	0.07 ± 0.01^{a} (0.01)	0.004 ± 0.002^{b} (0.0004)	0.002 ± 0.001^{b} (0.001)	
9-*Cis*-β-Carotene	0.020 ± 0.003^{a} (0.002)	0.009 ± 0.002^{b} (0.001)	0.0034 ± 0.0003^{c} (0.0009)	
13-*Cis*-β-carotene	ND	0.017±0.002 (0.002)	0.0049±0.0001 (0.0013)	0.1228
Unknown 1	ND	0.006±0.002 (0.001)	0.0024±0.0003 (0.0006)	0.0789
Unknown 2	ND	0.005±0.001 (0.001)	ND	
Unknown 3	ND	0.003±0.001 (0.0003)	0.0009±0.0004 (0.0002)	0.0731
Total	0.45±0.03 (0.05)	0.41±0.03 (0.04)	0.22±0.02 (0.06)	

ND: Not detected

Different letters within rows represent significant difference at $p < 0.05$, using LSD test. Means within rows followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

The P-value within rows was used to verify the presence or absence of significant difference between the individual carotenoids content in peel and flesh, using F test.

The carotenoids in the extracts from peel, flesh and seed were identified and measured by HPLC using a C_{30} column. The profiles of carotenoids are shown in Figures 5.3.a, 5.3.b and 5.3.c respectively. Using the reversed-phase HPLC, as shown in the profiles, dihydroxy lutein as a more polar xanthophyll was the first component eluted. Monohydroxy carotenoids, α-carotene and β-carotene were eluted after lutein, respectively. It is in agreement with the literature (Rodriguez-Amaya, 2004). However, elution of carotenes does not always follow the expected pattern and may vary depending on the type of column and the mobile phase. For example, β−carotene may elute before or after lycopene.

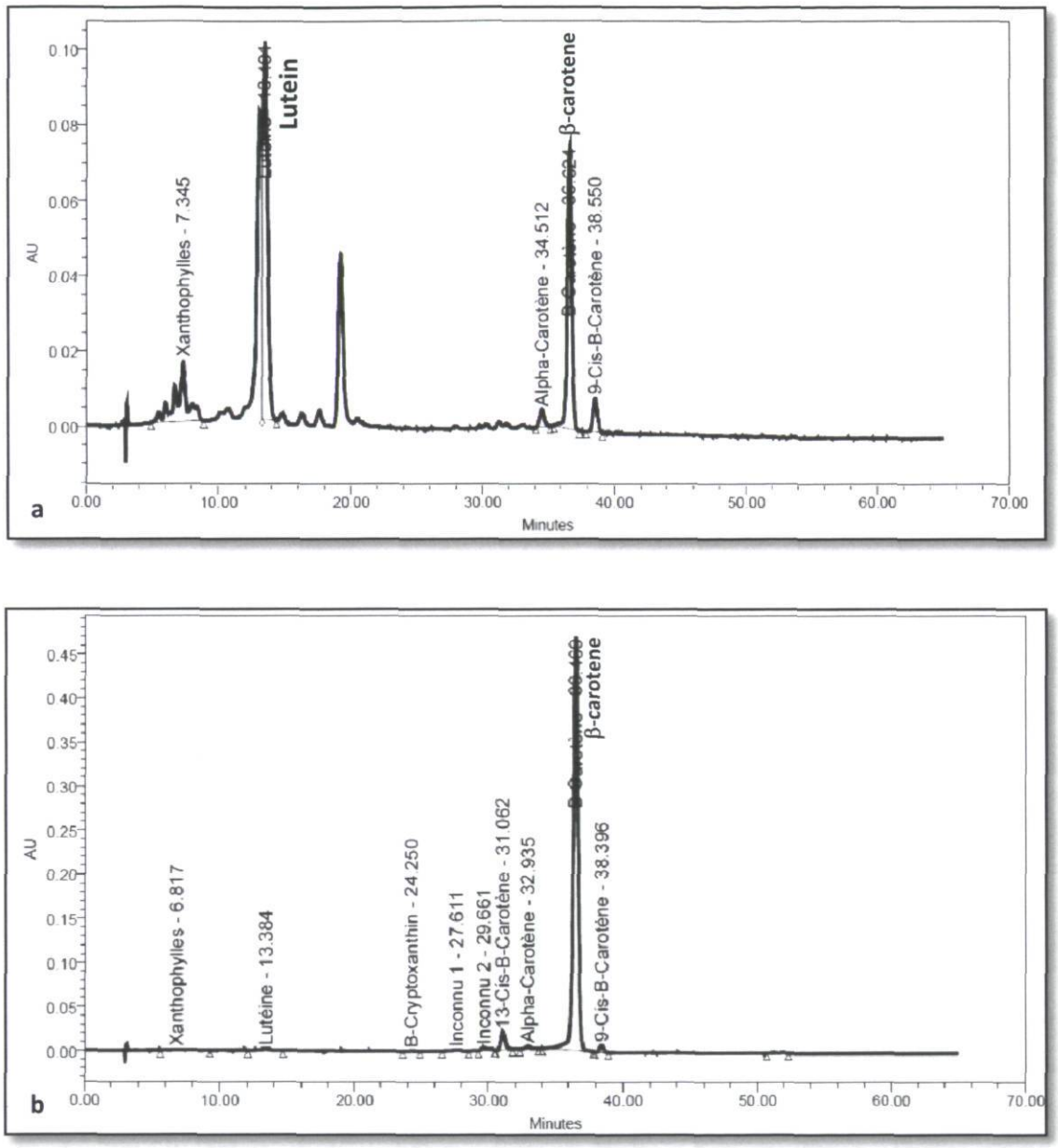


Figure 5.3. HPLC profile of carotenoids in the a) peel, b) flesh and c) seed extracts separated on a C_{30} column.

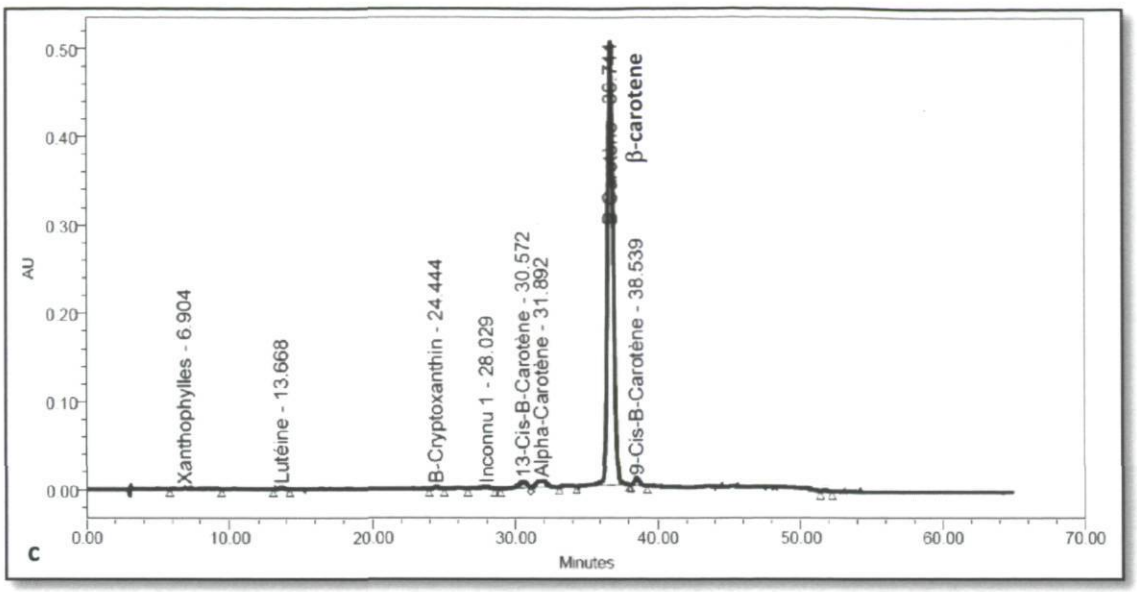


Figure 5.3. HPLC profile of carotenoids in the a) peel, b) flesh and c) seed extracts separated on a C_{30} column, continued.

Although *cis* isomers of β-carotene (9-*Cis*-β-carotene and 13-*Cis*-β-carotene) are present in the extracts, there is a probability that they may not be present in the fresh cantaloupe and are formed during sample preparation and storage. As explained in chapter 2, analysis of carotenoids is complicated by their diversity, instability, complexity and the presence of *cis* and *trans* isomers with their diverse spectrum of polarities. In nature, carotenoids are predominantly present in the *all-trans* configuration (Rodriguez-Amaya, 2001). However, the presence of 9-*cis*- and 13-*cis*-β-carotenes has been reported in raw and processed fruits and vegetables (Chandler and Schwartz, 1987; Forrest, 1987). Other authors have also reported that *cis* isomers exist in some plants, however they can also be formed during isolation procedures, storage and processing (Gortner and Singleton, 1961). Under these conditions, especially during thermal processing of foods, *trans* double bonds are susceptible to conformational isomerization, because of which some bonds take on a *cis* configuration (Chandler and Schwartz, 1987). However the approach used here, making use of an HPLC C_{30} column, was able to optimally separate a number of carotenoids with negligible sample preparation.

Lessin (Lessin, 1997) separated and quantitatively measured the geometric isomers of provitamin A carotenoids in fresh and processed fruits and vegetables, using a C_{30}

stationary phase for reversed phase HPLC. The isomers of *β*-Carotene, α-carotene and *β*-cryptoxanthin were determined isocratically. As shown in table 5.6, for most of the samples, the percentage of *all-trans* isomer was lower in the processed samples than that in the fresh samples. Thermal processing has caused *trans* to *cis* isomerization, which resulted in this change in isomeric composition. In the case of cantaloupe, no isomer of β-carotene, α-carotene and β-cryptoxanthin, except all-trans, were detected in fresh.

Table 5.6. Quantitative distribution of β-carotene, α-carotene, and β-cryptoxanthin isomers in fresh and processed fruits and vegetables. Concentrations are in μg/g of dry weight tissue (adapted from Lessin, 1997).

	β-carotene						*α-carotene*						*β-cryptoxanthin*				
Extract	*All trans*	*9-cis*	*13-cis*	*15 cis*	*Other cis*	*total*	*All-trans*	*9-cis*	*13-cis*	*13' cis*	*Other cis*	*Total*	*All-trans*	*13/13'-Cis*	*15-cis*	*total*	*total*
Broccoli																	
Fresh	29.2	5.0	3.3	1.9	2.0	41.4											41.4
Boiled	36.5	6.9	4.2	2.2	2.2	52.0											52.0
Cantaloupe																	
Fresh	162.8					162.8	0.9					0.9					163.7
Carrot																	
Fresh	534.4					534.4	372.7					372.7					907.1
Canned	420.4	32.7	90.5	30.4		574.0	290.9	6.1	91.0	55.9	37.1	481.0					1055.0
Peach																	
Fresh	2.2	0.3	0.5	tr		3.0							0.3	0.1	0.1	0.5	3.5
Canned	0.9	0.2	0.4	tr		1.5							0.2	0.1	tr	0.3	1.8
Spinach																	
Fresh	311.9	38.6	24.5	tr	22.5	397.5											397.5
Canned	309.8	96.9	28.6	14.9	22.9	473.1											473.1
Tomato																	
Fresh	71.0	4.8	5.8			81.6											81.6
Canned	49.1	5.5	12.0	4.8		71.4											71.4
Juice	40.0	4.5	10.1	4.8		59.4											59.4
Orange juice																	
Fresh	2.2	tr	0.4	tr		2.6	1.9	Tr	0.2	0.1		2.2	2.5	0.2	tr	2.7	7.5
Pasteurized	1.5	tr	0.3	tr		1.8	1.3	Tr	0.1	0.1		1.5	1.3	0.2	tr	1.5	4.8

tr: Trace

As a reference, carrots contain 0.066 mg/g fresh weight of *β*-carotene (Adams, 1975), as shown in table 5.7. The measured amount of *β*-carotene in table 5.4 is consistent with the given value in table 5.7. Among the commonly eaten fruits, orange-fleshed muskmelon is one of the richest source of β-carotene.

Table 5.7: Reported amount of β-carotene in some fruits (adapted from Adams, 1975).

Fruit	*β-carotene (mg/g fresh matter)*
Apple (Malus sylvestris Mill.)	0.0002
Apricot (Prunis armeniaca L.)	0.0162
Banana (Musa sp.)	0.0022
Grapes (Vitis vinifera L.)	0.0006
Mango (Mangifera indica L.)	0.0288
Muskmelon (Cucumis melo L. var. Reticulates Naud.)	0.0204
Nectarine (Prunis persica (L.) Batsch.)	0.0099
Oranges (Citrus sinenis (L.) Osb.)	0.0012
Peach (Prunis persica (L.) Batsch.)	0.008

Laur and Tian (Laur and Tian, 2011) used acetone:ethyl acetate with the ratio of 2:1 (v/v) as solvent and determined the amounts of lutein and β-carotene in edible portion of selected melons, by HPLC. β-carotene content varied from 3861.1 μg/100 g FW to 2447.9 μg/100 g FW, as shown in table 5.8. They also compared their results with the data reported in the USDA-ARS database (USDA-ARS, 2009). According to this database, the amount of β-carotene and lutein in cantaloupe is 2020±252.9 μg/100 g FW and 26±9.9 μg/100 g FW, respectively, which is in agreement with the results of Laur and Tian.

Table 5.8. β-Carotene content, the corresponding vitamin A activity, and lutein content in selected melons (adapted from Laur and Tian, 2011).

Variety (Origin)	*β-Crotene (μg/100 g FW)*	*Vitamin A (IU/100 g FW)*	*Vitamin A (μg RAE/100 g FW)*	*Lutein (μg/100 g FW)*
Cantaloupe				
Oro Rico (CA, USA)	3138±228.1[a]	5230±380.2[a]	261.5±19.0[a]	12.7±3.4[a]
Durango (CA, USA)	2448±291.8[b]	4080±486.3[b]	204.0±24.3[b]	17.3±6.6[a,d]
Caribbean Gold (Honduras)	3633±322.7[c]	6055±537.9[c]	302.7±26.9[c]	7.2±2.8[b,e]
Cantaloupe unknown variety (Guatemala)	3861±559.7[c]	6435±932.8[c]	321.8±46.6[c]	13.5±6[a]

Curl (1966) reported that β-carotene is the predominant carotenoid in orange-fleshed muskmelons. The total carotenoid (as β-carotene) in the edible portion of the melon was 0.02 mg/g FW. Vavich and Kemmerer used ethyl alcohol and petroleum ether as solvents

and measured the carotene content by spectrophotometer. They found that the carotene content of edible portion of cantaloupe is from 18 to 28 μg/g FW, at least 94% of the carotene was in the form of β-carotene (Vavich and Kemmerer, 1950).

Bureau and Bushway (1986), quantified the major vitamin A active compounds and total vitamin A activity of cantaloupe and carrot by using THF as solvent and HPLC which are revealed in table 5.9.

Table 5.9. Vitamin A activity from each of the provitamins analyzed (adapted from Bureau and Bushway, 1986).

	α-carotene in fresh matter (mg/g)	*β-carotene in fresh matter (mg/g)*	*β-cryptoxanthin in fresh matter (mg/g)*	*Total vitamin A activity (IU/100g)*
Cantaloupe	8.99×10^{-5}	0.016	5.85×10^{-5}	2750
Carrots	0.0379	0.076	0.00	15475

Lester (2008) determined the concentration of β-carotene in different tissues of mature orange-fleshed honeydew melon (*Cucumis melo* L.) using ice cold heptane as solvent. He determined that there is a gradient for β-carotene concentration. For both dry and fresh weight bases, β-carotene increased from the subpeel mesocarp tissues toward the inner tissues (seed cavity), as shown in table 5.10. High concentration of β-carotene in the inner tissues is due to chromophore conversion in ripening tissues. Table 5.10 shows the concentration of β-carotene in different mesocarp tissues of melon.

Table 5.10. β-carotene concentration gradients in different mesocarp tissues of mature orange-fleshed honeydew melon fruit, determined by HPLC (adapted from Lester, 2008).

Mesocarp tissue	*β-carotene in dry powder (mg/ g)*	*β-carotene in fresh matter (mg/ g)*
Hypodermal	0.18	0.009
Outer	0.27	0.016
Middle	0.22	0.023
Inner	0.21	0.027

Lester and Hodges (2008) determined the concentration of β-carotene of five different genotypes of cantaloupe, in two different seasons, using ice cold heptanes as solvent. According to his results, orange delight and orange dew have the highest β-carotene concentration. Table 5.11 shows the concentration of β-carotene in different genotypes of melon, determined by HPLC.

Table 5.11. Comparison of β-carotene levels in orange-fleshed cantaloupes (adapted from Lester and Hodges, 2008).

Genotypes	***β-carotene in fresh matter of flesh (mg/g)***	***β-carotene in fresh matter of flesh (mg/g)***
	Autumn	***Spring***
Honey Gold	0.013	0.010
Orange Delight	0.019	0.013
Orange Dew	0.018	0.016
SVR-03935152	0.008	0.011
Temptation	0.012	0.010

The measured total carotenoid in table 5.4 is in agreement with most of the references cited above. It should be noted that in some studies data is reported on a fresh weight basis while in other studies is reported on a dry weight basis with no moisture information included, thus making comparisons of individual studies difficult. In addition, the analyzed parts of the cantaloupe are not the same in references and are not clear in some cases.

There are several factors that can be attributed to these differences. The variety, length of storage after harvesting, type of storage, the packaging and handling during shipment, whether the product was mechanically or hand harvested, the cultivars and the method of analysis are the most important factors. The method of analysis, can create a lot of variations in the results, but can be controlled by a collaborative study to develop a standard method which could be chosen for all to use for obtaining nutritional values for food tables.

5.3. Extraction and characterization of polyphenols:

Double extraction of polyphenols was carried out by methanol and acetone as extraction solvent and spectrophotometer was used for determination of total polyphenol. The results

in table 5.12 show the concentration of total polyphenol (mg/g of dry powder) and the extraction yield in peel, flesh and seed for first and second extractions.

Table 5.12: Concentration of polyphenol in different parts of cantaloupe, determined by spectrophotometer.

Double extraction of polyphenol		*Polyphenol in dry powder (fresh matter) (mg/g)*	*Polyphenol in extract (mg/L)*	*Extraction yield (% W/W)*	*% Polyphenol in DM (dry matter)*
First extraction	Flesh	1.45 ± 0.08^{a} (0.14)	64.1 ± 9.3^{b}	78.5 ± 3.7^{a}	0.19 ± 0.01^{d}
First extraction	Peel	2.81 ± 0.10^{a} (0.29)	123.2 ± 7.0^{a}	33.6 ± 0.9^{b}	0.84 ± 0.03^{b}
First extraction	Seed	1.14 ± 0.02^{a} (0.31)	47.7 ± 2.5^{c}	31.0 ± 1.6^{b}	0.37 ± 0.01^{c}
Second extraction	Flesh	0.10 ± 0.04^{b} (0.01)	5.8 ± 2.8^{d}	17.3 ± 2.6^{c}	0.06 ± 0.02^{f}
Second extraction	Peel	0.93 ± 0.03^{a} (0.10)	51.2 ± 6.4^{c}	9.9 ± 0.4^{d}	0.94 ± 0.01^{a}
Second extraction	Seed	0.07 ± 0.01^{b} (0.02)	3.4 ± 0.2^{d}	5.2 ± 1.0^{e}	0.13 ± 0.01^{e}

Different letters within columns represent significant difference at $p < 0.05$, using LSD test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.
%Polyphenol in dry matter = (%polyphenol in dry powder) / (%extraction yield) × 100.

