CHAPTER 1
INTRODUCTION

1.1 THE PALM OIL INDUSTRY

1.1.1 General

Crude palm oil is derived from the mesocarp of the oil palm fruits. The oil palm is a monoecious plant and the female flowers give rise to fruits commonly referred to as fresh fruit bunches (FFB). Though palm forests are found in West Africa, oil palms are mostly cultivated in South America, East Africa, Malaysia and Indonesia.[1,2] The oil palm of commercial value today *Elaeis guineensis* Jacq., originated from West Africa. The current planting material in Malaysia is a cross of Dura and Pisifera varieties known as Tenera, all belonging to the *Elaeis guineensis* species.[3]

Each oil palm tree is capable of bearing about 10 to 12 fruit bunches per year. The number of fruits per bunch varies from 1000 to 3000 and the average weight of each bunch varies between 20 to 30 kg depending on the variety and age of the palm. The oil to bunch ratio is within the range of 25 to 28 percent depending on the variety and age of the palm.

In terms of productivity per unit area for oil crops, the oil palm provides the highest yield of oil per hectare of land, i.e. up to 3.8 tonnes per hectare per year. While crude palm oil is extracted from the mesocarp of the fruit, the kernel (endosperm) yields palm kernel oil at an annual yield of 11% of the crude palm oil production. Kernel oil has physical and chemical properties which are quite different from that of the palm oil.

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The production of palm oil in the world for 1991 was 11.414 million tonnes of which Malaysia (53.8%) and Indonesia (23.3%) are the two major producers.[4] Both countries are expected to increase production with Malaysia expecting to realise 8.380 million tonnes and Indonesia more than 4.475 million tonnes by 1995.[5] In 1984, the market share of palm oil in the world's oil supply was 10.1%, however, it is expected that by the year 2000, palm oil will be as important as soybean oil contributing to 21.1% of the world's supply of oils and fats.[6]

1.1.2 Characteristics of Palm Oil

Palm oil consists mainly of acylglycerols with a small amount of fatty acids. Triglycerides constitute the major component but a small proportion of free fatty acids, diglycerides and monoglycerides are also present.[7] Palm oil also contains other minor constituents, i.e. non-glyceride components (Table 1.1). This chemical composition determines the chemical and physical characteristics of palm oil.

Table 1.1 Percentage Composition of Palm Oil

Free Fatty Acids	3.5
Monoglycerides	trace
Diglycerides	5.0
Triglycerides	91.0
Unsaponifiable matter	0.5

The fatty acid composition of Malaysian crude palm oil is given in Table 1.2.[8] Palm oil has an iodine value of 50-56. Of the fatty acids present in palm oil, about 50% are saturated, 40% monounsaturated and 10% polyunsaturated. Adequate amount of the essential fatty acid 18:2

n-6 is present in palm oil. A high content of the monounsaturated acid 18:1 likens palm oil to olive oil which has been considered by nutritionists to be as effective as polyunsaturated oils in reducing blood cholesterol and the risk of coronary heart disease (CHD).[9]

Table 1.2 Fatty Acid Composition of Malaysian Palm Oil[8]

Fatty acid	% of Total acids	
	Range	Mean
C12:0	0.1 - 1.0	0.2
C14:0	0.9 - 1.5	1.1
C16:0	41.8 - 46.8	44.0
C16:1	0.1 - 0.3	0.1
C18:0	4.2 - 5.1	4.5
C18:1	37.3 - 40.8	39.2
C18:2	9.1 - 11.0	10.1
C18:3	0.0 - 0.6	0.4
C20:0	0.2 - 0.7	0.4

1.1.3 Minor Components in Crude Palm Oil

Crude palm oil contains approximately 1% of minor components and these are carotenoids, vitamin E (tocopherols and tocotrienols), sterols, phospholipids, glycolipids, terpenic and aliphatic hydrocarbons and other trace impurities.[10] The concentration of these components are shown in Table 1.3. Among these, the major minor components are carotenoids and vitamin E. Both of these classes of compounds possess important physiological properties.

Table 1.3 Minor Components of Crude Palm Oil

Minor Components	ppm
Carotenoids[11]	500 - 700
Tocopherols & Tocotrienols[8,12]	600 - 1000
Sterols[13]	326 - 527
Phospholipids[14,15]	5 - 130
Triterpene alcohol[16,17]	est. 40 - 80
Methylsterols[17]	est. 40 - 80
Squalene[18,19]	200 - 500
Aliphatic alcohols[11]	100 - 200
Aliphatic hydrocarbons[18,19]	50

1.1.4 Carotenoids in Palm Oil

The orange-red colour of crude palm oil is due to the presence of carotenoids. Crude palm oil exported from the Far East and the Belgian Congo typically contains 500 - 800 ppm of carotenoids. Oil from the Ivory Coast (especially Dahomey) contains higher amounts (1000 - 1600 ppm) but the oil yield is lower.[20] Crude palm oil derived from the Tenera variety which is widely planted in Malaysia has a carotenoid content of about 500 - 700 ppm.[10] This is in contrast to the generally low concentration (less than 100 ppm) in other vegetable oils such as yellow maize (corn), peanut oil, soyabean oil, rapeseed oil, linseed oil, olive oil, barley oil, sunflower seed oil and cottonseed oil.[21-27]

In fact, among the vegetable oils which are widely produced, palm oil contains the highest known concentration of agro-derived carotenoids[28]. Crude palm oil is also the world's richest natural plant source of carotenes in terms of retinol (pro-vitamin A) equivalent. It contains about 15 to 300 times more retinol equivalents than carrots, green leafy vegetables and tomatoes which are considered to have significant quantities of pro-vitamin A activities[29] (Table 1.4).