As indicated in table 5.12, in the first extraction, methanol could extract the majority of extractible polyphenol, while in the second extraction with acetone, the extracted amount of polyphenol from peel, flesh and seed was 33.1%, 6.9% and 6.1% of that for first extraction, respectively. The affinity of polyphenols with the solvent plays an essential role in their extraction. This affinity varies with the class of polyphenol. In contrast to the second extraction, statistical analysis (LSD test) shows that there is no significant difference between the amount of total polyphenol in the peel, flesh and seed after the first extraction.

The extraction yield from different parts of cantaloupe can be presented in the following order: flesh > peel > seed. Lower extraction yield for seed is may be due to the low solubility of its major components such as protein, fat and starch in methanol and acetone (Ismail et al., 2010).

Table 5.13 shows the sum of total polyphenol obtained by double extraction from peel, flesh and seed. The amount of total polyphenol in peel (3.73 mg/g dry powder) is significantly higher than that in flesh (1.55 mg/g dry powder) and seed (1.20 mg/g dry powder). This finding is in agreement with a previous study (Ismail, 2010) which reported a higher phenolic content in peel of cantaloupe than in flesh and seed, as shown in table

5.14. Probably, phenolic compounds accumulate in the epidermal tissue of plants due to their potential influence on the protection against UV radiation, acting as attractants to improve seeds dispersion, and as chemicals to defend against some pathogens and predators (Dixon and Paiva, 1995).

Table 5.13. Concentration of total polyphenol in different parts of cantaloupe, achieved after double extraction and determined by spectrophotometer.

Name	*Total polyphenol in dry powder (fresh matter) (mg/g)*	*Extraction yield (% W/W)*	*% Polyphenol in DM*
Flesh	1.55 ± 0.09^{b} (0.15)	95.8 ± 4.5^{a}	0.25 ± 0.01^{c}
Peel	3.74 ± 0.10^{a} (0.38)	43.5 ± 1.0^{b}	1.78 ± 0.03^{a}
Seed	1.21 ± 0.02^{c} (0.33)	36.2 ± 1.9^{b}	0.50 ± 0.01^{b}

Different letters within columns represent significant difference at $p < 0.05$, using LSD test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

Table 5.14. Extraction yield, total phenolic content and total flavonoid content of cantaloupe extracts (adapted from Ismail et al., 2010).

Part of cantaloupe	*Extraction yield (% W/W)*	*Total phenolic content (mg GAE/g extract)*	*Total flavonoid content (μg RE/g extract)*
Seed	13.66 ± 0.52^{a}	2.85 ± 0.21^{d}	1.62 ± 0.74^{d}
Flesh	89.62 ± 0.29^{b}	1.68 ± 0.14^{e}	2.03 ± 0.16^{d}
Leaf	16.29 ± 2.15^{c}	26.40 ± 0.34^{a}	69.70 ± 3.37^{a}
Skin	50.33 ± 4.35^{d}	4.70 ± 0.23^{c}	5.13 ± 1.32^{c}
Stem	23.78 ± 2.09^{e}	10.25 ± 0.40^{b}	9.68 ± 0.74^{b}

Wolfe et al (2008) determined the total phenolic content of edible portion of 25 commonly consumed fruits, using the Folin-Ciocalteu method. The total phenolic content of cantaloupe was 16.0±0.4 mg GAE/100 g FW, determined by spectrophotometer. This finding is in agreement with our results in which total phenolic content of flesh was 15 mg/100g FW.

Ultra performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS) was used in this study because of high resolution, short retention times and high sensitivity. The major polyphenol constituents separated from different parts of cantaloupe

and their quantitative distribution in the order of elution on a reversed phase column are shown in table 5.15. Eighty five components were shown to be present, which were separated and identified by comparing retention times and mass with commercial standard (gallic acid) and published literature. Different types of flavonoids and hydroxybenzoic acids were identified. One classe of phytoalexin was found in cantaloupe, for which the trivial generic name of viniferin was proposed. Among these polyphenols, viniferin and conidendrin were the main components. The quantity of viniferin (26.49 μg/g dry powder in flesh), conidendrin (23.01 μg/g dry powder in peel), ellagic acid acetyl-xyloside (12.27 μg/g dry powder in flesh), p-coumaroyl glucose (7.79 μg/g dry powder in peel) and cyanidin 3-O-galactoside (7.40 μg/g, dry powder in peel) are significantly higher than other components. It should be noted that these are preliminary findings and the presence of viniferin and conidendrin should be verified by other methods such as nuclear magnetic resonance spectroscopy (NMR). The values are approximative since the analysis method used is semi-quantitative. However they still provide a range for the values. According to this table, the amount of total polyphenol in peel (0.12 mg/g dry powder) is higher than that in flesh (0.06 mg/g dry powder) and seed (0.02 mg/g dry powder).

Table 5.15. Concentration of different types of polyphenol in peel, flesh and seed of cantaloupe, determined by UPLC-MS/MS.

No.	*Polyphenol*	*Peel (µg/100 g)*	*Flesh (µg/100 g)*	*Seed (µg/100g)*
1	4-Hydroxycoumarin	251.98±27.45	12.39±1.83	32.63±8.80
2	4-Hydroxybenzoic acid 4-O-glucoside	436.96±39.26	164.65±20.67	11.99±4.32
3	Hydroxybenzoylhexose	436.96±39.26	155.37±14.24	11.99±4.32
4	Protocatechuic acid	40.07±29.17	12.84±1.40	56.21±10.20
5	Galloyl-HHDP-glucose	5.45±1.37	7.79±1.45	6.46±1.11
6	m-Coumaric acid	67.99±5.27	0.28±0.04	10.35±4.03
7	Feruloyl tartaric acid	50.82±44.51	31.01±8.12	58.56±3.90
8	Apigenin 7-O-(6"-malonyl-apios yl-glucoside)	ND	5.25±1.14	0.75
9	1-caffeoylquinic acid (Pseudochlorogenic acid)	68.15±37.83	40.39±4.68	118.20±26.62
10	Caffeoyl glucose	209.02±35.56	110.50	ND
11	Dimere B (1)	35.55±4.05	3.10	17.91±8.12
12	Ferulic acid 4-O-glucoside	521.47±77.55	12.55±8.25	17.44±3.74
13	Cyanidin 3-O-galactoside	739.67±98.58	4.83±1.20	39.06±15.55
14	Caffeoyl glucose	79.37±5.26	ND	46.58±4.12
15	(+)-Catechin 3-O-gallate	ND	ND	6.58±1.82
16	Feruloyl glucose	521.47±77.55	14.12±0.07	6.41±0.22
17	5-Caffeoylquinic acid (Chlorogenic acid)	24.19±1.45	ND	0.06±0.002
18	o-coumaric acid	443.57±0.41	25.68	ND
19	p-coumaric acid 4-O-glucoside	282.27±22.30	10.73±2.70	0.53
20	2,4-hydroxybenzoic acid	28.59±2.44	5.10±0.14	7.45±0.98
21	p-coumaroyl glucose	779.17±37.20	9.67±4.20	ND
22	Dimere B (2)	6.72±0.09	16.38±4.67	73.39±1.12
23	Malvidin 3-O-arabinoside	19.32±4.04	6.21±1.23	18.14±2.74
24	p-Coumaroyl glucose	647.83±54.84	5.53±3.02	ND
25	Conidendrin	2301.05±211.64	23.48±3.94	47.02±12.65
26	Dimere B (3)	ND	ND	11.96±2.91
27	Catechin	356.40±68.63	21.50	2.70±0.10
28	Ferruloyl glucose	521.47±77.55	15.82±5.42	17.44±3.74
29	4-p-Coumaroylquinic acid	18.51±5.50	5.29±2.01	12.19±1.87
30	(-)-Epicatechin	17.43	43.47±4.80	2.72±0.81
31	Peonidin 3-O glucoside	26.93±3.42	17.34±1.84	5.41±0.50
32	(-)-Epicatechin 3-O-gallate	12.35±5.14	ND	ND
33	Myricetin	13.55±3.26	7.07±1.46	174.46±46.52
34	Sesamolinol	16.63±8.80	2.64±0.62	18.03±5.95
35	Peonidin 3-O-glucoside	26.93±3.42	17.34±1.84	5.41±0.50
36	5-p-coumaroylquinic acid	8.57±1.25	11.33±6.12	12.77
37	Quercetin 3-O-glucosyl-xyloside	1.09±0.19	2.83±0.04	5.58±0.77

Table 5.15. Concentration of different types of polyphenol in peel, flesh and seed of cantaloupe, determined by UPLC-MS/MS, continued.

No.	*Polyphenol*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Seed (μg/100 g)*
38	Trimere A (5)	2.89±0.29	0.06	6.24±1.35
39	Trimere A (6)	2.27±1.16	0.33	6.24±1.35
40	Myricetin 3-O-galactoside	8.67±1.43	1.18	8.90±2.24
41	p-Coumaric acid	293.83±4.64	75.95±15.34	133.73±20.99
42	3,4-DHPEA-EDA	10.26±1.43	0.68±0.13	3.71
43	p-coumaroyl glycolic acid	82.84±2.13	23.55±5.34	35.51±3.36
44	Xanthohumol	7.14	0.15±0.08	1.17±0.28
45	5-heneicosylresorcinol	25.81±5.81	4.03±0.09	3.74±1.32
46	Dihydroquercetin 3-O-rhamnoside	29.36±7.88	0.35	7.81±0.41
47	(-)-Epicatechin 3-O-gallate	74.03±11.51	ND	1.99±0.07
48	Sinapic acid	11.61±7.79	36.84±2.76	67.50±6.30
49	Quercetin 3-O-galactoside 7-O-rhamnoside	9.62±7.45	1.15±1.07	9.41
50	Myricetin 3-O-arabinoside	11.29±4.72	0.35	7.81±0.41
51	Kaempferol 3-O-xylosyl-glucoside	21.80±7.08	9.40±0.39	7.03±3.32
52	Ellagic acid	11.41±8.32	0.87	1.13
53	Quercetin 3-B-galactoside	7.12±0.53	12.00±1.04	9.64±1.18
54	Quercetin 3-O-glucuronide	179.42±40.18	4.17±0.57	5.43±3.98
55	Dimere A	60.02±14.96	21.19±2.25	29.91±6.10
56	Benzoic acid	4.53±1.16	1.53±0.11	6.51±0.32
57	Cyanidin 3-O-(6"-succinyl-glucoside)	14.19±0.63	4.73	11.13±1.93
58	Peonidin 3-O-(6"-malonyl-glucoside)	14.01±0.23	8.02±0.31	1.10±0.31
59	Naringenin 7-O-glucoside	40.79±0.11	0.12	3.54±0.59
60	Ellagitannin B	0.16	10.53±1.56	ND
61	Quercetin 3-O-glucoside	25.27±10.14	9.91±5.72	11.16±8.64
62	3-Hydroxyphloretin 2'-O-glucoside	7.93	1.92±0.58	3.40±0.97
63	Ac. Ellagic pentoside	4.98±1.72	1.62±0.23	2.92±0.29
64	Quercetin 3-O-arabinoside	12.21±0.009	5.75±0.16	2.22±0.70
65	Phloretin 2'-O-xylosyl-glucoside	6.25±0.97	25.20±0.27	17.78±1.76
66	Ellagic ac. Deoxyhexoside	81.34	6.13±0.03	4.20±0.23
67	Kaempferol 3-O-glucuronide	4.53±2.63	1.37±0.61	4.43±1.38
68	Kaempferol 3-O-glucoside	81.34	6.13±0.03	4.30±0.64
69	Myricetin 3-O-rhamnoside	26.50±8.41	9.91±5.72	11.16±8.64
70	3-Hydroxybenzoic acid	92.21±11.79	13.81±0.75	24.60±2.25
71	Phloretin	4.25±1.13	3.08	31.64±1.82
72	Isorhamnetin 4'-O-glucoside	246.71±32.16	21.58±2.06	49.70±4.34
73	Kaempferol 3-O-rhamnoside	13.66±0.46	5.31±0.83	2.49±0.34
74	Ellagitannin E	1.00	ND	48.71±8.06
75	Kaempferol 3-O-galactoside	48.09±2.48	37.22±2.90	2.51±0.42

Table 5.15. Concentration of different types of polyphenol in peel, flesh and seed of cantaloupe, determined by UPLC-MS/MS, continued.

No.	*Polyphenol*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Seed (μg/100 g)*
76	(+)-Gallocatechin	288.40±23.34	1.19±0.13	8.37±0.44
77	Dimere B (4)	253.70±26.44	5.41±3.20	5.97±0.49
78	Apigenin 7-O-glucuronide	40.85±4.04	26.00±2.38	26.37±2.46
79	Dimere B (5)	12.12±3.74	4.56±0.32	68.23±6.70
80	Quercetin 3-O-(6"-malonyl-glucoside)	24.48±3.34	7.99±1.94	20.92±0.13
81	Rhamnetin	28.82	ND	ND
82	Ellagic acid acetyl-arabinoside	29.30±7.72	318.70±13.08	13.69±1.80
83	Ellagic acid acetyl-xyloside	41.68±12.92	1227.36±72.49	58.35±8.39
84	Resveratrol	106.44±1.76	105.69±5.44	115.15±4.37
85	Viniferin	230.44±90.59	2649.06±135.00	269.49±24.06
	Sum (μg/100 g)	11653.07±324.39	5534.56±192.99	2033.32±72.41

ND: Not detected.

Resveratrol (3, 5, 4′-trihydroxystilbene) is a phytoalexin synthesized by grapevine leaf tissue following fungal infection and UV light irradiation (Langcake and Pryce, 1977). Resveratrol is responsible for some beneficial effects such as prevention of cardiovascular diseases (Constant, 1997). It has antioxidant and anticoagulant properties and inhibits the events associated with tumor initiation, promotion and progression (Jang et al., 1997). Langcake and Pryce (1977) have also identified oxidation products of resveratrol as ε-, α-, β-, and γ-viniferin, respectively, as a dimer, trimer, tetramer, and a more highly polymerized oligomer. δ-viniferin is an analogue of the resveratrol dehydrodimer, as a major resveratrol dimer with ε-viniferin, synthesized by P. Viticola-infected or UV-C-irradiated grapevine leaves (Pezet et al, 2003). Viniferin has beneficial effects, for instance, ε-viniferin (Figure 5.4) has a better antifungal activity and antioxidant capability than resveratrol (Piver et al., 2003).

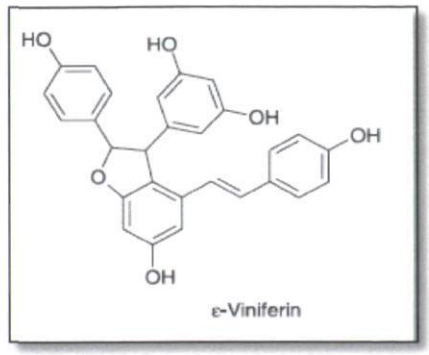


Figure 5.4. Structure of ε-viniferin (Piver et al., 2003).

Kolayli et al (2010), quantified seventeen different phenolic constituents in different varieties of melon by the Folin-Ciocalteau procedure and using spectrophotometer and reverse phase high-performance liquid chromatography (RP-HPLC). The amounts of total polyphenol are presented in table 5.16. The amounts of the phenolic acids as mg/100 g FW are presented in table 5.17.

Table 5.16. Total polyphenol contents of the tested melons, determined by spectrophotometer (adapted from Kolayli et al., 2010).

Total polyphenol mg gallic acid/100 g fresh matter of melon	***Standard***	***Grafted***	***Hybrid***
	115 ± 5.10	92.54 ± 4.00	96.00 ± 4.00

Table 5.17. Phenolic constituents of melon types determined by reverse phase high-performance liquid chromatography (mg /100 g of fresh matter) (adapted from Kolayli et al., 2010).

Compound	***standard***	***grafted***	***Hybrid***
	Phenolic acids		
gallic acid	1.74	2.23	1.34
protocatechuic acid	ND	ND	ND
p-hydroxybenzoic acid	0.72	ND	ND
chlorogenic acid	0.90	2.29	1.60
vanillic acid	7.24	6.36	7.83
syringic acid	0.52	ND	ND
caffeic acid	2.04	1.37	1.47
p-coumaric acid	3.24	4.16	3.07
ferulic acid	3.69	3.72	2.91
benzoic acid	30.06	5.55	9.06
o-coumaric acid	1.22	ND	ND
abscisic acid	15.39	11.71	8.35
t-cinnamic acid	2.99	5.72	6.85
	Flavanoids		
Quercetin	ND	2.26	ND
Catechin	ND	ND	ND
Epicatechin	3.71	ND	ND
Rutin	ND	12.73	10.75

The amount of phenolic acids of aqueous extracts of the melons varied widely from 0.5 to 30 mg/100 g FW. Table 5.17 shows that abscisic, benzoic, vanillic and trans-cinnamic acids were the major phenolic component in all samples. A small amount of P-coumaric, ferulic and gallic acids were found, while protocatechuic acid and cathecin were not detected in any of the melons. Among the melon varieties, only standard melon contained P-hydroxybenzoic acid, syringic acid, o-coumaric acid, and epicatechin, but in very low concentrations. The highest phenolic acid variability and content were found in standard melon. Quercetin was found only in grafted melon. Grafted and hybrid types had a high concentration of rutin. (Kolayli et al., 2010).

5.4. Optimization of carotenoid extraction parameters using ethanol as solvent:

The total carotenoids (TC) extraction from flesh and peel of cantaloupe was investigated to find the optimum extraction parameters. Solvent extraction method and UV-visible spectrophotometer were used for extraction and carotenoid determination, respectively. Extraction duration and extraction temperature are assumed to be the most important factors affecting solvent extraction for determination of TC. Four extraction temperatures of 25°C, 30°C, 40°C and 50°C and six extraction time of 0 min, 30 min, 1h, 2h, 3h and 6h were employed to choose the most suitable temperature and time for extraction of TC from peel and flesh. The results are shown in Figures 5.5 to 5.8. More details are provided in Appendix I. Two repeated measure analysis of variance were performed for the β-carotene and lutein data. The effect of time, temperature and type (peel/flesh) as well as their interactions were studied. According to statistical analysis, the effect of time ($P< 0.0001$), temperature ($P< 0.0001$), type ($P< 0.0001$), and the interaction of type × temperature ($P=0.0009$ for β-carotene and $P=0.0005$ for lutein), type × time ($P< 0.0001$), temperature × time ($P< 0.0001$) and type × temperature × time ($P< 0.0001$) were highly significant. More details on the interaction effects are provided in Appendix II.

Efficiency of ethanol for extraction of carotenoid was determined, using the following equation (Cacace and Mazza, 2003):

$$F(\%) = \frac{C_{eq}}{C_{fb}} \times 100 \qquad (5.1)$$

Where C_{eq} is the content of carotenoid extracted by ethanol 95% and C_{fb} is the content of carotenoid extracted by THF, both in mg/g of dry powder.

Figures 5.5.a and 5.5.b show, respectively, the concentration curves for total carotenoids as β-carotene and lutein in the flesh for different extraction times and temperatures.