Table 1.4 Retinol Equivalent of Red Palm Oil and other Foods[29]

Source	µg retinol equiv./100 g edible portion	Relative Quantity
Preformed Retinol*		
Halibut	900,000	30[#]
Shark	180,000	6[#]
Cod	8,000	1.7[@]
Carotene Derived**		
Red palm oil (RPO)	30,000	-
Carrots	2,000	15[@]
Most leafy green vegetables	685	44[@]
Apricots	250	120[@]
Tomatoes	100	300[@]
Bananas	30	1000[@]
Oranges/juices	8	750[@]

* Fish liver oils
** Fruits and vegetables
[#] Number of times above RPO
[@] Number of times below RPO

1.1.5 Extraction of Palm Oil

Crude palm oil is extracted from the oil palm fruits in the mill using screw presses. The process is presented diagrammatically in Fig. 1.1. This involves a few steps in order to maximise the recovery of the oil from the fruits, including sterilisation, stripping, digestion, oil extraction and clarification or purification.[30]

(a) Sterilisation

Fresh fruit bunches (FFB) from estates are transported to the mills and the FFB are sterilised in cages (2-5 tonnes capacity) in order to:- (i) prevent the increase of free fatty acids (FFA) due to enzyme action, (ii) facilitate mechanical stripping of fruitlets, (iii) prepare ("cook") the fruit pericarp for subsequent processing, (iv) precondition the nuts to minimise kernel breakage, and (v) coagulate the protein material and hydrolyse mucilaginous materials present in the palm fruits.

(b) Stripping

The sterilised fruits together with the associated calyx leaves are separated from the sterilised bunch stalks using strippers. There are two discharges from the strippers - sterilised fruits and empty bunch stalks. The fruits are then transferred to the digesters and the stalks are normally passed to an incinerator yielding ash rich in potash which can be used as fertiliser.

(c) Digestion

The sterilised fruits and accompanying calyx leaves from the strippers are reheated in steam-heated vessels provided with stirring arms known as digesters, where the pericarp is loosened from the nut. Adequate digestion is necessary to prevent oil losses in the subsequent pressing.

(d) Oil Extraction

The oil from the digested fruits are "extracted" by screw presses. Screw presses consist essentially of perforated cages in which are run single or double screws. Crude palm oil liquor and a matte of oily fibre and nuts are discharged from the screw presses. The fibre and nuts are conveyed to nut/fibre separator, where the nut is separated from the fibre, dried in the silo dryer and the shell is removed from the kernel in the nut crackers. The kernels are recovered for kernel oil extraction after the removal of shell in hydroclone and dried again in a silo dryer.

(e) Clarification and Purification of Crude Palm Oil

Crude oil extracted from the screw presses contains varying amounts of water together with impurities. The impurities consist of vegetable matter, some in the form of insoluble solids and some dissolved in the water. The impurities and water are removed in the clarification process using either "gravity settling" or the "direct centrifugation process". The oil recovered from either decanter (direct centrifugation process) or the settling tank (gravity settling) is passed to a centrifuge and then to a vacuum dryer. The final oil from both systems is pumped from the clarification plant to storage tanks.

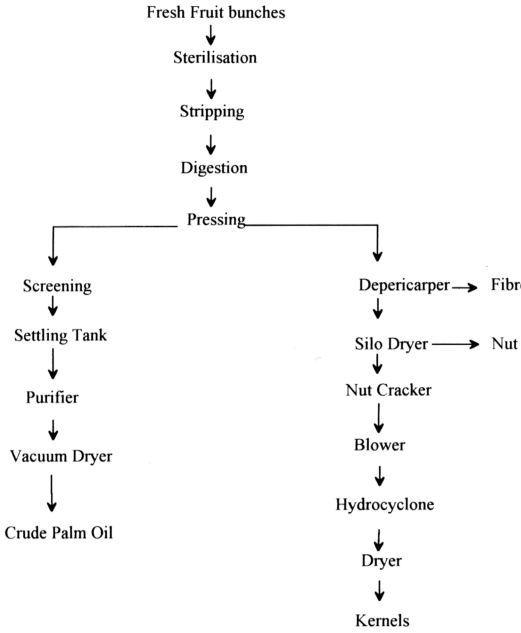


Figure 1.1 Extraction of crude palm oil

1.1.6 Refining of Palm Oil

Purification or refining processes are needed for crude palm oil to reduce as far as possible those contaminants of the crude oil which will adversely affect the quality of the end product and the efficient operation of modification technologies such as fractionation, hydrogenation and interesterification. Refining attempts to remove the causes and products of hydrolysis. e.g. moisture, insoluble impurities, free fatty acids, partial glycerides, enzymes, trace metals, volatile aldehydes and ketones, and pigments. Other impurities such as phosphatides, carbohydrates, pectins and glucosides need to be removed in order to improve refining and fractionation efficiency, and to obtain good quality refined oil.[31]

Two methods are being used to remove fatty acids; these are termed 'physical' method and 'chemical' method depending on the means by which free fatty acids are removed from the oil. Fatty acids are distilled off in the physical process, and in the chemical process they are neutralised. The processing steps involved in the two methods are depicted in Fig. 1.2 and the principal impurities removed are as shown in Table 1.5.