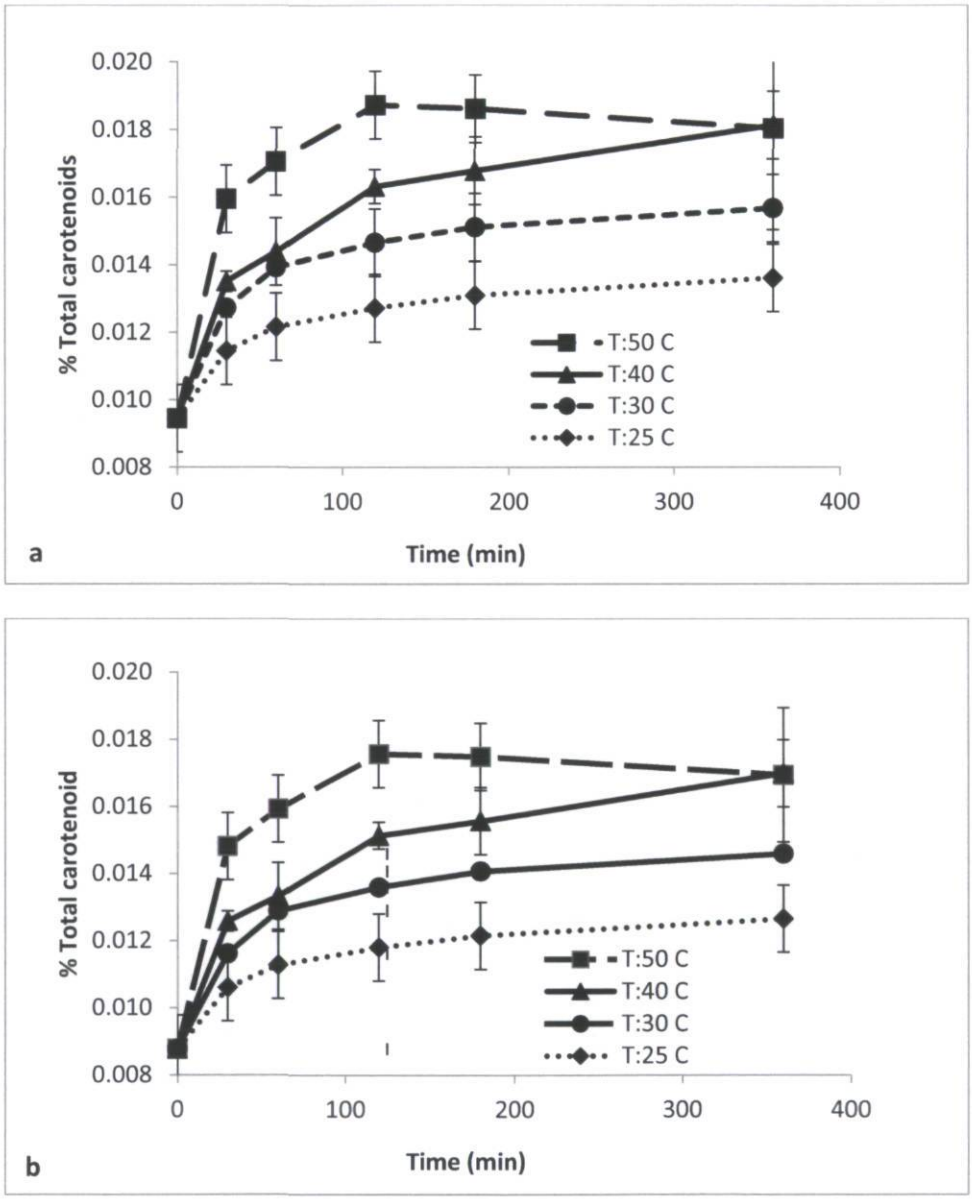


Figure 5.5. Concentration of total carotenoids as a) β-carotene and b) lutein in flesh for different extraction times and temperatures.

Figures 5.6.a and 5.6.b show the efficiency of ethanol for extraction of total carotenoids as β-carotene and lutein, respectively, in the flesh for different extraction times and temperatures.

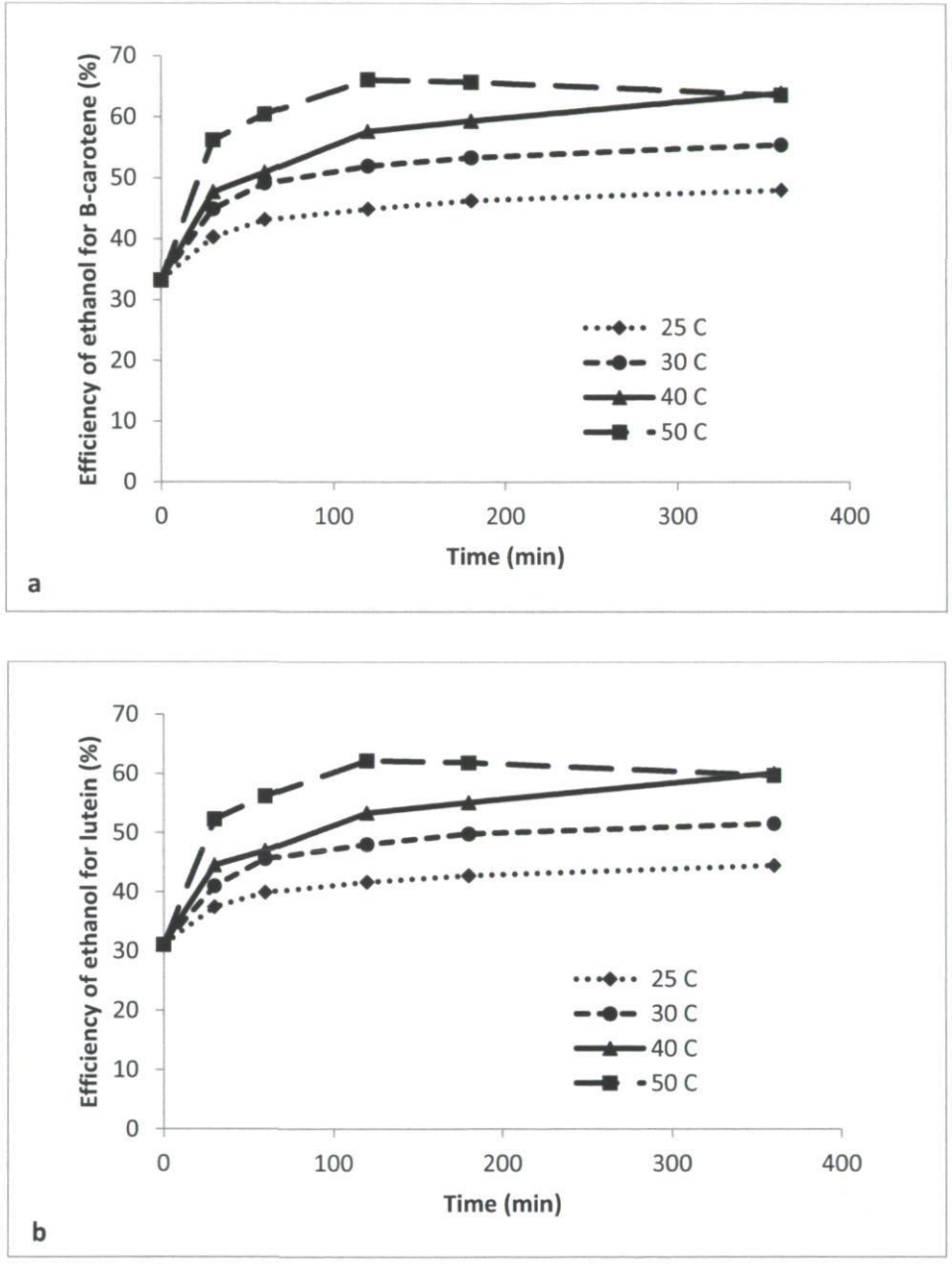


Figure 5.6. The efficiency of ethanol for extraction of total carotenoids as a) β-carotene and b) lutein in flesh for different extraction times and temperatures.

According to the Figures 5.5 and 5.6, it can be concluded that temperature has a significant effect on the extraction of carotenoids. For a constant extraction time of 120 minutes, the

efficiency of ethanol increased by 20% with increasing the temperature from 25°C to 50°C. For all extraction times, the amount of extracted carotenoids and the efficiency of ethanol were higher at 50°C compared to 25°C, 30°C and 40°C. Figure 5.5 show that at higher temperatures, shorter period is required to obtain a given concentration of carotenoids. This confirms that heat promotes the extraction of solutes. Increasing in carotenoid concentration in heated extract could be possibly due to an increase in its availability resulted from changes in the lipophilic membranes. Considering that carotenoids are enlosed within cells and the cell membrane is composed of a complex composition, temperature may facilitate the breakage of cell walls by ethanol (Calvo et al. 2007). As explained in chapter 2, the heat, below the degradation temperature, facilitates the extraction of solute by increasing the permeability of cell walls, the solubility of solutes and the diffusion coefficients, and also by decreasing the viscosity of the solvent. It should be noted that in addition to changes in temperature, mechanical stirring was also performed which contributed to the transfer in the solution. Mechanical stirring results in continued suspension of particles in the solvent and homogenization of the medium, and has a positive effect on extraction yield. It can reduce resistance to the transfer of solutes at the solid-liquid interface (boundary layer) and can increase the transfer coefficient.

Results indicate that for a constant extraction temperature, the carotenoid concentration increases slightly with extraction time. However, for the temperature of 50°C, after duration time of 2 hours the amounts of total carotenoids (both lutein and β-carotene) does not change significantly which may be due to isomerisation and degradation of carotenoids at high temperature and long time (Calvo et al. 2007; Rodriguez-Amaya, 2001).

Table 5.18 shows the percentage of carotenoids in dry matter (DM), the extraction yield and efficiency of ethanol for extraction of total carotenoids after 6 hours at different temperatures, in flesh. As shown in table 5.18, the efficiency of ethanol for carotenoid and the percentage of carotenoids in dry matter had the highest values at 40°C. However, it should be noted that the values in this table were calculated after 6 hours. Increasing the temperature from 25°C to 50°C, increased extraction yield. It may be an indicator that between 40°C and 50°C, some constituents other than carotenoids were still dissolving in the solvent without degradation.

Table 5.18. Extraction yield and dry matter in flesh, after 6h at 4 different temperatures.

Time & temperature	*Lutein in dry powder (mg/g)*	*β-carotene in dry powder (mg/g)*	*Extraction yield (% W/W)*	*% Lutein in DM*	*% β-carotene in DM*	*Efficiency of ethanol for lutein (%)*	*Efficiency of ethanol for β-carotene (%)*
6h-25°C	0.126 ± 0.008^{b}	0.136 ± 0.008^{b}	32.7 ± 2.9^{c}	0.039 ± 0.003^{a}	0.042 ± 0.003^{a}	44.5	48.1
6h-30°C	0.146 ± 0.006^{ab}	0.157 ± 0.006^{ab}	38.9 ± 0.4^{bc}	0.038 ± 0.001^{a}	0.040 ± 0.001^{a}	51.6	55.5
6h-40°C	0.170 ± 0.007^{a}	0.181 ± 0.008^{a}	42.1 ± 5.5^{ab}	0.040 ± 0.006^{a}	0.043 ± 0.006^{a}	60.1	64.0
6h-50°C	0.169 ± 0.024^{a}	0.180 ± 0.026^{a}	45.6 ± 3.4^{a}	0.037 ± 0.008^{a}	0.039 ± 0.008^{a}	59.7	63.6

Different letters within columns represent significant difference at $p < 0.05$, using Bonferroni test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

According to table 5.18 and Figures 5.5 and 5.6, it can be concluded that temperature of 50°C and duration time of 2 hours are the best parameters for extraction of carotenoids from the flesh.

Figures 5.7.a and 5.7.b show, respectively, the concentration curves for total carotenoids as β-carotene and lutein in the peel for different extraction times and temperatures. Figures 5.8.a and 5.8.b show the efficiency of ethanol for extraction of total carotenoids as β-carotene and lutein, respectively, in the peel for different extraction times and temperatures. Figures 5.7 and 5.8 show that extraction temperature has a slight effect on the extraction of carotenoids in peel. The amount of extracted carotenoids and efficiency of ethanol for carotenoids had the highest values at 30°C for 2 hours. When the temperature was increased from 25°C to 30°C, the efficiency of ethanol after 120 minutes was increased 15%. Comparing the results of peel with those of flesh, it can be said that the extraction of carotenoids from peel needs lower temperature. It may be due to the presence of more pectin and sugar in the flesh than in the peel which makes the extraction more difficult by complexing the structure of tissues. In addition, the carotenoid mixture of flesh is more complex due to the presence of a number of *cis*-isomers and minor pigments (Gross et al., 1973). Ethanol also caused the precipitation of pectin (Faravash and Ashtiani, 2008) which results in the sedimentation of carotenoids. Due to these reasons, higher extraction temperatures may be required to extract the carotenoids form the flesh compared to the peel.

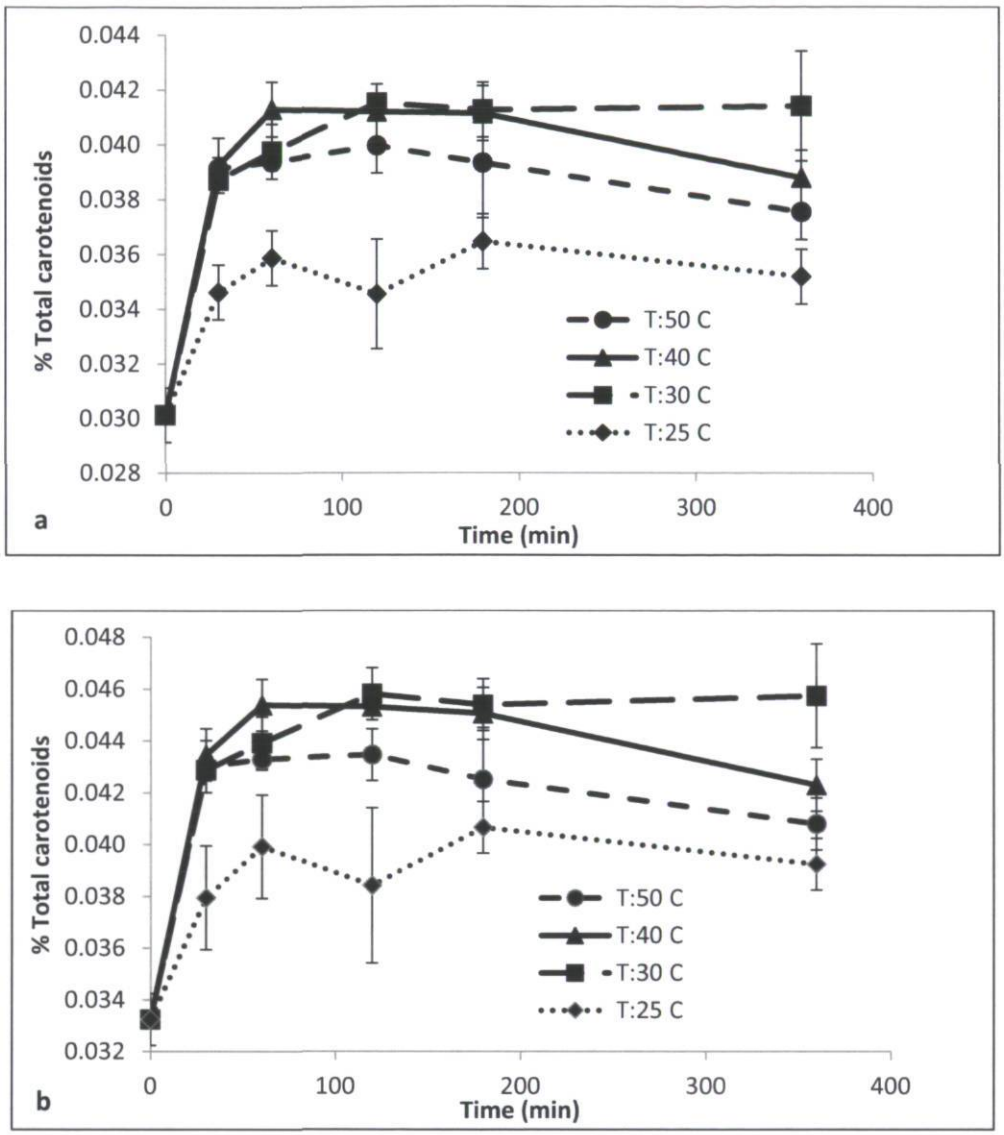
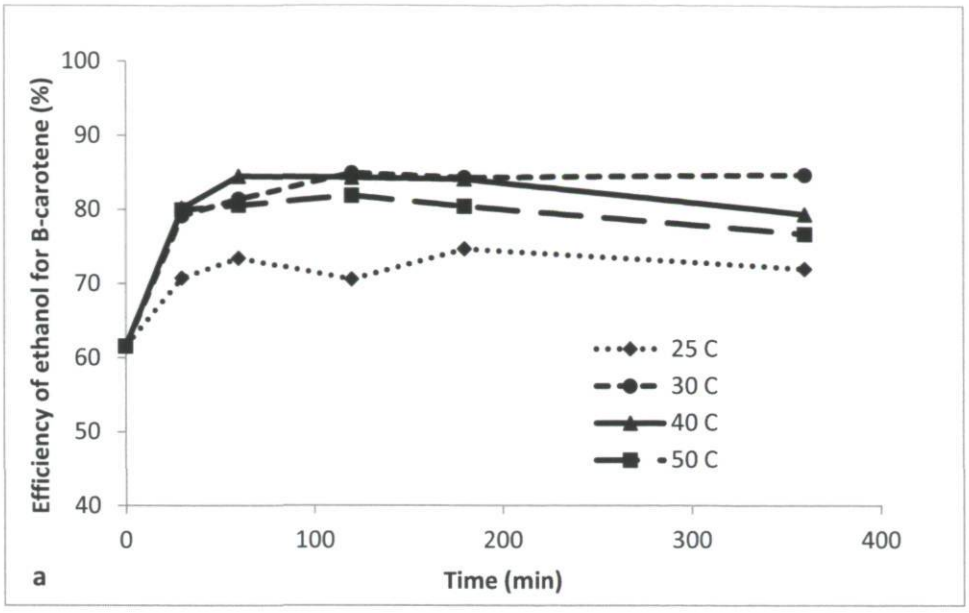


Figure 5.7. Concentration of total carotenoids as a) β-carotene and b) lutein in peel for different extraction times and temperatures.



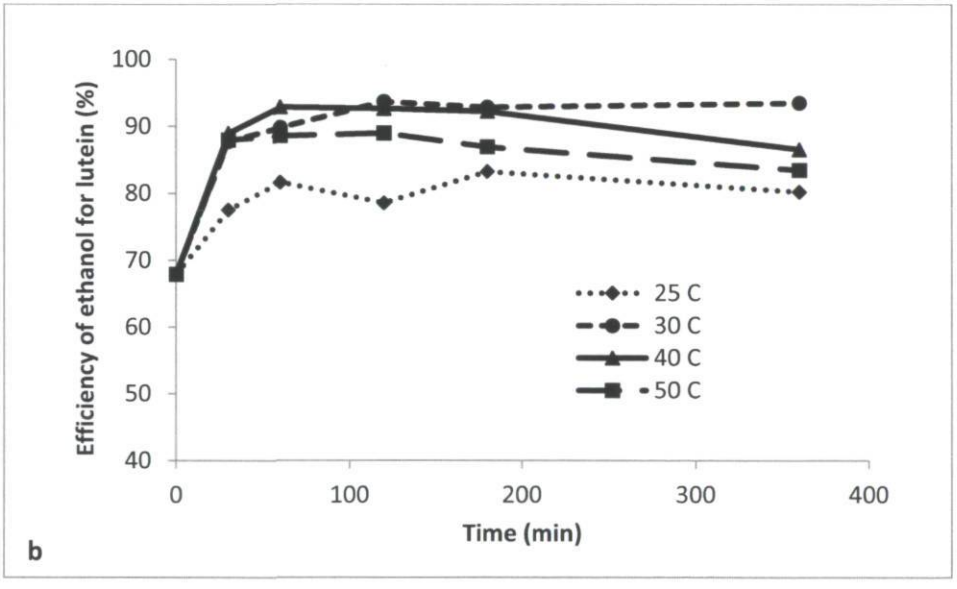


Figure 5.8. The efficiency of ethanol for extraction of total carotenoids as a) β-carotene and b) lutein in peel for different extraction times and temperatures.

Table 5.19 shows the extraction yield, percentage of carotenoids in dry matter and efficiency of ethanol for extraction of total carotenoids after 6 hours at different temperatures, in peel. According to table 5.19, the amount of total carotenoid and the percentage of carotenoid in dry matter have the highest values after 6 hours at 30°C. With increasing the temperature from 25°C to 50°C, the extraction yield increased.

Table 5.19. Extraction yield and dry matter in peel, after 6h at 4 different temperatures.