Of the two methods, the physical process is more efficient in terms of yield of products and energy (and materials) consumed. Additionally, physical refining presents few effluent treatment problems mainly because no soap is produced. As a result of these factors, the major part of the world's palm oil production is today refined using the physical method.

Table 1.6 shows the normal quality parameters measured before and after the refining process. The product after refining is normally referred to as refined, bleached and deodorised palm oil.

Table 1.5 Refining - Unit Processes

Stage	Principal impurities reduced or removed
Degumming	Phospholipids, trace metals, pigments, carbohydrates, proteins.
Neutralisation*	Fatty acids, phospholipids, pigments, trace metals, sulphur, oil insolubles, water solubles.
Washing*	Soap.
Drying	Water.
Bleaching	Pigments, oxidation products, trace metals, sulphur, traces of soap.
Filtration	Spent bleaching earth.
Deodorisation	Fatty acids, monoglycerides, oxidation products, pigment decomposition products.
Polishing	Removal of trace oil insolubles.

* in chemical refining

Table 1.6 Typical Specifications of Palm Oil during Physical Refining[31]

	Crude palm oil	Degummed & bleached	Refined, bleached & deodorised
FFA (%)	2-5	2-5	0.05
M&I (%)	0.15-0.3	0.02	0.02
Red colour	orange	orange	2.5
(5-25 in. Lovibond)	red	red	
PV (meq/kg)	1-5	0	0
AV	2-6	2-6	1-3
Carotenes (ppm)	500-700	-	-
DOBI	2-3.5	-	-
Phosphorus (ppm)	10-20	4	4
Fe (ppm)	+10	0.15	0.15
Cu (ppm)	0.05	0.05	0.05
Vitamin E (ppm)	600-1000	-	-
Diglycerides (%)	2-6	-	-

* FFA = free fatty acids, M&I = moisture and impurities, PV = peroxide value, AV = anisidine value, DOBI = deterioration of bleaching index, Vitamin E = total tocopherol and tocotrienols.

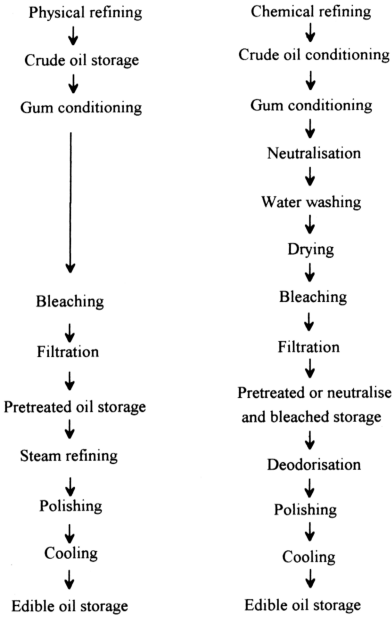


Figure 1.2 Refining of palm oil

1.2 CAROTENOIDS

1.2.1 Introduction

Of the various classes of pigments in living organisms, there can be no doubt that carotenoids are among the most widespread and important. The carotenoids are believed to have derived their names from the main representative of their group, β-carotene (*1.1*), the orange pigment first isolated from carrot root, *Daucus carota*, by Wackenroder in 1881.[32] They are found throughout the plant kingdom, though their presence is often masked by chlorophyll. They are responsible for many of the brilliant yellow and red colours in flowers and fruits. They are also found in animals, being responsible for the colour of birds, fishes, insects, and some invertebrates. Since carotenoids are synthesised only in plants, plant carotenoids constitute the source of all animal carotenoids. Animals may deposit them unaltered in their tissue or, as crustaceans and birds do, metabolise them further, usually to keto and hydroxyl derivatives.[20,33] In higher animals, a limited number of carotenoids are intestinally cleaved to yield retinal.[34,35]

β-Carotene is by far the most common of all carotenoids present in higher plants. In quantitative term, it is not as important as certain xanthophylls, such as lutein (*1.6*), violaxanthin (*1.7*), neoxanthin (*1.8*) and fucoxanthin (*1.9*) (widespread in marine algae). The three latter compounds occur, with β-carotene, universally in the leaves of higher plants.

1.2.2 Structure

Carotenoids belong to the class of polyenes and represent the most unsaturated mass products of biosynthesis. Most naturally occurring carotenoids contain 40 carbon atoms,

(tetraterpenoids), corresponding formalistically to 8 isoprene units, so that the linking of the units is reversed at the centre of the symmetrical molecule. The number of conjugated double bonds varies from 3 to 15 and is 10 or 11 in many common carotenoids. The bright colours of the carotenoids are due to the presence of a chain of conjugated double bonds constituting a chromophore. However, there are a few "colourless carotenoids" such as phytofluene (*1.10*) in which only 5 double bonds are conjugated. Phytofluene is practically colourless but fluoresce intensely in ultraviolet light. The lowest member of this series is phytoene (*1.4*) with 3 conjugated double bonds; it shows neither colour nor fluorescence. Cyclisation of the carbon skeleton, at one or both ends, also occurs in some carotenoid compounds.

There are two main groups of carotenoids, the hydrocarbons (carotenes) and the xanthophylls. The xanthophylls are formed from the hydrocarbon carotenes by the introduction of oxygen functionality, especially with hydroxy, oxo, and epoxy groups. Most of the substitutions are usually on the ring positions 1 - 6 and 1` - 6`. The structures of most known carotenoids are given by Straub,[36] while annual reviews of advances in carotenoid chemistry can be found in publications of chemical society (e.g. Britton[37]). The structural formulae of some important carotenoids are listed in Fig. 1.3.