Time & temperature	*Total carotenoid as lutein in dry powder (mg/g)*	*Total carotenoid as β-carotene in dry powder (mg/g)*	*Extraction yield (% W/W)*	*% Lutein in DM*	*% β-carotene in DM*	*Efficiency of ethanol for lutein (%)*	*Efficiency of ethanol for β-carotene (%)*
6h-25°C	0.392±0.014^{a}	0.352±0.014^{b}	15.6±2.6^{c}	0.251±0.044^{a}	0.226±0.039^{a}	80.2	72.0
6h-30°C	0.457±0.018^{a}	0.414±0.016^{a}	17.7±0.4^{bc}	0.258±0.007^{a}	0.234±0.006^{a}	93.5	84.7
6h-40°C	0.423±0.015^{a}	0.388±0.013^{ab}	19.5±0.8^{ab}	0.217±0.013^{a}	0.199±0.012^{a}	86.5	79.4
6h-50°C	0.408±0.006^{a}	0.375±0.005^{ab}	20.9±0.9^{a}	0.195±0.007^{a}	0.179±0.006^{a}	83.4	76.7

Different letters within columns represent significant difference at $p < 0.05$, using Bonferroni test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

At a temperatures higher than 30°C, the concentration of total carotenoids starts to decline. According to Calvo et al. (2007), it may be due to the auto oxidation of β-carotene. These authors evaluated the extraction yield of food grade ethanol by extracting β-carotene from tomato peel powder for 5 to 40 minutes at varying temperatures (25 °C, 35 °C, 50 °C and 60 °C). According to their results, for all extraction times, the highest concentrations of β-carotene were obtained at 25°C and the lowest values were found at 60°C. In general the extraction times did not have a great influence on the β-carotene yield. The (*Z*)-isomers were not detected and they concluded that the increase in the extraction temperature favoured β-carotene auto-oxidation (Calvo et al. 2007).

It can be concluded that extraction temperature of 30°C and the duration time of 2 hours are the best parameters for extraction of carotenoids from the peel. However, it should be considered that for industrial extraction, the mixture of peel and flesh of cantaloupe will be used; therefore the same temperature should be used for the entire mixture. On the other hand, flesh has the highest proportion in a cantaloupe. Therefore, the optimum extraction parameters for flesh, temperature of 50°C and the duration time of 2 hours, were chosen as the optimum parameters for the extraction of carotenoids from the peel.

5.5. Optimization of ethanol/water ratio for extraction of carotenoids and polyphenols

Different ratios of ethanol/water were used to verify its effect on the extraction of carotenoids and polyphenols. The factorial design structure was 2×5. An analysis of variance for two factors (ethanol ratio and type) was performed for seven dependent

variables. The effect of type and ethanol ratio as well as their interactions was studied. According to statistical analysis for seven variables in tables 5.20 and 5.22, the effect of type ($P< 0.0001$), ethanol ratio ($P< 0.0001$) and type × ethanol ratio ($P< 0.0001$) were highly significant.

Table 5.20 shows the total amount of polyphenols, extraction yield and efficiency of solvent for extraction of polyphenol in peel and flesh. Table 5.20 shows that with decreasing the percentage of ethanol from 95% to 0%, the concentration of polyphenols and efficiency of solvent increased. According to statistical analysis, it can be concluded that polyphenols extracted from peel and flesh using water as solvent is significantly higher than those extracted by ethanol. Ethanol:water ratio of 0:100 is the best ratio that allows optimal extraction of polyphenols.

Table 5.20. Concentration of polyphenols extracted from peel and flesh with different ratios of ethanol/water, determined by spectrophotometer.

		Polyphenol in dry powder (mg/g)	***Extraction yield (%W/W)***	***% Polyphenol in DM***	***Efficiency of solvent for polyphenol (%)***
95%	Flesh	0.92 ± 0.02^{Bc}	49.5 ± 2.4^{Ad}	0.19 ± 0.01^{Bb}	59.4
70%	Flesh	1.25 ± 0.05^{Bb}	82.6 ± 1.2^{Ac}	0.15 ± 0.01^{Bc}	80.7
50%	Flesh	1.28 ± 0.05^{Bb}	96.3 ± 0.6^{Aa}	0.13 ± 0.01^{Bc}	82.6
30%	Flesh	1.30 ± 0.02^{Bb}	89.6 ± 1.6^{Ab}	0.15 ± 0.01^{Bc}	83.9
0%	Flesh	1.75 ± 0.07^{Ba}	83.4 ± 0.6^{Ac}	0.21 ± 0.01^{Ba}	112.9
95%	Peel	2.12 ± 0.16^{Ac}	21.3 ± 0.4^{Bc}	1.00 ± 0.06^{Aa}	56.8
70%	Peel	3.35 ± 0.10^{Ab}	34.6 ± 1.5^{Bb}	0.97 ± 0.07^{Aa}	89.8
50%	Peel	3.57 ± 0.11^{Ab}	47.7 ± 2.1^{Ba}	0.75 ± 0.02^{Ab}	95.7
30%	Peel	3.64 ± 0.17^{Aab}	48.5 ± 2.6^{Ba}	0.75 ± 0.06^{Ab}	97.6
0%	Peel	3.96 ± 0.03^{Aa}	45.0 ± 1.0^{Ba}	0.88 ± 0.02^{Aab}	106.2

For each type, different letters (a-c) within columns represent significant difference at $p < 0.05$, using Bonferroni test. For each percentage of ethanol, different uppercase letters (A-B) within columns represent significant difference at $p < 0.05$. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

Table 5.21 indicates that the highest concentration of viniferin was extracted with ethanol 70% from the flesh which is equal to 29.44 μg/g, while the highest concentration of conidendrin was extracted with water from the peel and it is equal to 5.68 μg/g. The values of this table are approximative since the analysis method used is semi-quantitative.

Table 5.21. Concentration of different types of polyphenol, extracted from peel and flesh with different ratios of ethanol/water, determined by UPLC.

Polyphenol	*Ethanol 95%*		*Ethanol 70%*		*Ethanol 50%*		*Ethanol 30%*		*Water 100%*	
	Peel (μg/100 g)	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*
4-Hydroxycoumarin	37.83±9.17	5.81±0.15	16.26±0.25	5.66±0.60	5.31±0.38	5.94±0.42	6.07±0.74	5.62±0.62	42.38±0.18	21.10±2.06
4-Hydroxybenzoic acid 4-O-glucoside	116.27±16.81	41.35±5.24	53.03±7.50	12.73±1.27	24.41±1.00	8.04±0.43	37.79±1.99	11.19±0.85	15.02±0.31	14.41±1.17
Hydroxybenzoylhexose	116.27±16.81	33.73±5.54	53.03±7.50	16.28±6.20	22.80±1.23	8.58±0.87	35.88±3.59	11.43±0.52	13.87±2.92	14.88±1.18
Protocatechuic acid	178.62±19.87	17.88±2.71	145.63±50.32	5.67±0.41	34.89±3.77	6.06±0.38	42.59±3.22	5.61±0.46	292.17±7.98	19.02±0.65
m-Coumaric acid	33.24±1.78	6.48±0.20	28.57±1.17	6.13±0.12	5.33±0.78	7.31±0.96	6.14±0.26	5.72±0.56	1.76±1.20	5.91±0.41
Feruloyl tartaric acid	26.32±1.42	9.92±0.40	15.71±0.19	5.60±0.56	5.61±0.21	5.60±0.60	5.46±0.45	5.52±0.41	33.22±2.25	26.84±0.04
1-caffeoylquinic acid (Pseudochlorogenic acid)	29.88±4.59	7.82±0.69	13.75±0.50	5.16±0.39	6.34±1.17	5.55±0.56	7.79±0.67	5.85±0.25	49.79±4.75	11.53±0.49
Caffeoyl glucose	14.93±6.73	6.35±0.40	30.97±7.15	6.83±0.70	16.43±0.24	5.87±0.53	16.80±2.30	5.78±0.63	146.17±24.46	8.12±0.20
Hydroxycinnamic acid	16.48±0.91	6.03±0.09	18.38±0.62	5.82±0.57	5.81±0.39	5.71±0.50	12.01±0.41	5.94±0.47	17.05±1.70	6.05±0.08
5-O-galloylquinic acid	5.72±0.64	5.59±0.53	6.29±0.31	5.62±0.74	5.56±0.71	11.69±1.61	5.14±0.37	5.60±0.51	13.27±1.42	5.58±0.60
Dimere B (1)	12.27±0.12	5.46±0.51	24.33±1.27	5.50±0.48	5.43±0.54	5.67±0.57	5.99±0.33	5.47±0.54	25.83±1.63	6.39±0.81
Ferulic acid 4-O-glucoside	108.73±3.83	5.80±0.45	174.09±0.06	5.67±0.64	32.57±2.69	5.59±0.64	32.57±2.69	5.59±0.64	33.40±3.34	5.91±0.49
Cyanidin 3-O-galactoside	92.01±8.55	10.44±0.43	142.28±5.89	7.26±0.03	64.05±5.69	6.97±0.28	103.57±5.78	8.36±0.37	141.08±1.56	10.43±1.02
Caffeoyl glucose	6.51±1.26	5.83±0.37	14.89±3.07	6.39±0.21	7.86±0.04	6.41±0.10	8.19±0.59	5.71±0.63	77.08±5.05	6.27±0.22
(+)-Catechin 3-O-gallate	6.57±1.31	5.85±0.17	5.82±0.59	5.85±0.56	6.40±0.52	6.24±0.84	7.81±0.22	5.58±0.17	6.65±0.66	5.76±0.61
Feruloyl glucose	108.73±3.83	5.49±0.25	179.73±8.76	5.90±0.41	31.95±3.61	6.02±0.63	33.18±3.66	5.79±0.45	157.07±2.56	6.09±0.60
5-Caffeoylquinic acid (Chlorogenic acid)	5.46±0.47	5.71±0.50	7.92±0.41	5.95±0.59	6.14±0.50	6.10±0.57	6.24±0.13	5.58±0.50	11.03	5.63±0.44
o-coumaric acid	119.55±13.99	5.71±0.20	71.16±7.80	7.30±0.61	12.26±10.65	7.31±0.06	6.74±0.08	5.66±0.52	171.40±13.14	6.67±0.87
p-coumaric acid 4-O-glucoside	183.11±8.06	7.01±1.31	137.29±8.81	5.52±0.61	115.86±30.55	5.71±0.40	108.04±2.06	5.76±0.63	6.32±5.51	6.90±0.50

Table 5.21. Concentration of different types of polyphenol, extracted from peel and flesh with different ratios of ethanol/water, determined by UPLC, continued.

Polyphenol	*Ethanol 95%*		*Ethanol 70%*		*Ethanol 50%*		*Ethanol 30%*		*Water 100%*	
	Peel (μg/100 g)	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*	*Peel (μg/100 g)*	*Flesh (μg/100 g)*
2,4-hydroxybenzoic acid	83.85±5.02	5.68±0.79	129.57±24.92	7.93±3.79	23.77±4.34	6.08±0.38	35.27±20.92	6.16±0.62	278.09±10.54	21.08±1.15
p-coumaroyl glucose	184.28±5.71	5.73±0.26	13.12±1.94	5.65±0.43	115.09±31.63	5.64±0.40	12.14±1.69	5.48±0.62	11.52±1.78	5.30±0.36
p-Coumaroyl glucose	45.27±3.75	6.12±0.39	33.68±2.03	5.58±0.62	28.20±3.40	5.79±0.56	35.54±0.60	6.24±0.01	12.52±0.77	6.24±0.47
Conidendrin	377.64±36.60	5.60±0.41	473.76±20.80	5.23±0.15	190.33±29.16	5.20±0.41	191.26±1.81	5.36±0.54	567.83±16.75	5.59±0.30
3,4-Dyhydroxyphenylacetic acid	26.87±1.20	10.07±2.18	111.12±10.76	5.62±0.54	17.31±2.71	5.42±0.53	18.43±1.51	5.46±0.47	51.76±2.53	6.13±0.35
Catechin	110.70±3.83	6.24±0.59	208.90±14.30	5.77±0.79	106.60±27.37	5.67±0.17	40.69±3.24	5.47±0.43	48.48±2.91	6.05±0.39
Ferruloyl glucose	108.73±0.04	5.68±0.24	174.68±3.47	5.88±0.45	32.19±0.24	6.02±0.63	23.80±7.70	6.00±0.42	157.07±2.56	6.62±0.20
4-p-Coumaroylquinic acid	17.29±2.31	9.33±0.23	25.50±2.46	5.82±0.59	12.15±1.02	5.54±0.35	13.92±0.81	5.62±0.32	33.09±0.88	12.66±0.29
(-)-Epicatechin	6.85±1.39	5.99±0.58	8.86±3.40	5.91±0.51	5.68±0.51	5.59±0.59	5.41±0.75	5.74±0.60	5.76±0.54	5.65±0.39
Sesamolinol	6.05±0.35	5.52±0.82	7.15±0.26	5.45±0.53	5.89±0.60	5.49±0.90	6.72±0.59	5.55±0.82	15.37±0.29	5.59±0.53
5-p-coumaroylquinic acid	5.91±0.47	5.73±0.58	7.59±0.54	5.57±0.48	5.73±0.59	5.77±0.33	6.73±0.79	5.22±0.29	6.93±1.18	6.08±0.41
Quercetin 3-O-glucosyl-xyloside	6.63±0.04	5.27±0.27	11.45±0.18	6.60±0.42	7.70±2.22	5.65±0.66	13.91±2.15	3.77±3.28	10.80±0.23	1.91
Myricetin 3-O-galactoside	17.11±1.54	9.99±0.51	27.60±0.38	11.65±1.20	26.32±1.97	11.77±1.03	19.40±0.33	10.22±0.32	21.86±1.44	11.78±0.59
p-Coumaric acid	42.05±1.64	7.28±0.35	148.14±0.79	6.97±0.48	65.08±4.97	6.52±0.22	66.95±5.48	8.24±0.74	34.18±1.20	6.74±0.50
p-coumaroyl glycolic acid	32.27±1.38	19.38±0.42	145.56±2.86	7.68±1.49	67.58±1.43	6.63±0.22	65.22±3.03	6.97±0.57	31.71±2.80	6.22±0.32
Xanthohumol	7.05±0.69	6.59±0.62	8.70±0.30	6.67±0.08	5.21±0.27	6.77±0.19	9.52±0.65	6.19±0.49	9.25±0.97	6.27±0.57
3-Hydroxybenzoic acid	24.10±14.22	9.39±0.84	37.62±8.77	11.21±0.93	15.80±1.17	9.80±0.82	19.27±0.74	13.60±3.20	72.35±5.70	7.18±1.47
Dimere B (4)	18.22±0.91	5.52±0.46	31.02±0.47	5.73±0.62	28.26±0.93	5.92±0.58	30.56±1.39	6.29±0.30	22.48±2.83	6.02±0.14

Table 5.21. Concentration of different types of polyphenol, extracted from peel and flesh with different ratios of ethanol/water, determined by UPLC, continued.

	Ethanol 95%		*Ethanol 70%*		*Ethanol 50%*		*Ethanol 30%*		*Water 100%*	
Polyphenol	***Peel (μg/100 g)***	***Flesh (μg /100g)***	***Peel (μg/100 g)***	***Flesh (μg/100 g)***	***Peel (μg/100 g)***	***Flesh (μg/100 g)***	***Peel (μg/100 g)***	***Flesh (μg/100 g)***	***Peel (μg/100 g)***	***Flesh (μg/100 g)***
Dimere B (5)	7.22±0.12	5.39±0.18	36.10±3.37	5.64±0.44	47.95±5.20	5.56±0.40	41.23±1.99	5.44±0.47	33.96±3.28	5.69±0.50
Ellagic acid acetyl-arabinoside	5.48±0.51	159.00±12.81	6.30±1.23	93.75±1.57	40.55±3.55	75.00±3.28	5.07±0.30	16.21±1.44	5.38±0.54	5.45±0.52
Ellagic acid acetyl-xyloside	5.43±0.53	262.73±9.53	5.61±0.92	586.00±33.81	30.83±2.02	597.24±32.71	5.38±0.50	16.96±0.85	5.42±0.51	5.11±0.44
Resveratrol	260.26±25.48	341.73±33.94	329.43±25.20	426.37±20.12	396.38±30.86	549.00±10.76	520.15±41.29	555.94±58.79	425.50±24.32	627.22±39.45
Viniferin	5.47±0.45	2277.59±111.84	70.98±20.65	2944.36±185.26	269.70±0.46	2573.10±195.74	12.46±2.69	26.28±7.81	7.56±1.28	10.15±0.60
Sum (μg/100 g)	2623.23±61.47	3379.81±118.30	3191.57±74.38	4307.88±189.58	1959.31±69.24	4041.54±198.81	1687.03±48.73	860.17±59.59	3123.43±45.09	980.22±39.69

The amount of carotenoid, the extraction yield and efficiency of ethanol for extraction of carotenoids in peel and flesh are shown in table 5.22.

Table 5.22: Concentration of lutein and β-carotene in different ratios of ethanol/water in peel and flesh, determined by spectrophotometer.

% Ethanol	*Total carotenoid as lutein in dry powder (mg/g)*	*Total carotenoid as β-carotene in dry powder (mg/g)*	*Extraction yield (% W/W)*	*% Lutein in DM*	*% β-carotene in DM*	*Efficiency of ethanol for lutein (%)*	*Efficiency of ethanol for β-carotene (%)*
Flesh-95%	0.192 ± 0.006^{Ba}	0.206 ± 0.036^{Aa}	49.5 ± 2.4^{Ad}	0.039 ± 0.003^{Ba}	0.042 ± 0.001^{Ba}	67.8	72.8
Flesh-70%	0.012 ± 0.001^{Bb}	0.011 ± 0.001^{Ab}	82.6 ± 1.2^{Ac}	0.0015 ± 0.0002^{Bb}	0.0013 ± 0.0002^{Bb}	4.2	3.9
Flesh-50%	0.0053 ± 0.0003^{Bc}	0.0047 ± 0.0002^{Ac}	96.3 ± 0.6^{Aa}	0.00060 ± 0.00003^{Bc}	0.00049 ± 0.00003^{Bc}	1.9	1.7
Flesh-30%	0.008 ± 0.003^{Bbc}	0.008 ± 0.003^{Abc}	89.6 ± 1.6^{Ab}	0.0009 ± 0.0003^{Bbc}	0.0009 ± 0.0003^{Bbc}	2.8	2.8
Flesh-0%	0.009 ± 0.002^{Abc}	0.009 ± 0.002^{Ab}	83.4 ± 0.6^{Ac}	0.0011 ± 0.0002^{Bb}	0.0011 ± 0.0002^{Bb}	3.2	3.2
Peel-95%	0.389 ± 0.029^{Aa}	0.365 ± 0.103^{Aa}	21.3 ± 0.4^{Bc}	0.183 ± 0.014^{Aa}	0.171 ± 0.051^{Aa}	79.6	74.6
Peel-70%	0.118 ± 0.003^{Ab}	0.121 ± 0.003^{Bb}	34.6 ± 1.5^{Bb}	0.034 ± 0.002^{Ab}	0.035 ± 0.002^{Ab}	24.1	24.7
Peel-50%	0.057 ± 0.003^{Ac}	0.054 ± 0.003^{Bc}	47.7 ± 2.1^{Ba}	0.012 ± 0.001^{Ac}	0.011 ± 0.001^{Ac}	11.7	11.0
Peel-30%	0.018 ± 0.001^{Ad}	0.015 ± 0.001^{Ad}	48.5 ± 2.6^{Ba}	0.0037 ± 0.0002^{Ad}	0.0031 ± 0.0001^{Ad}	3.7	3.1
Peel-0%	0.011 ± 0.001^{Ae}	0.009 ± 0.001^{Ad}	45.0 ± 1.0^{Ba}	0.0024 ± 0.0003^{Ad}	0.0020 ± 0.0002^{Ad}	2.3	1.8

For each type, different letters (a-c) within columns represent significant difference at $p < 0.05$, using Bonferroni test. For each percentage of ethanol, different uppercase letters (A-B) within columns represent significant difference at $p < 0.05$. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

Statistical analysis shows that a significant difference exists between the amount of total carotenoid extracted from peel and flesh with different ratios of ethanol:water (95%, 70%, 50% and 30%). As shown in table 5.22, the amount of total carotenoid as β-carotene and lutein decreased rapidly with decreasing ethanol concentration. For example, the amount of lutein extracted from peel decreased from 0.389 mg/g to 0.011 mg/g when the percentage of ethanol in the solvent decreased from 95% to 0%. Maximum and minimum extraction of carotenoids from peel and flesh, were obtaind by ethanol 95% and water, respectively. In other words, the ratio of ethanol:water equal to 95:5 is the ratio that allows optimal extraction of carotenoid. The highest amount of extraction yield for flesh and peel were obtained with ethanol 50% and 30%, respectively. Although the extraction yield increased with decreasing the percentage of ethanol, the amount of carotenoids and consequently the efficiency of ethanol for carotenoid extraction reduced. It shows that with reducing the percentage of ethanol, other components such as polyphenol were extracted.