Owing to the double bonds in the molecule, all carotenoids exhibit *cis-trans* isomerisation. In nature, the carotenoids are predominantly by all-*trans* isomers, which are more stable but naturally occurring *cis* and poly-*cis* isomers have been isolated.[38] A detailed study on *cis-trans* isomerisation of carotenoids was reported by Zechmeister.[39]

1.2.3 Nomenclature

Rules for the nomenclature of carotenoids (semi-systematic names) have been published by the International Union of Pure and Applied Chemistry (IUPAC) and IUPAC-International Union of Biochemists (IUB) Commissions on nomenclature.[40] For the most common types of

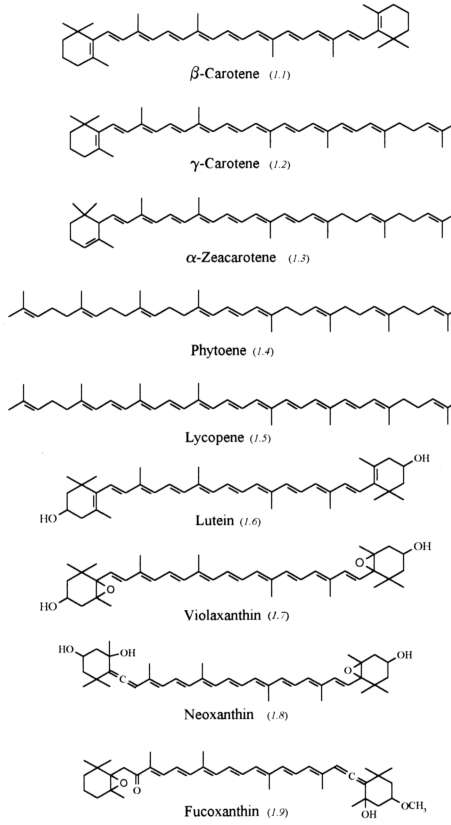


Figure 1.3 Structural formulae of some important carotenoids

carotenoids, trivial names are normally used. All specific names are based on the stem name, carotene, which corresponds to the structure and numbering in Fig. 1.4. The name of a specific compound is constructed by adding two Greek letters as prefixes (Table 1.7) to the stem name, carotene; the Greek letter prefixes are cited in alphabetical order. About 600 carotenoids have been isolated from natural sources; they are listed with their trivial and semi-systematic names in Key to Carotenoids.[41]

1.2.4 Biosynthesis

Extensive studies over the past 30 years have established that the general features of carotenoid formation are similar in higher plants, algae, fungi and bacteria. Comprehensive reviews of the biogenesis of carotenoids have been published.[42-45]

In general, the first specific precursor of all terpenoids is mevalonic acid (MVA; C6), which is converted to the universal C5 biological isoprene precursor, isopentenyl pyrophosphate (IPP). After the isomerisation, followed by a series of condensation reactions, and a dimerisation of carbon 20 intermediate, the first basic carotene, phytoene (*1.4*) is formed.[43]

The sequential desaturation of phytoene to lycopene (*1.13*) involves a series of didehydrogenations alternatively to the left and right of the central phytoene to produce, in succession, phytofluene (*1.10*), ζ-carotene (*1.11*), neurosporene (*1.12*) and lycopene (*1.13*) as shown in Fig. 1.5.

Cyclisation can take place at one end of the molecule after desaturation of the 7,8-double bond (formation of neurosporene) and the second cyclisation at the opposite end of the carotene molecule must await corresponding desaturation. On the other hand, lycopene can be cyclised to form γ-carotene and β-carotene directly. An overall scheme for the formation of monocyclic- and bicyclic-carotenes is shown in Fig. 1.6.

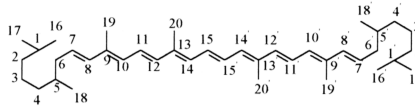
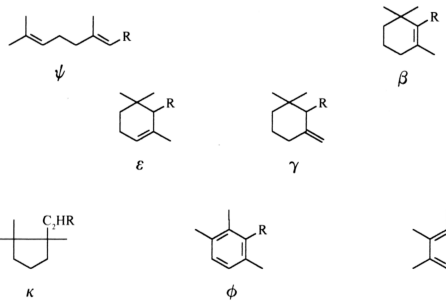


Figure 1.4 Structure and numbering of the parent carotene

Table 1.7 End Group Designation of Carotenes

Type	Prefix
Acyclic	ψ
Cyclohexene	β,ε
Methylenecyclohexane	γ
Cyclopentene	κ
Aryl	ϕ,χ



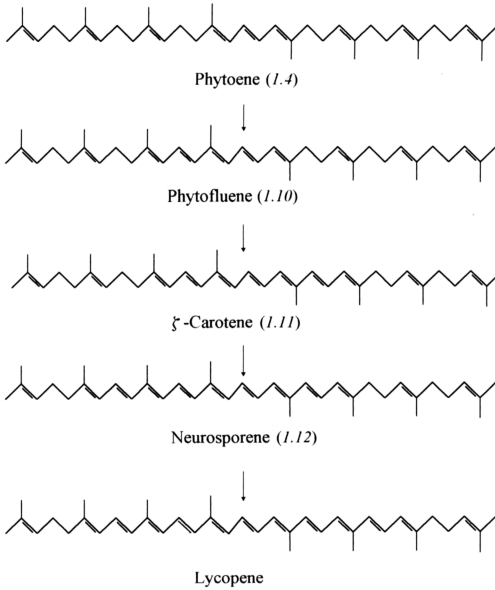


Figure 1.5 Formation of hydrocarbon carotenes

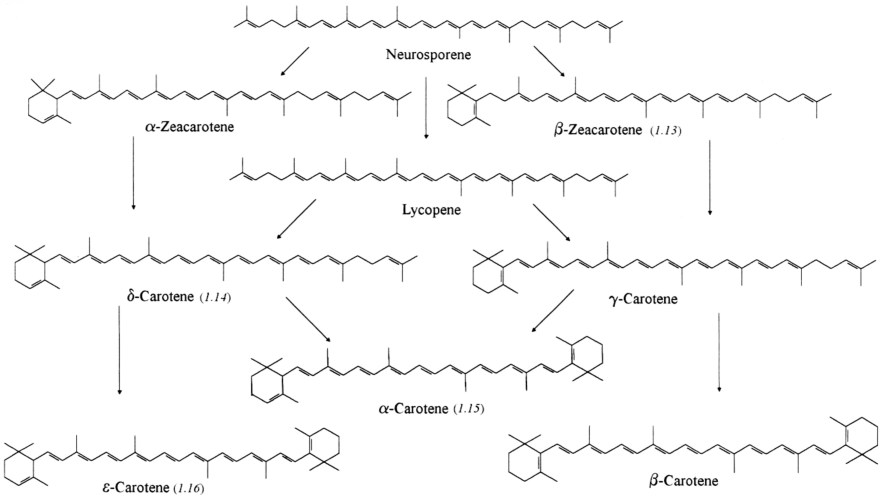


Figure 1.6 Overall scheme for the biosynthesis of cyclic carotenes

1.2.5 Physical Properties

(a) Absorption Spectroscopy

One of the most important characteristics of a carotenoid is its electronic absorption spectrum. This technique has been the cornerstone for identifying carotenoids ever since it was introduced into biochemical research some fifty years ago.[43] This is a function of the chromophore; the initial contribution of the polyene chain is influenced both by the nature of certain other structural features and by the solvent. The position of the long-wave absorption bands (usually three) of carotenoid is a function of the number of conjugated double bonds in the molecule. An increase in this number results in an increase in the wavelengths of maximal absorption, but this effect may be modified by cyclisation and the number and the type of oxygen substituents. The effects of the substituents and solvent used on the main absorption maxima of carotenoids are shown in Tables 1.8 and 1.9 as discussed in detail by Davies.[46]

Table 1.8 Effect of Substituents on the Main Absorption Maxima of Carotenoids[47]

Substituent	Position	nm
Double bond	chain	+7 - 35
Double bond	ring	+5 - 9
Carbonyl (first)	chain	28
Carbonyl (second)	chain	+1 - 7
Carbonyl (first)	ring	7
Carbonyl (second)	ring	+5 - 9
Epoxide 5,6	-	-8
Epoxide 5,8	-	-20
Trans — *cis*		-4
Normal — *retro*		-10

Table 1.9 Effect of Solvent on Absorption Maxima and $A^{1\%}/_{1cm}$ of β-Carotene[48]

Solvent	Position of main absorption maximum (nm)	$A^{1\%}/_{1cm}$
Hexane (light petroleum)	453	2592
Ethanol	453	2620
Cyclohexane	457	2505
Benzene	465	2337
Chloroform	465	2396
Carbon disulphide	484	2008

Structural variations also affect the degree of fine structure (persistence) and the intensity of the absorption. The introduction of a ring double bond into conjugation with the acyclic polyene system and the conjugation of the polyene chain with another chromophore, e.g. a carbonyl group, twist the chromophore out of plane and reduce both fine structure and intensity (hypochromic effect).[43]

Carotenoid spectra are also affected by the geometrical isomerisation. The formation of a *cis*-isomer from a parent *trans* compound results in a hypsochromic shift accompanied by a reduction in fine structure and a hypochromic effect on absorbance. These changes are accompanied by the appearance of a characteristic "*cis* peak" in the ultraviolet region of the spectrum.

(b) Other Spectroscopic Methods

At present, various advanced spectroscopic methods have been used to determine the complex structure and configuration of new carotenoids. These include infrared spectroscopy (IR), mass spectrometry (MS), proton magnetic resonance spectroscopy, carbon magnetic resonance spectroscopy, optical rotary dispersion and circular dischroism. Details on the application of these methods on carotenoid analysis have been reported.[47,49,50]

1.2.6 Chemical Properties of Carotenoids

There are a number of simple chemical tests which are useful in the determination of carotenoid structure, especially on the oxygen functional groups. Carefully chosen chemical tests are able to confirm suggestions of structure arising from spectroscopic evidence and are often crucial in the recognition and differentiation of oxygen functions. Common chemical tests include epoxide, carbonyl reduction and hydroxyl functional group tests (which include esterification, silylation and allylic hydroxyl tests).[43,47,51]

1.2.7 Applications of Carotenoids

(a) Colourant

Carotenoids are important constituents of most foods and act as colouring agents, additives or stabilisers. They are also useful in the pharmaceutical industry as coating agents for drugs to give appetising colours and flavours.[52]

Some natural plant pigments used as food colours are annatto, paprika extracts, alfalfa and tagetes extracts, tomato extracts, and carrot extracts. These natural extracts are gradually being replaced by synthetic carotenoids, namely β-carotene, β-apo-8'-carotenal, and

canthaxanthin. β-Carotene, which has vitamin A value, is the most recommended colourant, producing yellow to orange colours; the other two colourants produce a red hue. These carotenoids are added directly to food for human consumption. Other carotenoids, called pigmenters, when added to animal food will colour either the body tissue (skin and fat) or animal products such as milk, eggs, butter, and cheese.