Table 5.23 shows the amount of different types of carotenoids which were extracted from peel and flesh by using different ratios of ethanol:water as solvent extraction.

Table 5.23: Concentration of lutein, β-carotene, β-cryptoxanthin, Xanthophylles, 9-*Cis*-β-Carotene and 13-*Cis*-β-carotene in peel and flesh extracted with different ratios of ethanol:water and determined by HPLC.

Name	*β-carotene (mg/g)*	*β-cryptoxanthin (mg/g)*	*Lutein (mg/g)*	*Xanthophylles (mg/g)*	*9-Cis-β-Carotene (mg/g)*	*13-Cis-β-carotene (mg/g)*	*Total carotenoid in dry powder (mg/g)*
Peel-95%	0.07±0.01	0.006±0.002	0.08±0.02	0.024±0.002	0.015±0.001	ND	0.20±0.02
Peel-70%	0.005±0.001	0.005±0.001	0.07±0.01	0.02±0.01	ND	ND	0.10±0.01
Peel-50%	0.01	ND	ND	ND	ND	ND	0.01
Peel-30%	0.003±0.001	ND	ND	ND	ND	ND	0.003±0.001
Peel-0%	ND	ND	ND	ND	ND	ND	ND
Flesh-95%	0.1	0.009±0.002	0.0019±0.0001	ND	0.05±0.04	0.0023±0.0004	0.16±0.04
Flesh-70%	0.002	ND	0.0020±0.0004	ND	ND	ND	0.004±0.0004
Flesh-50%	ND	ND	ND	ND	ND	ND	ND
Flesh-30%	ND	ND	ND	ND	ND	ND	ND
Flesh-0%	ND	ND	ND	ND	ND	ND	ND

ND: Not detected.

The carotenoids which were extracted by different ratios of ethanol:water from peel and flesh, were identified and measured by HPLC using a C_{30} column. The profiles of carotenoids in flesh and peel are shown in Figures 5.9 and 5.10, respectively.

The results obtained by HPLC show that the solubility of carotenoids is decreased by increasing the concentration of water. As shown in table 5.23, no carotenoid is detected in ethanol 30% and water. The amount of total carotenoid detected by HPLC is almost half of that detected by spectrophotometer. Since the dry matter obtained after evaporation of the extract, did not dissolve well in Methanol:MTBE of 80:20.

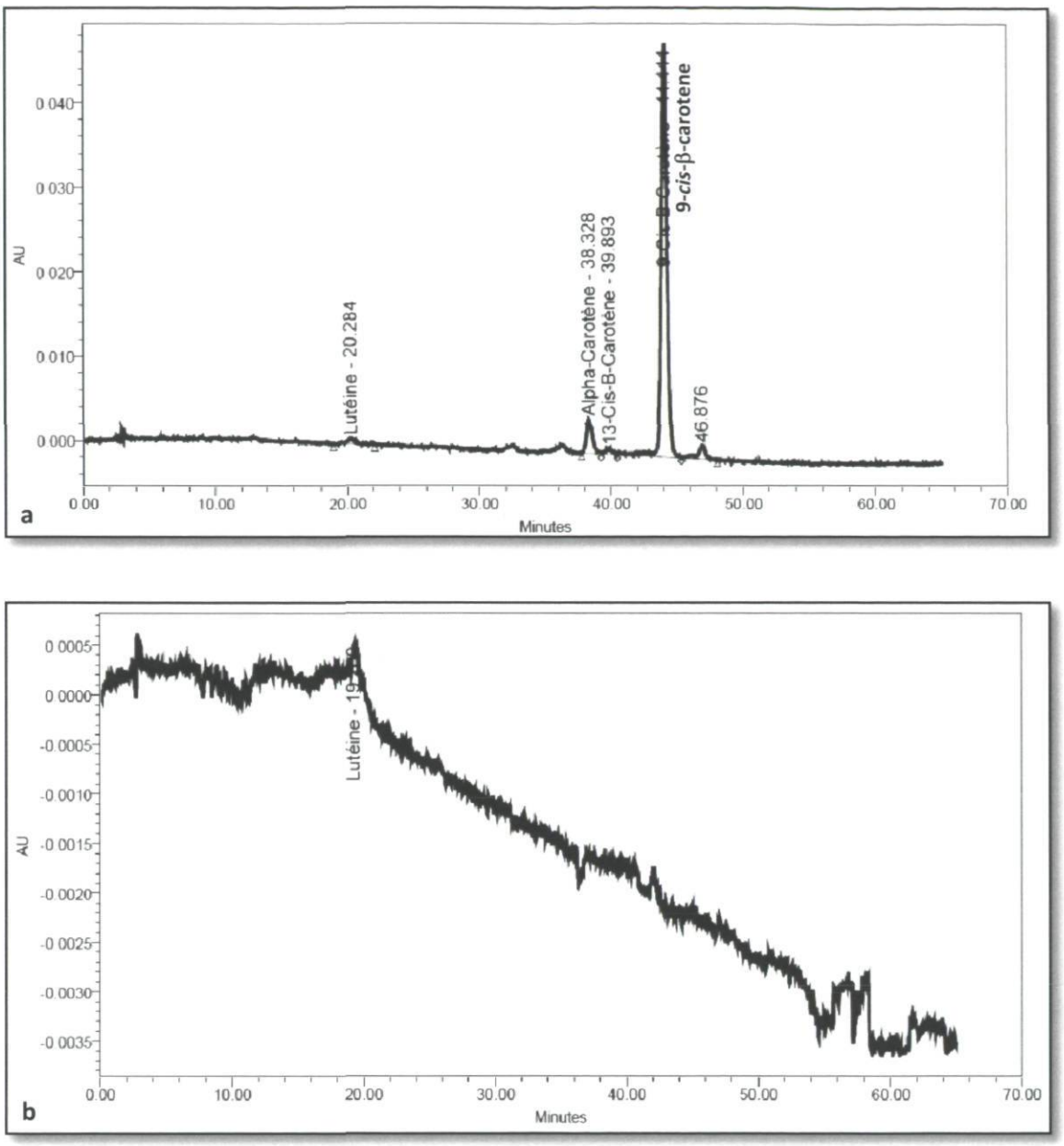


Figure 5.9. HPLC profile of carotenoids in flesh extracted by ethanol a) 95%, and b) 70%, separated on a C_{30} column. The same patterns as (b) were obtained by ethanol 50%, 30% and water.

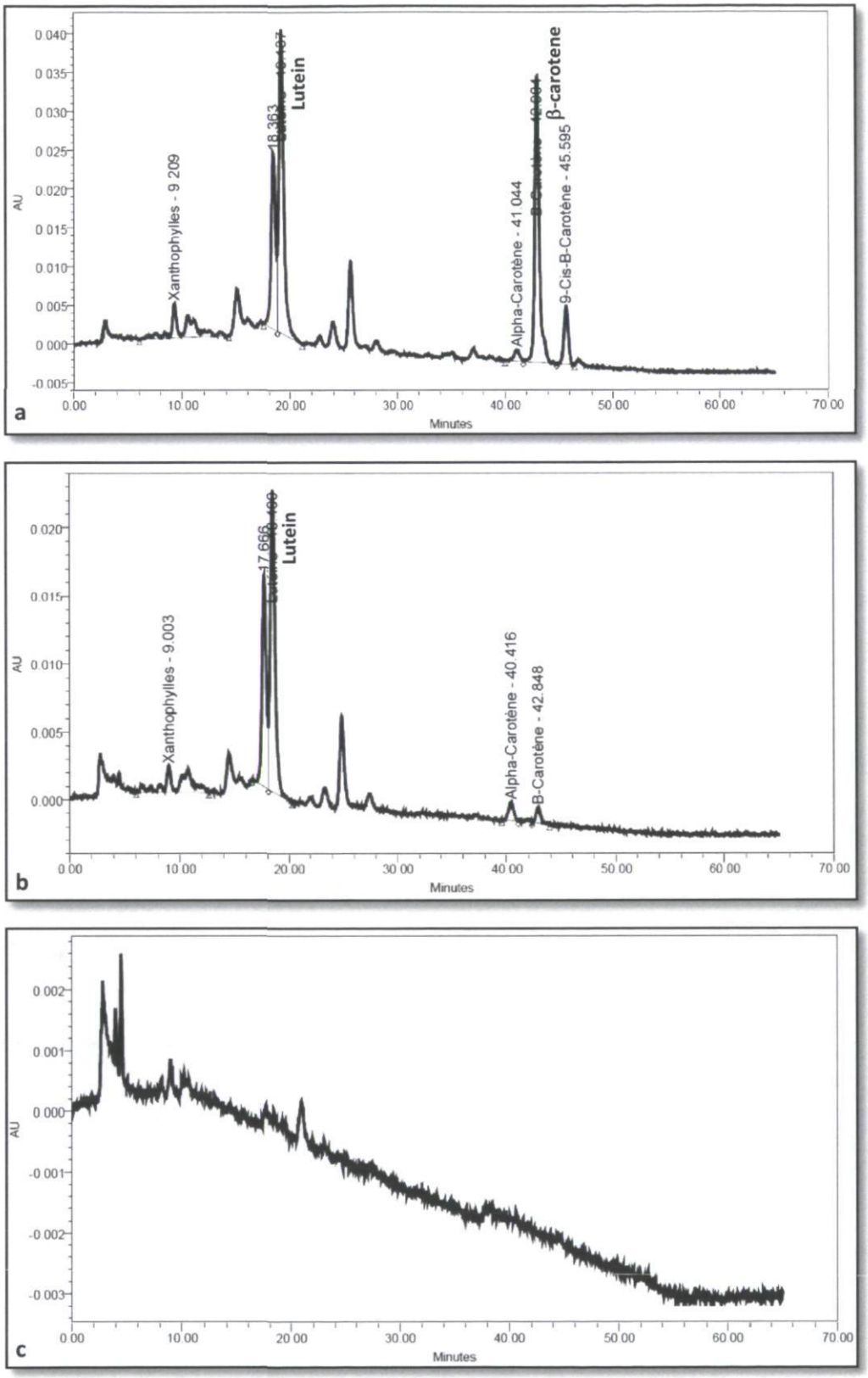


Figure 5.10. HPLC profile of carotenoids in peel extracted by ethanol a) 95%, b) 70% and c) 50% separated on a C_{30} column. The same patterns as (c) were obtained by ethanol 30% and water.

It can be concluded that ethanol 95% is the best solvent for extraction of carotenoid from peel and flesh, as indicated in tables 5.22 and 5.23. This may be due to a change in the solvent polarity due to addition of ethanol. As mentioned before in chapter 2, most of the carotenoids are lipophilic and insoluble in water (Rodriguez-Amaya, 2001). Takahashi et al. (2006) also found that water is not effective for the extraction of β-carotene.

5.6. Ethanolic extraction of carotenoids:

Table 5.24 shows the concentration of total carotenoids, extraction yield and percentage of carotenoids in dry matter for peel and flesh. The probability (p) of 0.0003 (using F test) indicates that there is a significant difference between the amount of lutein extracted from the peel and flesh, while this difference for β-carotene is not significant (p = 0.0653). Peel has a higher concentration of carotenoids, as lutein and β-carotene, than flesh, however the extraction yield of flesh is more than that of peel (p = 0.0001).

Table 5.24. Concentration of Lutein and β-carotene in the flesh and peel of cantaloupe by using ethanol 95% as extraction solvent, determined by spectrophotometer.

Name	*Total carotenoid as lutein in dry powder (mg/g)*	*Total carotenoid as β-carotene in dry powder (mg/g)*	*Extraction yield (% W/W)*	*% Lutein in DM*	*% β-carotene in DM*
Flesh	0.19±0.01	0.21±0.04	49.5±2.4	0.038±0.003	0.04±0.01
Peel	0.39±0.03	0.37±0.10	21.3±0.4	0.18±0.01	0.17±0.05
P-value	0.0003	0.0653	0.0001	< 0.0001	0.012

The P-value within columns was used to verify the presence or absence of significant difference between the TC content and extraction yield in peel and flesh, using F test.

Table 5.25 shows the concentration of total carotenoids extracted from peel and flesh by THF and ethanol 95%. Using the optimum extraction parameters such as time, temperature and ethanol:water ratio, the efficiency of ethanol for extraction of carotenoids as lutein was increased to 79.6% for peel and 67.9% for flesh. The p-values of 0.1098 and 0.1752 (using F test) respectively show that there is no significant difference between the amount of total carotenoid as lutein and β-carotene extracted by ethanol and that obtained by THF in the peel. The p-values of 0.0540 and 0.1218 respectively show that there is no significant difference between the amount of total carotenoid as lutein and β-carotene extracted by

ethanol and that obtained by THF in flesh. The p-value of 0.0653 indicates that there is no significant difference between the amount of total carotenoid as β-carotene extracted by ethanol from the peel and flesh. The p-value of 0.0003 (α=0.01) shows that there is a significant difference between the amount of total carotenoid as lutein extracted by ethanol from the peel and flesh.

Table 5.25. Comparison of the concentration of total carotenoid in flesh and peel, extracted by THF and ethanol 95%, determined by spectrophotometer.

Name	*Total carotenoid in dry powder, extracted by THF (mg/g)*	*Total carotenoid as lutein in dry powder (fresh), extracted by ethanol 95% (mg/g)*	*Total carotenoid as β-carotene in dry powder (fresh), extracted by ethanol 95% (mg/g)*	*Efficiency of ethanol for lutein (%)*	*Efficiency of ethanol for β-carotene (%)*
Peel	0.49±0.08	0.39±0.03 (0.04)	0.37±0.10 (0.04)	79.6	75.5
Flesh	0.28±0.06	0.19±0.01 (0.02)	0.21±0.04 (0.02)	67.9	75.0
P value	0.0225	0.0003	0.0653		

The P-value within columns was used to verify the presence or absence of significant difference between the TC content in peel and flesh, using F test.

Table 5.26 shows the concentration of different types of carotenoid in peel and flesh, extracted by ethanol 95% and determined by HPLC using a C_{30} column. To compare the results with those obtained by THF, the results of table 5.5 are represented again in this table. Five components were present which were separated and identified by comparing retention times and UV/visible absorbtion spectra with commercial standard (β-carotene). The identified carotenoids were β-carotene, lutein, 9-*Cis*-β-carotene, xanthophilles and α-carotene. The major carotenoids were identified as β-carotene and Lutein. The comparison between the HPLC results of the carotenoids extracted from peel and flesh indicates that the highest level of β-carotene was detected in flesh (0.20 mg/g compared to 0.12 mg/g). On the contrary, the highest level of lutein was detected in the peel (0.12 mg/g compared to 0.004 mg/g). As indicated in table 5.26, 85.7% and 55.6% of β-Carotene, 57.1% and 80% of lutein could be extracted by ethanol 95% from peel and flesh, respectively. In general, 73.3% and 53.7% of total carotenoid could be extracted by ethanol 95% from peel and flesh, respectively.

Table 5.26. Concentration of Lutein, β-carotene, α-Carotene, Xanthophylles and 9-*Cis*-β-Carotene in flesh and peel, extracted by ethanol 95% and THF, determined by HPLC.

Carotenoid	*Carotenoid in dry powder of peel, extracted by ethanol 95% (mg/g)*	*Carotenoid in dry powder of peel, extracted by THF (mg/g)*	*Efficiency of ethanol for peel (%)*	*Carotenoid in dry powder of flesh, extracted by ethanol 95% (mg/g)*	*Carotenoid in dry powder of flesh, extracted by THF (mg/g)*	*Efficiency of ethanol for flesh (%)*
β-Carotene	0.12 ± 0.01	0.14 ± 0.01	85.7	0.20 ± 0.04	0.36±0.03	55.6
α-Carotene	0.017 ± 0.002	0.009 ± 0.001	188.9	0.010 ± 0.002	0.004 ± 0.001	250
Lutein	0.12 ± 0.02	0.21 ± 0.03	57.1	0.004 ± 0.001	0.005 ± 0.002	80
Xanthophylles	0.05 ± 0.01	0.07 ± 0.01	71.4	0.002 ± 0.001	0.004 ± 0.002	50
9-*Cis*-β-Carotene	0.022 ± 0.002	0.020 ± 0.003	110	0.008 ± 0.003	0.009± 0.002	88.9
13-*Cis*-β-carotene	ND	ND	ND	ND	0.017 ± 0.002	ND
β-crypthoxanthin	ND	ND	ND	ND	0.0013±0.0004	ND
Unknown 1	ND	ND	ND	ND	0.006 ± 0.002	ND
Unknown 2	ND	ND	ND	ND	0.005 ± 0.001	ND
Unknown 3	ND	ND	ND	ND	0.003 ± 0.001	ND
Total	0.33 ± 0.02	0.45 ± 0.03	73.3	0.22 ± 0.04	0.41±0.03	53.7
P-value	0.0185			0.1533		

ND: not detected.
The P-value within columns was used to verify the presence or absence of significant difference between the TC content obtained by ethanol and THF, using F test.

The carotenoids extracted from peel and flesh by ethanol 95% were identified and measured by HPLC using a C_{30} column. The profiles of carotenoids in peel and flesh are shown in Figures 5.11.a and 5.11.b, respectively.

Table 5.27 shows the concentration of total polyphenol, extraction yield and polyphenol/dry matter in peel and flesh in the solution obtained after extraction. According to this table, the amount of polyphenol for the peel was higher than those for flesh. The p-values of 0.0001 show that the difference between the amount of polyphenol and extraction yield for the peel and flesh is significant. Extraction yield for flesh is significantly more than those for peel.