(b) Medical Applications

The medical applications of carotenoids have been reviewed by Mathews Roth.[53,54] The established and primary use of food carotenoids is for the prevention or correction of vitamin A deficiency in man. β-Carotene is also being used in the therapy of erythropoietic protoporphyria, in which sensitivity to visible light is a cardinal symptom.[55] Recently, carotenoids are being used as potential prevention agent for a number of cancers, especially lung, skin and stomach cancers.[56,57]

1.3 ANALYTICAL TECHNIQUES

1.3.1 Introduction

In recent years, there has been special emphasis on understanding the types and concentration of carotenoids in fruits, vegetables, foods and also in the plasma. This is due to the recent epidemiological evidence that have suggested an inverse relationship between the consumption of carotenes and the incidence of several types of human cancers.[58] There is also doubt that previously reported values of vitamin A activity in food composition tables may not be accurate. Data on β-carotene content in food or reported in food consumption tables may be too high because they are based on methods that determine the total carotenes without separation of β-carotene from other carotenoids which have lower or are without vitamin A activity.[59] As only 50 out of 600 types of natural carotenoids have pro-vitamin A activity and the methodologies used were not sufficiently discriminative, most of the reported values had included carotenoids which do not possess vitamin A activity.

On the other hand, some carotenoids devoid of vitamin A activity such as lycopene have been reported to be associated with lower cancer risk.[60] Thus improved analytical methods for the various carotenoids would greatly help to determine the compounds of interest not only in food but also in the plasma.

Detailed analytical methods and general precautions to be taken for carotenoid analysis have been described by Britton and Goodwin[61] and De Ritter and Purcell,[62] but the most detailed coverage is by Davies.[46] Updated reviews have been done by Taylor[63] and Britton.[64]

In general, the analyses of carotenoids involved extraction, treatment of the extract by saponification, sterols removal, phase separation and finally chromatographic separation of individual carotenoid. Further purification may be necessary for spectroscopic identification, e.g. crystallisation or chemical modification.

The main problem associated with work on carotenoids arises from the inherent instability of the pigment. They are especially sensitive to light, heat, oxygen, acid and in some instances (e.g. astaxanthin, fucoxanthin etc.) to alkali. Any failure to strictly observe a number of general precautions on the handling of carotenoids in the laboratory may result not only in low overall quantitative recovery, but also in the possible loss of certain carotenoids particularly labile carotenoids. It may also result in their conversion to other carotenoids and in the appearance of *cis-trans* isomers as artefacts. Solvent containing acids and acidic chromatography absorbents (e.g. acid-washed alumina and untreated silica gel) should be avoided. Due to the evaporation of large volumes of solvent in carotenoids recovery, the purification of solvent for carotenoids analysis and separation need to be particularly vigorous. Carotenoids should always be stored in the dark, under nitrogen (or in case of solids, in vacuum) at about -20°C. Pure carotenoids are best kept as crystallise solids, but the pigments may be stored in solution using a hydrocarbon solvent.

1.3.2 Extraction

Since carotenoids have such a wide natural distribution and occur in a large variety of types of tissues, no one method of extraction can be said to be universally applicable and adopted as a standard technique. Details of the extraction of carotenoids from various sources have been published.[65,66]

To avoid pigment decomposition, air-drying of the biological material is normally avoided. Instead, instant hypotilisation or dehydration by treatment with aqueous methanol are being used. Dry material can be extracted with water-miscible solvent, however, extraction is more efficient and easier to carry out with most samples using water-miscible solvent such as mixtures of acetone and methanol; for certain cases mechanical grinding of material could be useful for complete extraction.

1.3.3 Saponification

Due to the presence of large quantities of unwanted lipids and chlorophylls in most of the plant extracts, saponification is normally included in the purification procedure to simplify the chromatographic analysis in later stages, but this of course cannot be carried out for the alkali-unstable carotenoids and carotenol-esters. Many examples have been described in detail elsewhere.[46,67]

In addition to carotenoids, the unsaponifiable fraction also contains other components (e.g. sterols) which could be removed through crystallisation from light petroleum at -10°C. Liquid-liquid partition could be useful for both crude extracts or the unsaponifiable fraction to separate the polar and non-polar fractions of carotenoids.

1.3.4 Separation of Carotenoids

The most important technique in the isolation of individual carotenoid is based on various chromatography techniques. There has been a lot of work on the development and improvement on the separation, detection and quantification of carotenoids mainly based on

chromatography, especially high performance liquid chromatography. In certain cases, pre-separation through phase separation was found to be useful.

(a) Phase Separation

The separation of carotenes from xanthophylls can be achieved by solvent partition between petroleum ether and aqueous methanol. This method is still being used to obtain a preliminary fractionation before chromatography.[68]

A method based on liquid-liquid partition by counter-current distribution was used with the Craig apparatus for carotenoid fractionation.[69] However, the method was tedious and not being further used, this was later replaced with droplet counter current chromatography which was used to separate the carotenoids of pasley.[70]

(b) Open Column Chromatography

Ever since the classical separation of carotene from chlorophylls by Tswett[71] and the development of absorption column chromatography by Kuhn,[72] Zechmeister[73] and Strain,[74] the separation of carotenoids using open column chromatography has provided one of the best samples of the technique with different absorbents being used according to the type of carotenoid to be separated.[46,75] Although recently this method has been superseded by high-performance liquid chromatography, it is still useful for pre-separation.