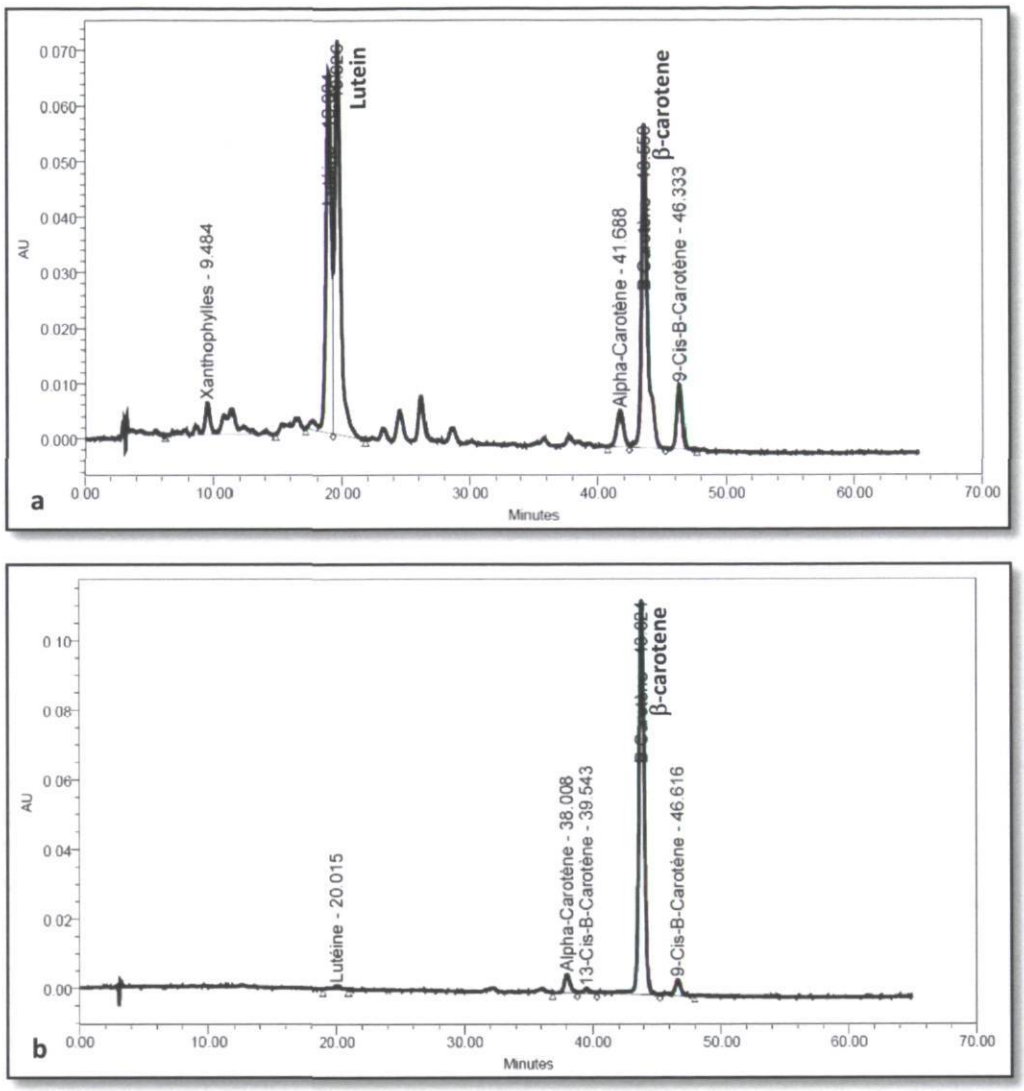


Figure 5.11. HPLC profile of carotenoids in a) peel and b) flesh extracts separated on a C_{30} column.

Table 5.27. Concentration of polyphenol in flesh and peel, determined by spectrophotometer.

	Polyphenol in dry powder (mg/g)	*Polyphenol in extract (mg/l)*	*Extraction yield (% W/W)*	*% Polyphenol in DM*	*Efficiency of ethanol for polyphenol*
Flesh	0.80±0.03	40.25±5.46	49.5±2.4	0.16±0.01	51.6
Peel	2.23±0.16	111.43±19.19	21.3±0.4	1.05±0.09	59.5
P- value	0.0001	0.0035	< 0.0001	< 0.0001	

The P-value within columns was used to verify the presence or absence of significant difference between the polyphenols content in peel and flesh, using F test.

Table 5.28 shows the amount of total carotenoids which were remained in the residue and could not be extracted by ethanol 95%. The remained carotenoids were extracted by THF

and determined by spectrophotometer. A low amount of carotenoid is remained in the residue. The p-value of 0.5664 shows that the difference between the remained carotenoid in the peel and flesh is not significant.

Table 5.28. Concentration of carotenoids in the residue of flesh and peel, determined by spectrophotometer.

Name	*Total carotenoid in dry powder, extracted by THF (mg/g)*	*Lutein in dry powder, extracted by ethanol 95% (mg/g)*	*β-carotene in dry powder, extracted by ethanol 95% (mg/g)*	*Carotenoid in residue (mg/g)*	*% Non-extracted*
Peel	0.49±0.08	0.39±0.03	0.37±0.10	0.04±0.01	8.2
Flesh	0.28±0.06	0.19±0.01	0.21±0.04	0.05±0.01	17.9
P- value	0.0225	0.0003	0.0653	0.5664	

The P-value within columns was used to verify the presence or absence of significant difference between the TC content in peel and flesh, using F test.

The carotenoids which were remained in the residue of peel and flesh, were identified and measured by HPLC using a C_{30} column. Table 5.29 shows the amount of different types of remained carotenoids. The p-value of 0.0001 reveals that the amount of β-carotene in the residue of flesh is significantly higher than that of peel.

Table 5.29. Concentration of individual carotenoids in the residue of flesh and peel, extracted by THF and determined by HPLC.

Carotenoid	*Carotenoid in dry powder of peel (mg/g)*	*Carotenoid in dry powder of flesh (mg/g)*	*P- value*
β-Carotene	0.0020 ± 0.0004	0.088 ± 0.003	0.0001
α-Carotene	0.011 ± 0.001	ND	
Lutein	0.016 ± 0.003	ND	
Xanthophylles	0.004 ± 0.001	ND	
9-*Cis*-β-Carotene	0.013 ± 0.002	0.002 ± 0.001	0.0142
Total	0.046 ± 0.004	0.090 ± 0.003	0.0142

ND: Not detected.
The P-value within columns was used to verify the presence or absence of significant difference between the individual carotenoids in peel and flesh, using F test.

The profiles of the remained carotenoids are shown in Figures 5.12.a and 5.12.b, respectively.

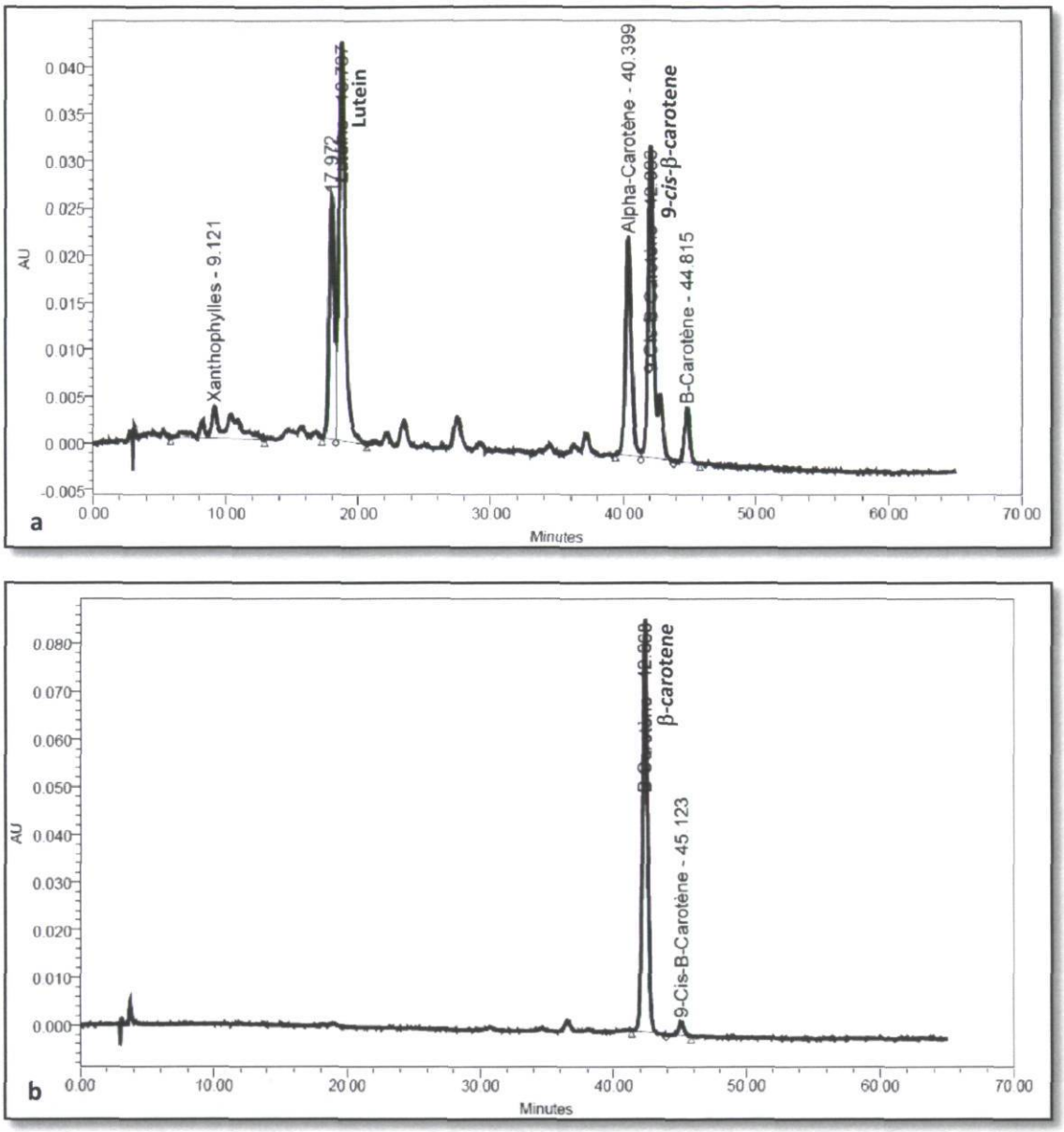


Figure 5.12. HPLC profile of carotenoids in the residue of a) peel and b) flesh separated on a C_{30} column.

According to the results of table 5.28 and their comparison with the results of table 5.4, which were obtained by THF, it can be concluded that the majority of the carotenoids could be extracted by ethanol 95%. In fact, 75% of β-carotene and 67.9% of lutein in flesh and 75.5% of β-carotene and 79.6% of lutein in peel were extracted after optimizing the parameters of extraction and using ethanol 95% as solvent.

5.7. Determination of the concentration of total carotenoid and total polyphenol in the whole cantaloupe

Table 5.30 shows the amount of total carotenoid in whole cantaloupe. Total carotenoid in whole cantaloupe, extracted by THF, is 0.30 mg/g of dry powder (0.03 mg/g FW). The sum of total carotenoid as β-carotene in the peel and flesh, extracted by ethanol 95%, is 0.19 mg/g of dry powder (0.02 mg/g FW).

Table 5.30. Concentration of total carotenoid in whole cantaloupe.

Name	*Total carotenoid in dry powder, extracted by THF (mg/g)*	*Total carotenoid in fresh matter, extracted by THF (mg/g)*	*Total carotenoid as lutein in dry powder, extracted by ethanol 95% (mg/g)*	*Total carotenoid as β-carotene in dry powder, extracted by ethanol 95% (mg/g)*	*Total carotenoid as lutein in fresh matter, extracted by ethanol 95% (mg/g)*	*Total carotenoid as β-carotene in fresh matter, extracted by ethanol 95% (mg/g)*
Peel	0.09	0.01	0.07	0.07	0.01	0.01
Flesh	0.16	0.02	0.11	0.12	0.01	0.01
Seed	0.05	0.01	ND	ND	ND	ND
Sum	0.30	0.04	0.18	0.19	0.02	0.02

ND: Not determined.

Table 5.31 shows the amount of total polyphenol in whole cantaloupe. Polyphenol content in whole cantaloupe, extracted by double extraction using methanol and acetone, is 1.46 mg/g of dry powder (0.21 mg/g FW). The sum of polyphenol in peel and flesh, extracted by ethanol 95%, is 0.87 mg/g of dry powder (0.10 mg/g FW).

Table 5.31. Concentration of polyphenol in whole cantaloupe.

Name	*Polyphenol in dry powder, determined by double extraction (mg/g)*	*Polyphenol in fresh matter, determined by double extraction (mg/g)*	*Polyphenol in dry powder, determined by ethanol 95% (mg/g)*	*Polyphenol in fresh matter, determined by ethanol 95% (mg/g)*
Peel	0.29	0.08	0.42	0.05
Flesh	0.88	0.11	0.45	0.06
Seed	0.30	0.03	ND	ND
Sum	1.46	0.21	0.87	0.10

ND: Not determined

5.8. Extraction of proanthocyanidins from peel, flesh and seed of cantaloupe

Table 5.32 shows the concentration of proanthocyanidins in seed, peel and flesh of cantaloupe. Statistical analysis (LSD test) shows that there is a significant difference between the amount of proanthocyanidins in the seed, peel and flesh. Proanthocyanidins content in the peel is significantly more than that of seed and flesh respectively. Li et al. (2006) also indicated that the proanthocyanidins content in skin was higher than in pulp extract of pomegranate. It can be due to accumulation of phenolic compounds in the epidermal tissue of plants to protect against ultraviolet radiation and to resist against pathogens and predators (Dixon and Paiva, 1995).

Table 5.32. % proanthocyanidin in seed, peel and flesh of cantaloupe.

Name	*Proanthocyanidin (mg/g)*
Seed	0.79±0.35 [b]
Peal	1.40±0.14 [a]
Flesh	0.18±0.03 [c]

Different letters within columns represent significant difference at $p < 0.05$, using LSD test. Data are expressed as average of triplicate measurements with standard deviation.

5.9. Semi-industrial extraction of carotenoids with ethanol

Table 5.33 shows the amount of polyphenol and carotenoids in the presence or absence of enzymes (Kleryme and Viscozyme). According to the results indicated in this table and LSD test, the amount of polyphenols and carotenoids in the presence of Viscozyme is higher than those in the presence of Kleryme and in the control specimen, respectively. Extraction yield with ethanol was lower in the presence of Viscosyme. Because higher amounts of polyphenol, sugar and pectin have been separated by Viscozyme and brought into the water. Therefore a lower extraction yield for ethanolic extraction was obtained.

Table 5.33. Concentration of polyphenol and carotenoid in the presence or absence of enzymes.

Solvant	*Enzyme*	*Polyphenol (mg/g dry powder)*	*Total Carotenoid (TC)*	*Extraction yield (W/W %)*	*% polyphenol in DM*	*%TC in DM*
Water	Control	1.42 ± 0.02^{b}	ND	74.1 ± 3.6^{b}	0.19 ± 0.01^{b}	ND
Water	Viscozyme	1.99 ± 0.05^{a}	ND	85.4 ± 0.2^{a}	0.23 ± 0.01^{a}	ND
Water	Kleryme	1.46 ± 0.09^{b}	ND	73.17 ± 4.7^{b}	0.207 ± 0.004^{ab}	ND
Ethanol	Control	0.50 ± 0.06^{c}	0.14 ± 0.01^{a}	36.9 ± 4.4^{c}	0.14 ± 0.04^{c}	0.04 ± 0.01^{ab}
Ethanol	Viscozyme	0.36 ± 0.08^{d}	0.17 ± 0.01^{a}	31.7 ± 5.4^{c}	0.11 ± 0.03^{c}	0.06 ± 0.01^{a}
Ethanol	Kleryme	0.39 ± 0.05^{d}	0.15 ± 0.03^{a}	36.0 ± 5.4^{c}	0.102 ± 0.002^{c}	0.04 ± 0.01^{ab}

ND: Not determined

Different letters within columns represent significant difference at $p < 0.05$, using LSD test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

Table 5.34 shows the concentration of total carotenoid, after 30 min, 1 h and 2 h of ethanolic extraction. This table indicates that the amount of TC (0.16 mg/g) is higher for extraction at 50°C after 2 hours. This result is in agreement with the results of laboratory extraction presented in table 5.30, in which, the sum of carotenoids in the peel and flesh is 0.19 mg/g of dry powder.

Table 5.34. Concentration of total carotenoids, after different times of ethanolic extraction.

Ethanolic extraction		
Time	*Total carotenoid (mg/g of dry powder)*	*Polyphenol (mg/g of dry powder)*
30 min	0.13	ND
1h	0.15	ND
2h	0.16	0.43

ND: Not determined.

After extraction, ethanol was evaporated and a resin was formed. When ethanol was evaporated, the carotenoids were insoluble in the remained water and were sticked around the balloon as a resin. After drying the water in spray dryer, a powder was formed. Total carotenoid and polyphenol in the resin and powder were determined. According to the results in table 3.35, the powder obtained by spray dryer in pilot scale is not a good source of carotenoid and polyphenols but the resin obtained after evaporation of ethanol could be a rich source of carotenoids and chlorophylls (9.39 mg/g). The resin was dried using two methods to determine the carotenoid content in dry mass and to verify the influence of drying method on degradation of carotenoids. None of the methods resulted in a completely dried resin. Total carotenoid in dried resin (11.11 mg/g and 11.33 mg/g) was very close to that

found after direct analysis of resin (11.62 mg/g) indicating that degradation of carotenoids was not considerable. The resin was lipophile and was dissolved easily in the canola oil. It can be concluded that, instead of a powder rich in carotenoid, it is possible to produce a resin which is a good source of carotenoid and can be offered as dissolved in oil. In contrast to the powder, the juice of cantaloupe was a good source of polyphenols.

Table 3.35. Concentration of polyphenol and carotenoid in different steps of semi-industrial procedure.

	Total carotenoid (mg/g)	***Poylyphenol (mg/g)***
Analysis of solution (resin+water)	ND	0.22±0.01
Direct analysis of resin without drying / based on dry mass	9.39 / 11.62	ND
Analysis of resin after drying in oven at 45 °C	11.11	ND
Analysis of resin after freeze drying	11.33	ND
Analysis of powder after spray drying	0.10±0.001	0.50±0.02
Analysis of juice of cantaloupe after freeze drying	0.001	1.39±0.15

ND: Not determined

Althouth viniferin and conidendrin were present in the powder obtained in laboratory scale extraction, their presence in the powder obtained from the juice of cantaloupe in pilot scale was not identified. It can be due to the different extraction method used in the pilot scale.

5.10. Conclusion

Lypophilic solvents are generally used to extract carotenoids and may remain in the extracted product. This research aimed to extract carotenoids and polyphenols from peel and flesh of cantaloupe using ethanol with limited health concerns. The efficiency of ethanol can be improved using optimum extraction conditions including time, temperature and ethanol:water ratio. Extraction conditions of 2 h/50°C and 2 h/30°C were found to be the optimum extraction conditions for flesh and peel, respectively. β-carotene and lutein were the major carotenoids extracted using ethanol 95%, but in the case of polyphenol, ethanol was not as efficient as water. Using ethanol 95% as solvent for the extraction of carotenoids may result in lower toxicity compared to other solvents used for carotenoid extraction and may reduce the toxic residues that would be derived from strong solvents such as acetone.

β-carotene and lutein were the major carotenoids found in peel and flesh of cantaloupe. Results of this study show that the amount of lutein was higher in the peel while the major carotenoid in flesh was β-carotene. Many different types of polyphenols as flavonoids, hydroxybenzoic and hydroxylcinnamic acids were found in cantaloupe. Overall, the amount of total polyphenols exceeded that of total carotenoids in peel and flesh of cantaloupe. High carotenoid, polyphenol and proanthocyanidin contents were observed in the peel as compared to the flesh. The results of the present study suggest that peel extracts can be potentially considered as new sources of natural antioxidants as carotenoids and polyphenols for food and nutraceutical products. In fact, phytochemicals that contribute to health are abundant in cantaloupe peel which is a by-product of cantaloupe juice processing. If the peels are not processed, they become waste.

In pilot scale extraction, a resin rich of carotenoids and chlorophylls was obtained after evaporation of ethanol. In contrast, the powder obtained after spray drying was not a good source of carotenoid and polyphenols. The resin was easily dissolved in vegetable oil and can be used as nutritional additive which may have the potential to reduce the risk of disease as cancer, cardiovascular, cataract and macular degenerescence.

Bibliographie

Acevedo, P. and J.S. Bertram, *Liarozole potentiates the cancer chemopreventive activity of and the up-regulation of gap junctional communication and connexin43 expression by retinoic acid and β-carotene in 10t1/2 cells.* Carcinogenesis, 1995. **16**(9):p. 2215-2222.

Adams C.F., *Nutritive value of american foods: In common units.* Agriculture Handbook No. 456, 1975, Washington, DC: US Dept. of Agriculture.

Ames, B.N., M.K. Shigenaga, and T.M. Hagen, *Oxidants, antioxidants, and the degenerative diseases of aging.* Proceedings of the National Academy of Sciences, 1993. **90**(17): p. 7915-7922.