(c) Thin Layer Chromatography

Thin layer chromatography (TLC) and paper chromatography have been succesfully used in carotenoid separation and purification.[44,76-79] They may be used alone for quantitation of small samples or used in combination with column chromatography. The methods permit rapid and sharp separation and are generally applied on a micro or semimicro scale. They were shown

to be invaluable for quantitative analyses and identification of many types of carotenoids. Choice of various absorbents and solvent systems as well as the R_f values for some major carotenoids have been published for both thin layer and paper chromatography.[77]

(d) Gas Liquid Chromatography

In the 1970's a few studies of carotenoids were carried out using gas-liquid chromatography. However, the thermal instability of carotenoids precludes their direct examination by gas-liquid chromatography and this technique is only applicable to the perhydro derivatives which have limited application.[80]

(e) High-Performance Liquid Chromatography

From the late 70's, the advent of high-performance liquid chromatography (HPLC) has brought to liquid-solid chromatography the elegance and precision of separation that have previously been the characteristics of gas-liquid chromatography. Rapid separation, high resolution capacity and its non-destructiveness have shown to be a method of choice for modern carotenoid analysis, and HPLC has now replaced the standard methods of open column chromatography and TLC.[81,82] Recently, there have been rapid advances in the development of the separation techniques and instrumentation for more accurate identification and quantitation of carotenoids from various sources using HPLC. HPLC has shown to be able to minimise the isomerisation and decomposition of these light, heat, and air-sensitive compounds. With this rapid, reproducible and highly sensitive technique, the separation, identification and estimation of carotenoids are achieved simultaneously.[83]

There are many recent publications discussing the application of both normal-phase and reversed-phase HPLC to resolve complex carotenoid mixtures. HPLC systems using silica, alumina or magnesia have been shown to be of particular benefit for the separation of

certain *cis-trans* isomers and diastereoisomers but fail to distinguish between positional isomers such as α- and β-carotenes.[84,85] These supports have also been suspected to catalyse carotenoid degradation[86] and the silica also causes isomerisation of *cis* isomers to the more stable all *trans* isomers.[87]

In recent developments, reversed-phase (RP) materials have superseded their normal-phase counterparts for carotenoid chromatography. It has been shown to give better separation of hydrocarbon carotenes, and when compared to silica, reversed-phase materials show enhanced stability and improved reproducibility, hence, RP is preferable for quantitative work.[83,88] A drawback of several "conventional" RP materials, however, is their relatively low retentivity toward the more polar derivatives (xanthophylls).[89] As a result, substantial amounts of water have to be included in the eluent to ensure sufficient solute retention. However, this could cause a solubility problem and affect the peak shape.[83]

In order to cover the whole range of polar and non-polar carotenoids, attempts have been made using gradient elution. In general, it has a number of disadvantages, the most notable is the long equilibration periods needed between runs.[83,88]

In the 80's, the concept of nonaqueous reversed-phase chromatography on highly retentive packing material was recommended as a useful approach for the chromatography of nonpolar compounds.[88] It has been shown to have provided a lot of benefits which include enhanced sample solubility, chromatographic efficiency, good recovery, increased sample capacity and column lifetime. This method has been successfully applied to carotenoid profiling of extracts from human serum and almost all samples from animal and vegetable origin.[90,91] Many other researchers have since developed nonaqueous reversed-phase high-performance liquid chromatography conditions employing a variety of organic solvents and various types of HPLC columns.[92-96]

1.3.5 Detection

Carotenoids strongly absorb light in the 400-500 nm region except for the less conjugated carotenes such as phytoene and phytofluene which absorb at the 280-374 nm region. Absorption detection at these high wavelengths is particularly favourable in terms of selectivity (little interference from biological materials) and sensitivity (molar absorption coefficients of 10^5 and more). So far, other detectors have not found any application.

A major problem that is to be faced is the identification of chromatographic peaks in complex pigment mixtures. Recently, on-line recording of the characteristic absorption spectra using a modern scanning detector, such as photodiode array detector, have provided useful structural information, and it has been shown to be a suitable analytical tool for identification of carotenoids.[97,98]

1.4 RECOVERY OF CAROTENOIDS

1.4.1 Introduction

Before the commercial production of synthetic β-carotene, the carotenoids were commercially extracted primarily from three natural sources, i.e. carrots (*Daucus carota*), alfalfa (*Medicago Sativa*) and palm oil. It involved different processes for each different sources.[99-104] However, all these processes were rapidly overtaken by total organic synthesis of β-carotene developed by Imhoffen and Bohlmann.[105] This synthesis was later developed into an industrial process by Hoffmann La Roche and the synthetic β-carotene has been produced commercially since 1954.[46] Today, β-carotene manufacturers are also producing some oxidised derivatives such as canthaxanthin and astaxanthin from β-carotene by synthetic procedures.[106] In 1989, synthetic carotenoids accounts for almost 90% of the total market, as it can be produced at a reasonable price with high purity and uniform colour.

1.4.2 Natural Carotenoids

It is estimated that global commercial carotenoid production in 1989 was about 300 metric tonnes. Natural carotenoids accounted for approximately 10% of the total market and the demand for natural carotenoids appears to exceed supply. As the recent perception of carotenoids as an anticarcinogenic material or of other medicinal value, it was forecasted that there will be increased demand of carotenoids, especially from natural sources which are valued for their perceived safety.