Alves-Rodrigues, A. and A. Shao, *The science behind lutein.* Toxicology Letters, 2004. **150**(1): p. 57–83.

Azevedo-Meleiro, C.H. and D.B. Rodriguez-Amaya, *Confirmation of the identity of the carotenoids of tropical fruits by HPLC-DAD and HPLC-MS.* Journal of Food Composition and Analysis, 2004. **17**(3–4): p. 385-396.

Balassa, L.L., G.O. Fanger, and O.B. Wurzburg, *Microencapsulation in the food industry.* C R C Critical Reviews in Food Technology, 1971. **2**(2): p. 245-265.

Barth, M.M., C. Zhou, K.M. Kute, and G.A. Rosenthal, *Determination of optimum conditions for supercritical fluid extraction of carotenoids from carrot (Daucus carota l.) tissue.* Journal of Agricultural and Food Chemistry, 1995. **43**(11): p. 2876-2878.

Becker, E.M., L.R. Nissen, and L.H. Skibsted, *Antioxidant evaluation protocols: food quality or health effects.* European Food Research and Technology, 2004. **219**(6): p. 561-571.

Bengoechea, M.L., A.I. Sancho, B. Bartolomé, I. Estrella, C. Gómez-Cordovés, and M.T. Hernández, *Phenolic composition of industrially manufactured purées and concentrates from peach and apple fruits.* Journal of Agricultural and Food Chemistry, 1997. **45**(10): p. 4071-4075.

Bergquist, S.A., U.E. Gertsson, and M.E. Olsson, *Influence of growth stage and postharvest storage on ascorbic acid and carotenoid content and visual quality of baby spinach (Spinacia oleracea L.).* Journal of the Science of Food and Agriculture, 2006. **86**(3): p. 346–355.

Bhandari, B.R., E.D. Dumoulin, H.M.J. Richard, I. Noleau, and A.M. Lebert, *Flavor encapsulation by spray drying: application to citral and linalyl acetate.* Journal of Food Science, 1992. **57**(1): p. 217-221.

Bravo, L., *Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance.* Nutrition Reviews, 1998. **56**(11): p. 317-333.

Brianceau S., *Extraction des molecules bioactives du marc de pomme*, 2010.

Britton G, *Carotenoids.* Methods in Plant Biochemistry, 1991. **7**: p. 473-518.

Bucić-Kojić, A., M. Planinić, S. Tomas, L. Jakobek, and M. Šeruga, *Influence of solvent and temperature on extraction of phenolic compounds from grape seed, antioxidant activity*

and colour of extract. International Journal of Food Science and Technology, 2009. **44**(12): p. 2394-2401.

Bureau J.L. and Bushway R.J., *HPLC Determination of carotenoids in fruits and vegetables in the united states.* Journal of Food Science, 1986. **51**(1): p. 128-130.

Cacace, J.E. and G. Mazza, *Optimization of extraction of anthocyanins from black currants with aqueous ethanol.* Journal of Food Science, 2003. **68**(1): p. 240-248.

Calo, P., J.B. Velazquez, C. Sieiro, P. Blanco, E. Longo, and T.G. Villa, *Analysis of astaxanthin and other carotenoids from several Phaffia rhodozyma mutants.* Journal of Agricultural and Food Chemistry, 1995. **43**(5): p. 1396-1399.

Calvo, M., D. Dado, and G. Santa-María, *influence of extraction with ethanol or ethyl acetate on the yield of lycopene, β-carotene, phytoene and phytofluene from tomato peel powder.* European Food Research and Technology, 2007. **224**(5): p. 567-571.

Canals, R., M.C. Llaudy, J. Valls, J.M. Canals, and F. Zamora, *Influence of ethanol concentration on the extraction of color and phenolic compounds from the skin and seeds of tempranillo grapes at different stages of ripening.* Journal of Agricultural and Food Chemistry, 2005. **53**(10): p. 4019-4025.

Castenmiller, J.J.M., C.E. West, J.P.H. Linssen, K.H. van het Hof, and A.G.J. Voragen, *The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans.* The Journal of Nutrition, 1999. **129**(2): p. 349-355.

Cavalcante ML and Rodriguez-Amaya DB. *Carotenoid composition of the tropical fruits eugenia uniflora and malpighia glabra.* In charalambous G (ed), Food science and human nutrition. Elsevier Science Publishers, Amsterdam, 1992. p. 643-650.

Chandler, L.A., and Schwartz, S.J., *HPLC separation of cis-trans carotene isomers in fresh and processed fruits and vegetables. Journal of Food Science*, 1987. **52**: p. 669-672.

Chen B.H. and Tang Y.C., *Processing and stability of carotenoid powder from carrot pulp waste*. Journal of Agricultural and Food Chemistry, 1998. **46**(6): p. 2312−2318.

Chen, B.H., S.H. Yang, and L.H. Han, *characterization of major carotenoids in water convolvulus (ipomoea aquatica) by open-column, thin-layer and high-performance liquid chromatography.* Journal of Chromatography A, 1991. **543**(0): p. 147-155.

Cheynier, V., *Polyphenols in foods are more complex than often thought.* The American Journal of Clinical Nutrition, 2005. **81**(1): p. 223S-229S.

Constant, J., *Alcohol, ischemic heart disease, and the french paradox.* Clinical Cardiology, 1997. **20**(5): p. 420-424

Craft, N.E. and J.H. Soares, *Relative solubility, stability, and absorptivity of lutein and beta.-carotene in organic solvents.* Journal of Agricultural and Food Chemistry, 1992. **40**(3): p. 431-434.

Craft, N.E., S.A. Wise, and J.H. Soares Jr, *Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids.* Journal of Chromatography A, 1992. **589**(1–2): p. 171-176.

Craft, N.E., S.A. Wise, and J.H. Soares, *Individual carotenoid content of srm 1548 total diet and influence of storage temperature, lyophilization, and irradiation on dietary carotenoids*. Journal of Agricultural and Food Chemistry, 1993. **41**(2): p. 208-213.

Curl, A.L., *The carotenoids of muskmelons.* Journal of Food Science, 1966. **31**(5): p. 759-761.

De Melo, M.L.S., N. Narain, and P.S. Bora, *Characterisation of some nutritional constituents of melon (Cucumis melo hybrid af-522) seeds.* Food Chemistry, 2000. **68**(4): p. 411-414.

De Mello, M.L.S., P.S. Bora, and N. Narain, *Fatty and amino acids composition of melon (Cucumis melo var. saccharinus) seeds.* Journal of Food Composition and Analysis, 2001. **14**(1): p. 69-74.

Demir, N., J. Acar, K. Sarıoğlu, and M. Mutlu, *The use of commercial pectinase in fruit juice industry. Part 3: Immobilized pectinase for mash treatment.* Journal of Food Engineering, 2001. **47**(4): p. 275-280.

Desjardins, Chapter 9- *Physiological and ecological functions and biosynthesis of health-promoting compounds in fruit and vegetables. In. Improving the health-promoting compounds in fruit and vegetables*. 2008: Eds. Tomas-Barberan, T. and Gil, M.I., Woodhead Publishing Limited, Cambridge . p. 201-248.

Desobry, S.A., F.M. Netto, and T.P. Labuza, *Comparison of spray-drying, drum-drying and freeze-drying for β-carotene encapsulation and preservation.* Journal of Food Science, 1997. **62**(6): p. 1158-1162.

Desobry S.A., F.M. Netto and T.P., Labuza, *Influence of maltodextrin systems at an loss during storage equivalent 25de on encapsulated β-carotene*. Journal of Food Processing and Preservation, 1999. **23**(1): p. 39-55.

Dixon, R.A., and Paiva, N.I., *Stress-induced phenylpropanoid metabolism*. The Plant Cell, 1995. **7**(7): p. 1085–1097.

EL-Qudah J.M., *Identification and quantification of major carotenoids in some vegetables*. American Journal of Applied Sciences, 2009. **6**(3): p. 492-497.

Escribano-Bailon, M.T.and Santos-Buelga. *Polyphenols extraction from foods. Methods in polyphenol analysis*. (edited by C. Santos-Buelga and Williamson G). 1–16. Cambridge, UK: Royal Society of Chemistry, 2003.

Faravash, R.S. and F.Z. Ashtiani, *The influence of acid volume, ethanol-to-extract ratio and acid-washing time on the yield of pectic substances extraction from peach pomace.* Food Hydrocolloids, 2008. **22**(1): p. 196-202.

Forrest, W., *Reverse phase HPLC separation of cis-and trans-carotenoids and its application to β-carotenes in food materials.* Journal of Liquid Chromatography, 1987. **10**(4): p. 643-653.

Gartner, C., Stahl, W., and Sies, H., *Lycopene is more bioavailable from tomato paste than from fresh tomatoes*. American Journal of Clinical Nutrition, 1997. **66**(1): p. 116–122.

Gehm, B.D., J.M. McAndrews, P.-Y. Chien, and J.L. Jameson, *Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor.* Proceedings of the National Academy of Sciences, 1997. **94**(25): p. 14138-14143.

Gibbs F., S.K., Inteaz Alli, Catherine N. Mulligan and Bernard, *Encapsulation in the food industry: a review.* International Journal of Food Sciences and Nutrition, 1999. **50**(3): p. 213-224.

Gil, M.I., F.A. Tomás-Barberán, B. Hess-Pierce, and A.A. Kader, *Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and plum cultivars from california.* Journal of Agricultural and Food Chemistry, 2002. **50**(17): p. 4976-4982.

Gortner, W.A. and V.L. Singleton, *Carotenoid pigments of pineapple fruit. II. Influence of fruit ripeness, handling and processing on pigment isomerizationa.* Journal of Food Science, 1961. **26**(1): p. 53-55.

Granado, F., Olmedilla, B., Blanco, I., *Nutritional and clinical relevance of lutein in human health.* British Journal of Nutrition, 2003, **90**: p. 487–502.

Gregory G.K. T.S. Chen, and T. Philip, *Quantitative analysis of carotenoids and caritenoid esters in fruits by HPLC: red bell peppers*. Journal of Food Science. 1987. **52**: p. 1071.

Grohmann, K. and E.A. Baldwin, *Hydrolysis of orange peel with pectinase and cellulase enzymes.* Biotechnology Letters, 1992. **14**(12): p. 1169-1174.

Gross, J., M. Gabai, A. Lifshitz, and B. Sklarz, *Carotenoids in pulp, peel and leaves of Persea americana.* Phytochemistry, 1973. **12**(9): p. 2259-2263.

Gruszecki, W., *Carotenoids in membranes.the photochemistry of carotenoids*, H. Frank, et al., Editors. 2004, Springer Netherlands, p. 363-379.

Gruz J., O. Novák, and M. Strnad, *rapid analysis of phenolic acids in beverages by UPLC-MS/MS.* Food Chemistry, 2008. **111**(3): p. 789-794.

Hart, D.J. and K.J. Scott, *Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK.* Food Chemistry, 1995. **54**(1): p. 101-111.

Hedrén, E., V. Diaz, and U. Svanberg, *Estimation of carotenoid accessibility from carrots determined by an in vitro digestion method.* European Journal of Clinical Nutrition, 2002. **56**(5): p. 425.

Henriques M., Silva A. and Rocha J., *Extraction and quantification of pigments from a marine microalga: A simple and reproducible method.* Formatex, 2007. p. 586-593.

Hof, K.H.v.h., B.C.J. de Boer, L.B.M. Tijburg, B.R.H.M. Lucius, I. Zijp, C.E. West, J.G.A.J. Hautvast, and J.A. Weststrate, *Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the triglyceride-rich lipoprotein fraction of plasma after a single consumption and in plasma after four days of consumption.* The Journal of Nutrition, 2000. **130**(5): p. 1189-1196.

Ismail, H.I., K.W. Chan, A.A. Mariod, and M. Ismail, *Phenolic content and antioxidant activity of cantaloupe (Cucumis melo) methanolic extracts.* Food Chemistry, 2010. **119**(2): p. 643-647.

Jang, M., L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W.W. Beecher, H.H.S. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, and J.M. Pezzuto, *Cancer chemopreventive activity of resveratrol, a natural product derived from grapes.* Science, 1997. **275**(5297): p. 218-220.

Johnson, E.J., *The role of lutein in disease prevention.* Nutrition in Clinical Care, 2000. **3**(5): p. 289-296.

Khachik, F., G.R. Beecher, and N.F. Whittaker, *Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography.* Journal of Agricultural and Food Chemistry, 1986. **34**(4): p. 603-616.

Kimura M, Rodriguez-Amaya DB and Yokoyama SM. *Cultivar differences and geographic effects on the carotenoid composition and vitamin a value of papaya.* Lebens Wissen Technol, 1991. **24**: p. 415-418.

Kolayli, S., M. Kara, F. Tezcan, F.B. Erim, H. Sahin, E. Ulusoy, and R. Aliyazicioglu, *Comparative study of chemical and biochemical properties of different melon cultivars: standard, hybrid, and grafted melons.* Journal of Agricultural and Food Chemistry, 2010. **58**(17): p. 9764-9769.

Kondratyuk, T.P. and J.M. Pezzuto, *Natural product polyphenols of relevance to human health.* Pharmaceutical Biology, 2004. **42**(s1): p. 46-63.

Konings, E.J.M. and H.H.S. Roomans, *Evaluation and validation of an LC method for the analysis of carotenoids in vegetables and fruit.* Food Chemistry, 1997, **59**(4): p. 599-603.

Kris-Etherton, P.M., K.D. Hecker, A. Bonanome, S.M. Coval, A.E. Binkoski, K.F. Hilpert, A.E. Griel, and T.D. Etherton, *Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer.* The American Journal of Medicine, 2002. **113**(9, Supplement 2): p. 71-88.

Landrum J.T., Bone R.A., Joa H., Kilburn M.D., Moore L.L. and Sprague K.E., *A one-year study of the macular pigment: The effect of 140 days of a lutein supplement.* Experimental Eye Research, 1997. **65**: p. 57-62.

Langcake, P. and R. Pryce, *A new class of phytoalexins from grapevines.* Cellular and Molecular Life Sciences, 1977. **33**(2): p. 151-152.

Laur, L.M. and L. Tian, *Provitamin A and vitamin C contents in selected california-grown cantaloupe and honeydew melons and imported melons.* Journal of Food Composition and Analysis, 2011. **24**(2): p. 194-201.

Lessin, W.J. and S.J. Schwartz, *Quantification of cis-trans isomers of provitamin A carotenoids in fresh and processed fruits and vegetables.* Journal of Agricultural and Food Chemistry, 1997. **45**(10): p. 3728-3732.

Lester, G.E. and F. Eischen, *Beta-carotene content of postharvest orange-fleshed muskmelon fruit: Effect of cultivar, growing location and fruit size.* Plant Foods for Human Nutrition, 1996. **49**(3): p. 191-197.

Lester G.E., *Antioxidant, sugar, mineral and phytonutrient concentrations across edible fruit tissues of orange-fleshed honeydew melon (Cucumis melo L.).* Journal of Agricultural and Food Chemistry, 2008. **56**(10): p. 3694–3698.

Lester, G.E. and D.M. Hodges, *Antioxidants associated with fruit senescence and human health: novel orange-fleshed non-netted honey dew melon genotype comparisons following different seasonal productions and cold storage durations.* Postharvest Biology and Technology, 2008. **48**(3): p. 347-354.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., and Cheng, S., *Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract.* Food Chemistry, 2006. **96**: p. 254–260.

Machlin, L.J., *Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease.* Critical Reviews in Food Science and Nutrition, 1995. **35**(1-2): p. 41-49.

Manach, C., A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, *Polyphenols: food sources and bioavailability.* The American Journal of Clinical Nutrition, 2004. **79**(5): p. 727-747.

Mayne, S., *Beta-carotene, carotenoids, and disease prevention in humans.* The Faseb Journal, 1996. **10**(7): p. 690-701.

Mattila, P., and Kumpulainen, J. *Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection.* Journal of Agricultural and Food Chemistry, 2002. **50**(13): p. 3660–3667.

Maznah I. Abdalbasit M. Gururaj B. and Ling H.S., *Fatty acid composition and antioxidant activity of oils from two cultivars of cantaloupe extracted by supercritical fluid extraction.* Grasas Y Aceites, 2010. **61**(1): p. 37-44.

Michel, H., *Carotenoids as membrane stabilizers in chloroplasts.* Trends in Plant Science, 1998. **3**(4): p. 147-151.

Mouly, P.P., E.M. Gaydou, and J. Corsetti, *Characterization of Paprika (Capsicum annuum) extract in orange juices by liquid chromatography of carotenoid profiles.* Journal of Agricultural and Food Chemistry, 1999. **47**(3): p. 968-976.

Müller H., *Determination of the carotenoid content in selected vegetables and fruit by HPLC and photodiode array detection.* Z Lebensm Unters Forsch A, 1997. **204**: p. 88-94.

Naczk, M. and F. Shahidi, *Extraction and analysis of phenolics in food.* Journal of Chromatography A, 2004. **1054**(1-2): p. 95-111.

Naczk, M. and Shahidi, F. *Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis.* Journal of Pharmaceutical and Biomedical Analysis, 2006. **41**: p. 1523–1542.

(NCBE) National Centre for Biotechnology Education. *Carbohydrase mix: novozymes viscozyme*. 2011 (December 1, 2011); Available from: http://www.ncbe.reading.ac.uk/NCBE/MATERIALS/ENZYMES/viscozyme.html.

Nguyen, M., D. Francis, and S. Schwartz, *Thermal isomerization susceptibility of carotenoids in different tomato varieties*. Journal of the Science of Food and Agriculture, 2001. **81**(9): p. 910-917.

Nun˜ez-Palenius, H., Gomez-Lim, M., Ochoa-Alejo, N., Grumet, R., Lester, G. and Cantliffe, D., Melon Fruits: Genetic diversity, physiology, and biotechnology features. Critical Reviews in Biotechnology, 2008, **28**: p. 13–55.

Oliver, J., A. Palou, and A. Pons, *Semi-quantification of carotenoids by high-performance liquid chromatography: saponification-induced losses in fatty foods.* Journal of Chromatography A, 1998. **829**(1-2): p. 393-399.

Oliver, J. and A. Palou, *Chromatographic determination of carotenoids in foods.* Journal of Chromatography A, 2000. **881**(1–2): p. 543-555.

Omenn, G.S., G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, B. Valanis, J.H. Williams, S. Barnhart, and S. Hammar, *Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease.* New England Journal of Medicine, 1996. **334**(18): p. 1150-1155.

Packer L. and Colman C., *The antioxidant miracle*, John Wiley and Sons Inc., 2000.

Paredes-López, O., M. Cervantes-Ceja, M. Vigna-Pérez, and T. Hernández-Pérez, *Berries: Improving human health and healthy aging, and promoting quality life—A review.* Plant Foods for Human Nutrition (Formerly Qualitas Plantarum), 2010. **65**(3): p. 299-308.

Park, Y.W., *Effect of freezing, thawing, drying, and cooking on carotene retention in carrots, broccoli and spinach.* Journal of Food Science, 1987. **52**(4): p. 1022-1025.

Pezet, R., C. Perret, J. B. Jean-Denis, R. Tabacchi, K. Gindro, and O. Viret, *δ-Viniferin, a resveratrol dehydrodimer: one of the major stilbenes synthesized by stressed grapevine leaves.* Journal of Agricultural and Food Chemistry, 2003. **51**(18): p. 5488-5492.

Pilnik, W. and A.G.J. Voragen, *Effect of enzyme treatment on the quality of processed fruit and vegetables*, in *quality factors of fruits and vegetables*. 1989, American Chemical Society. p. 250-269.

Piver, B., F. Berthou, Y. Dreano, and D. Lucas, *Differential inhibition of human cytochrome P450 enzymes by ε-viniferin, the dimer of resveratrol: comparison with resveratrol and polyphenols from alcoholized beverages.* Life Sciences, 2003. **73**(9): p. 1199-1213.

Remorini, D., S. Tavarini, E. Degl'Innocenti, F. Loreti, R. Massai, and L. Guidi, *Effect of rootstocks and harvesting time on the nutritional quality of peel and flesh of peach fruits.* Food Chemistry, 2008. **110**(2): p. 361-367.

Riso, P., A. Brusamolino, L. Scalfi, and M. Porrini, *Bioavailability of carotenoids from spinach and tomatoes.* Nutrition, Metabolism and Cardiovascular Diseases, 2004. **14**(3): p. 150-156.

Robert, P., R. Carlsson, N. Romero, and L. Masson, *Stability of spray-dried encapsulated carotenoid pigments from Rosa mosqueta (Rosa rubiginosa) oleoresin.* Journal of the American Oil Chemists' Society, 2003. **80**(11): p. 1115-1120.

Rodriguez-Amaya, D.B., M. Kimura, H.T. Godoy, and H.K. Arima, *Assessment of provitamin A determination by open column chromatography/visible absorption spectrophotometry.* Journal of Chromatographic Science, 1988. **26**(12): p. 624-629.

Rodriguez-Amaya D.B., *Nature and distribution of carotenoids in foods. In charalambous g (ed), shelflife studies of foods and beverages. Chemical, biological, physical and nutritional aspects*. Elsevier Science Publishers, Amsterdam, 1993. p. 547-589.

Rodriguez-Amaya D.B., *Carotenoids and food preparation: the retention of provitamin A carotenoids in prepared, processed, and stored foods.* 1997: John Snow Inc.

Rodriguez-Amaya D.B., *A guide to carotenoid analysis in foods.* 2001: International Life Sciences Institute, Washington D.C.

Rodriguez-Amaya D.B. and Mieko Kimura, *Harvest plus handbook for carotenoid analysis.* International Food Policy Research Institute (IFPRI) and International Center for Tropical Agriculture (CIAT), 2004.

Sander, L.C., K.E. Sharpless, N.E. Craft, and S.A. Wise, *Development of engineered stationary phases for the separation of carotenoid isomers.* Analytical Chemistry, 1994. **66**(10): p. 1667-1674.

Schieber, A., P. Hilt, P. Streker, H.-U. Endreß, C. Rentschler, and R. Carle, *A new process for the combined recovery of pectin and phenolic compounds from apple pomace.* Innovative Food Science and amp; Emerging Technologies, 2003. **4**(1): p. 99-107.

Schmitz H.H., W.E. Artz, C. L. Poor, J. M. Dietz and J. W. Erdman, Jr, *High-performance liquid chromatography and capillary supercritical-fluid chromatography separation of vegetable carotenoids and carotenoid isomers.* Journal of Chromatography, 1989. **479**: p. 261-268.

Stahl, W. and H. Sies, *Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans.* The Journal of Nutrition, 1992. **122**(11): p. 2161-6.

Stewart, J.R., N.E. Ward, C.G. Ioannides, and C.A. O'Brian, *Resveratrol preferentially inhibits protein kinase c-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism.* Biochemistry, 1999. **38**(40): p. 13244-13251.

Straatsma, J., G. Van Houwelingen, A.E. Steenbergen, and P. De Jong, *Spray drying of food products: 1. Simulation model.* Journal of Food Engineering, 1999. **42**(2): p. 67-72.

Sun, M. and F. Temelli, *Supercritical carbon dioxide extraction of carotenoids from carrot using canola oil as a continuous co-solvent.* The Journal of Supercritical Fluids, 2006. **37** (3): p. 397-408.

Sun, T., J.R. Powers, and J. Tang, *Effect of enzymatic macerate treatment on rutin content, antioxidant activity, yield, and physical properties of asparagus juice.* Journal of Food Science, 2007. **72**(4): p. S267-S271.

Swain, T. and W.E. Hillis, *The phenolic constituents of prunus domestica. i.—the quantitative analysis of phenolic constituents.* Journal of the Science of Food and Agriculture, 1959. **10**(1): p. 63-68.

Takahashi, M., H. Watanabe, J. Kikkawa, M. Ota, M. Watanabe, Y. Sato, H. Inomata, and N. Sato, *Carotenoids extraction from japanese persimmon (hachiyakaki) peels by supercritical co2 with ethanol*. Analytical Sciences, 2006. 22.

Taungbodhitham, A.K., G.P. Jones, M.L. Wahlqvist, and D.R. Briggs, *Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables.* Food Chemistry, 1998. **63**(4): p. 577-584.

Thies M, Fischer R., New reaction for microchemical detection and the quantitative determination of catechins. Mikrochim Acta, 1971. **1**: p. 9-13.

Tomás-Barberán, F.A., M.I. Gil, P. Cremin, A.L. Waterhouse, B. Hess-Pierce, and A.A. Kader, *HPLC−DAD−ESIMS analysis of phenolic compounds in nectarines, peaches, and plums.* Journal of Agricultural and Food Chemistry, 2001. **49**(10): p. 4748-4760.

Tonucci, L.H., J.M. Holden, G.R. Beecher, F. Khachik, C.S. Davis, and G. Mulokozi, *Carotenoid content of thermally processed tomato-based food products.* Journal of Agricultural and Food Chemistry, 1995. **43**(3): p. 579-586.

Toor, R.K. and G.P. Savage, *Antioxidant activity in different fractions of tomatoes.* Food Research International, 2005. **38**(5): p. 487-494.

Unknown Author, *nomenclature of carotenoids (rules approved 1974) issued by the iupac commission on the nomenclature of organic chemistry and the iupac—iub commission on biochemical nomenclature,* The journal of Biological Chemistry, 1972. **247**(9).

USDA-ARS, 2009. USDA national nutrient database for standard reference, Release 22. Nutrient Data Laboratory Home Page: http://www.ars.usda.gov/ba/bhnrc/ndl.

Valko, M., D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, and J. Telser, *Free radicals and antioxidants in normal physiological functions and human disease.* The International Journal of Biochemistry & Cell Biology, 2007. **39**(1): p. 44-84.

Vavich, M.G. and A.R. Kemmerer, *The carotenes of cantaloupes.* Journal of Food Science, 1950. **15**(6): p. 494-497.

Velioglu, Y.S., G. Mazza, L. Gao, and B.D. Oomah, *Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products.* Journal of Agricultural and Food Chemistry, 1998. **46**(10): p. 4113-4117.

Vouldoukis, I., D. Lacan, C. Kamate, P. Coste, A. Calenda, D. Mazier, M. Conti, and B. Dugas, *Antioxidant and anti-inflammatory properties of a Cucumis melo LC. extract rich in superoxide dismutase activity.* Journal of Ethnopharmacology, 2004. **94**(1): p. 67-75.

Wagner, L.A. and J.J. Warthesen, *Stability of spray-dried encapsulated carrot carotenes.* Journal of Food Science, 1995. **60**(5): p. 1048-1053.

Wallace, T.C. and M.M. Giusti, *Evaluation of parameters that affect the 4-dimethylaminocinnamaldehyde assay for flavanols and proanthocyanidins.* Journal of Food Science, 2010. **75**(7): p. C619-C625.

Wang L. and Liu Y., *Optimization of solvent extraction conditions for total carotenoids in rapeseed using response surface methodology.* Natural Science, 2009. **1**: p. 23-29.

Weissenberg M., Schaeffler I., Menagem E., Barzilai M. and Levy A., *Isocratic non-aqueous reversed-phase high-performance liquid chromatographic separation of capsanthin and capsorubin in red peppers (Capsicum annuum l.), paprika and oleoresin.* Journal of Chromatography A, 1997. **757**: p. 89-95.

Will, F., M. Mischler, and K. Dörreich, *Pilot scale isolation and separation of apple juice colloids.* LWT - Food Science and Technology, 1994. **27**(3): p. 292-294.

Wolfe, K., X. Wu, and R.H. Liu, Antioxidant activity of apple peels. Journal of Agricultural and Food Chemistry, 2003. **51**(3): p. 609–614.

Wolfe, K.L., X. Kang, X. He, M. Dong, Q. Zhang, and R.H. Liu, *Cellular antioxidant activity of common fruits.* Journal of Agricultural and Food Chemistry, 2008. **56**(18): p. 8418-8426.

World Cancer Research Fund/American Institute for Cancer Research. *Food, nutrition, physical activities, and the prevention of cancer: A Global Perspective.* 2007: American Institute for Cancer Research, Washington, DC.

Wrolstad R.E. , T.E. Acree, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Schwartz, C.F. Shoemaker, D. Smith, and P. Sporns, *Carotenoids, in handbook of food analytical chemistry pigments, colorants, flavors, components texture and bioactive food,* 2005, John Wiley and Sons, Inc: New Jersey.

Yu, K., D. Little, R. Plumb, and B. Smith, *High-throughput quantification for a drug mixture in rat plasma – a comparison of Ultra Performance™ liquid chromatography/tandem mass spectrometry with high-performance liquid chromatography/tandem mass spectrometry.* Rapid Communications in Mass Spectrometry, 2006. **20**(4): p. 544-552.

Zakaria, M., K. Simpson, P.R. Brown, and A. Krstulovic, *Use of reversed-phase high-performance liquid chromatographic analysis for the determination of provitamin A carotenes in tomatoes.* Journal of Chromatography A, 1979. **176**(1): p. 109-117.

Appendix I

Concentration of lutein and β-carotene in flesh for different extraction times and temperatures.

Temperature	*Time (min.)*	*Lutein in dry powder (mg/g)*	*β-carotene in dry powder (mg/g)*	*Efficiency of ethanol for lutein (%)*	*Efficiency of ethanol for β-carotene (%)*
25°C	0	0.088 ± 0.008^{d}	0.094 ± 0.008^{b}	31.10	33.22
	30	0.106 ± 0.006^{c}	0.114 ± 0.007^{a}	37.46	40.28
	60	0.113 ± 0.007^{bc}	0.122 ± 0.008^{a}	39.93	43.11
	120	0.118 ± 0.006^{ab}	0.127 ± 0.006^{a}	41.70	44.88
	180	0.121 ± 0.006^{ab}	0.131 ± 0.006^{a}	42.76	46.29
	360	0.126 ± 0.008^{a}	0.136 ± 0.008^{a}	44.52	48.06
30°C	0	0.088 ± 0.008^{d}	0.094 ± 0.008^{c}	31.10	33.22
	30	0.116 ± 0.002^{c}	0.127 ± 0.001^{b}	40.99	44.88
	60	0.129 ± 0.002^{b}	0.139 ± 0.002^{ab}	45.58	49.12
	120	0.136 ± 0.007^{ab}	0.147 ± 0.007^{ab}	48.06	51.94
	180	0.141 ± 0.008^{a}	0.151 ± 0.009^{a}	49.82	53.36
	360	0.146 ± 0.006^{a}	0.157 ± 0.006^{a}	51.59	55.48
40°C	0	0.088 ± 0.008^{d}	0.094 ± 0.008^{d}	31.10	33.22
	30	0.126 ± 0.003^{c}	0.135 ± 0.003^{c}	44.52	47.70
	60	0.133 ± 0.006^{c}	0.144 ± 0.007^{bc}	47.00	50.88
	120	0.151 ± 0.004^{b}	0.163 ± 0.005^{ab}	53.36	57.60
	180	0.156 ± 0.009^{ab}	0.168 ± 0.010^{a}	55.12	59.36
	360	0.170 ± 0.007^{a}	0.181 ± 0.008^{a}	60.07	63.96
50°C	0	0.088 ± 0.008^{d}	0.094 ± 0.008^{c}	31.10	33.22
	30	0.148 ± 0.009^{c}	0.159 ± 0.010^{b}	52.30	56.18
	60	0.159 ± 0.007^{b}	0.171 ± 0.008^{ab}	56.18	60.42
	120	0.176 ± 0.007^{a}	0.187 ± 0.008^{a}	62.19	66.08
	180	0.175 ± 0.014^{a}	0.186 ± 0.015^{a}	61.84	65.72
	360	0.169 ± 0.024^{ab}	0.180 ± 0.026^{ab}	59.72	63.60

For each temperature, different letters within columns represent significant difference at $p < 0.05$, using Bonferroni test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

Concentration of lutein and β-carotene in peel for different extraction times and temperatures.

Temperature	*Time (min.)*	*Total carotenoid as lutein in dry powder (mg/g)*	*Total carotenoid as β-carotene in dry powder (mg/g)*	*Efficiency of ethanol for lutein (%)*	*Efficiency of ethanol for β-carotene (%)*
25°C	0	0.332±0.006^{b}	0.301±0.006^{b}	67.89	61.55
	30	0.379±0.016^{a}	0.346±0.011^{a}	77.51	70.76
	60	0.399±0.016^{a}	0.359±0.015^{a}	81.60	73.42
	120	0.384±0.025^{a}	0.345±0.022^{a}	78.53	70.55
	180	0.407±0.011^{a}	0.365±0.007^{a}	83.23	74.64
	360	0.392±0.014^{a}	0.352±0.014^{a}	80.16	71.98
30°C	0	0.332±0.006^{b}	0.301±0.006^{c}	67.89	61.55
	30	0.429±0.003^{a}	0.387±0.003^{b}	87.73	79.14
	60	0.439±0.007^{a}	0.398±0.006^{ab}	89.78	81.39
	120	0.458±0.005^{a}	0.415±0.003^{a}	93.66	84.87
	180	0.454±0.009^{a}	0.412±0.008^{a}	92.84	84.25
	360	0.457±0.018^{a}	0.414±0.016^{a}	93.46	84.66
40°C	0	0.332±0.006^{b}	0.301±0.006^{c}	67.89	61.55
	30	0.435±0.005^{a}	0.392±0.005^{b}	88.96	80.16
	60	0.454±0.008^{a}	0.413±0.007^{a}	92.84	84.46
	120	0.453±0.002^{a}	0.412±0.001^{ab}	92.64	84.25
	180	0.451±0.014^{a}	0.411±0.015^{ab}	92.23	84.05
	360	0.423±0.015^{a}	0.388±0.013^{ab}	86.50	79.35
50°C	0	0.332±0.006^{b}	0.301±0.006^{b}	67.89	61.55
	30	0.430±0.005^{a}	0.391±0.004^{a}	87.94	79.96
	60	0.433±0.004^{a}	0.394±0.003^{a}	88.55	80.57
	120	0.435±0.013^{a}	0.400±0.010^{a}	88.96	81.80
	180	0.425±0.019^{a}	0.393±0.017^{a}	86.91	80.37
	360	0.408±0.006^{a}	0.375±0.005^{a}	83.44	76.69

For each temperature, different letters within columns represent significant difference at $p < 0.05$, using Bonferroni test. Means within a column followed by the same letter are not significantly different at $P \leq 0.05$. Data are expressed as average of triplicate measurements with standard deviation.

Appendix II

Repeated measure analysis of variance for β-carotene according to type and temperature

The Univariate procedure
Variable. Scaled Resid (Scaled Residual)

Moments			
N	144	**Sum Weights**	144
Mean	0	**Sum Observations**	0
Std Deviation	0.81934698	**Variance**	0.67132948
Skewness	-0.4273193	**Kurtosis**	1.93564704
Uncorrected SS	96.0001154	**Corrected SS**	96.0001154
Coeff Variation	.	**Std Error Mean**	0.06827892

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.971046	**Pr < W**	0.0038
Kolmogorov-Smirnov	**D**	0.079628	**Pr > D**	0.0240
Cramer-von Mises	**W-Sq**	0.140764	**Pr > W-Sq**	0.0327
Anderson-Darling	**A-Sq**	0.835676	**Pr > A-Sq**	0.0318

Effect = type*Temperature Method = Step down Bonferroni (P<.05) Set=1

Obs	type	Temperature	Temps	Estimate	Standard Error	Letter Group
1	Flesh	50	_	0.1630	0.004011	**A**
2	Flesh	40	_	0.1476	0.004011	**AB**
3	Flesh	30	_	0.1359	0.004011	**BC**
4	Flesh	25	_	0.1208	0.004011	**C**

Effect = type*Temperature Method = Step down Bonferroni (P<.05) Set=2

Obs	type	Temperature	Temps	Estimate	Standard Error	Letter Group
5	Peel	30	_	0.3879	0.004011	A
6	Peel	40	_	0.3863	0.004011	A
7	Peel	50	_	0.3758	0.004011	A
8	Peel	25	_	0.3447	0.004011	B

Effect = type*temps Method = Step down Bonferroni (P<.05) Set=1

Obs	type	Temperature	temps	Estimate	Standard Error	Letter Group
1	Flesh	_	360	0.1636	0.003659	A
2	Flesh	_	180	0.1590	0.003189	A
3	Flesh	_	120	0.1560	0.002954	A
4	Flesh	_	60	0.1438	0.002573	B
5	Flesh	_	30	0.1341	0.001754	C
6	Flesh	_	0	0.09446	0.002061	D

Effect = type*temps Method = Step down Bonferroni (P<.05) Set=2

Obs	type	Temperature	temps	Estimate	Standard Error	Letter Group
7	Peel	_	180	0.3953	0.003189	A
8	Peel	_	120	0.3930	0.002954	A
9	Peel	_	60	0.3907	0.002573	AB
10	Peel	_	360	0.3824	0.003659	BC
11	Peel	_	30	0.3793	0.001754	C
12	Peel	_	0	0.3013	0.002061	D

Repeated measure analysis of variance for lutein according to type and temperature Square root transformation

The Univariate procedure
Variable. Scaled Resid (Scaled Residual)

Moments			
N	144	**Sum Weights**	144
Mean	0	**Sum Observations**	0
Std Deviation	0.8193505	**Variance**	0.67133524
Skewness	-0.3088851	**Kurtosis**	0.9944343
Uncorrected SS	96.0009395	**Corrected SS**	96.0009395
Coeff Variation	.	**Std Error Mean**	0.06827921

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	**W**	0.973035	**Pr < W**	0.0061
Kolmogorov-Smirnov	**D**	0.079238	**Pr > D**	0.0249
Cramer-von Mises	**W-Sq**	0.178497	**Pr > W-Sq**	0.0098
Anderson-Darling	**A-Sq**	1.099981	**Pr > A-Sq**	0.0071

Effect = type*Temperature Method = Step down Bonferroni (P<.05) Set=1

Obs	type	Temperature	temps	Estimate	Standard Error	Letter Group
1	Flesh	50	_	-1.9090	0.02277	**A**
2	Flesh	40	_	-2.0081	0.02277	**B**
3	Flesh	30	_	-2.0870	0.02277	**B**
4	Flesh	25	_	-2.1969	0.02277	**C**

Effect = type*Temperature Method = Step down Bonferroni (P<.05) Set=2

Obs	type	Temperature	temps	Estimate	Standard Error	Letter Group
5	Peel	30	_	-0.8540	0.02277	A
6	Peel	40	_	-0.8625	0.02277	A
7	Peel	50	_	-0.8948	0.02277	AB
8	Peel	25	_	-0.9639	0.02277	B

Effect = type*temps Method = Step down Bonferroni (P<.05) Set=1

Obs	Type	Temperature	temps	Estimate	Standard Error	Letter Group
1	Flesh	_	360	-1.8876	0.01741	A
2	Flesh	_	180	-1.9198	0.01455	AB
3	Flesh	_	120	-1.9408	0.01194	B
4	Flesh	_	60	-2.0217	0.01093	C
5	Flesh	_	30	-2.0957	0.01008	D
6	Flesh	_	0	-2.4358	0.01747	E

Effect = type*temps Method = Step down Bonferroni (P<.05) Set=2

Obs	type	Temperature	temps	Estimate	Standard Error	Letter Group
7	Peel	_	180	-0.8359	0.01455	A
8	Peel	_	120	-0.8409	0.01194	A
9	Peel	_	60	-0.8422	0.01093	A
10	Peel	_	360	-0.8690	0.01741	AB
11	Peel	_	30	-0.8733	0.01008	B
12	Peel	_	0	-1.1015	0.01747	C