The natural carotenoids in the market available now are mainly from carrot oil and microalgae. The production costs for the extraction of carotenoids from carrots are relatively high, as it involves solvent (*n*-hexane) extraction and the carotene content of carrot is relatively low (50-100 ppm). Moreover, there is a strong seasonality in the supply of fresh carrots. Presently, carrot oil is produced on a small scale and the carrot carotene contains 30-50% of α-carotene in addition to β-carotene.

Most of the natural β-carotene is produced through the mass cultivation of two species of microalgae[106,107] namely *Dunaliella saline* and *D. bardawilli*. This offers two advantages over other natural sources of compounds such as carrots, apricots and other green plants, as carotenes are produced in relatively high concentration in the cell mass and there is little cellulosic tissue to hinder extraction.

Culture methods have evolved along two different lines: extensive and intensive. Extensive algaculture involves harvesting natural halotolerent algae from saline water. Intensive algaculture involves the growth of the microalgae in bioreactors. Carotenes from *Dunaliella* contains 6-10% of α-carotene in addition to β-carotene (Table1.10).

1.4.3 Recovery of Carotenoids from Palm Oil

Besides carrot and microalgae, crude palm oil has been known to be a good source for carotenoid recovery as the oil contains the highest known concentration of agro-derived carotenoids.[28] The production of palm oil is increasing rapidly in Southeast Asia and it is expected that by the year 2000, palm oil will be contributing to 21.1% of the world's supply of oils and fats. Since palm oil is derived from a perennial crop, a reliable supply is available throughout the year, as opposed to most vegetable oils, which are from annual seasonal crops.

Apart from the older methods mentioned earlier, numerous extraction methods have been developed to recover the carotenoids from palm oil. These include urea process,[108] adsorption,[109-113] selective solvent extraction[114] and molecular distillation.[115] However, most of the reported methods are difficult, inefficient or costly.

Recently, preparation of palm oil methyl esters on a large scale for oleochemicals or diesel substitutes has been carried out.[116,117] The mild reaction converts palm oil (triglycerides) to volatile methyl esters and results in little, if any, loss of carotenoids. This allows for a unique opportunity for the recovery of carotenoids in palm oil. The carotenoids have been concentrated or recovered from the esters through a few routes; these include adsorption,[118] solvent-solvent extraction[119] and distillation.[120]

A commercial oleochemical plant constructed by Lion Corporation in Japan had made efforts to recover carotenoids through transesterification, solvent extraction and purification by liquid chromatography. The palm carotene obtained after liquid chromatography contains more than 95% of carotenoids.[106]

Table 1.10 shows the major carotene compositions of palm oil as compared to other carotenes in the market. Synthetic β-carotene consists of only β-carotene, *Dunaliella* carotene consists about 90% β-carotene. On the other hand, palm oil carotene consists of 60-65% of β-carotene which is quite similar to carrot carotene.

Table 1.10 Carotenoid Compositions of Various Carotenes[106]

	α-Carotene	β-Carotene	Other carotenoids
Carrot carotene	30-50	40-60	10-15
Synthetic β-carotene	0	100	0
Dunaliella carotene	6-10	84-90	0
Palm oil carotene	20-35	60-65	5-10

1.5 PROPERTIES OF CAROTENOIDS

The functions of carotenoids can readily be attributed to their chemical structures. They have the capacity to quench or inactivate excited states of molecules, for example, the quenching of excited state molecules formed in photosensitised reactions. This property depends on the length and rigidity of the molecule, the length of the conjugated chromophore, and hence its quenching and light absorbing ability, the nature of the end groups and the presence of substituents.

1.5.1 Antioxidant and Singlet-Oxygen Quenching Properties

(a) A Singlet-Oxygen Quencher

Carotenoids were first shown to quench excited singlet oxygen in 1968 by Christopher Foote and Robert Denny,[121] and the maximum quenching effect was found in carotenoids having nine or more double bonds. β-Carotene, for example, with 11 conjugated double bonds, can distribute energy over all its double and single bonds. The energy transferred to β-carotene can be released as heat and the β-carotene eventually reverted to its original energy level. The reaction does not destroy the β-carotene molecule but transforms singlet oxygen into a stable oxygen species lacking the energy to engage in harmful reactions. Carotenoids with five and three conjugated double bonds (phytoene and phytofluene) offer no protection. Recently, Di Masscio *et al*[122] have demonstrated that lycopene was approximately twice as effective as β-carotene in quenching $^1O_2$. Their data suggested that the quenching properties of carotenoids reside not only in the length of the conjugated double bond system but also in the structure of the pigment.

(b) Antioxidant Activity of Carotenoids

In addition to preventing free radical formation resulting from reactions involving singlet oxygen, β-carotene can react with or scavenge free radicals directly and thus act as an antioxidant.[123,124] These activities were shown by a number of *in vitro* studies, including lipids in homogeneous solutions,[125] liposomes,[126-127] isolated membranes[128,129] and intact cells.[130,131] Additionally, direct evidence of carotenoids functioning as antioxidants *in vivo* has been reported in animal models.[132-137]

Carotenoids are known to be able to limit the oxidative damage induced by oxy radical-generating systems. This protection involves both nuclear and lipid molecules. β-carotene is able to reduce the extent of nuclear damage[138] as well as to inhibit the lipid peroxidation induced by enzymatic sources of oxy radical[139-142] or nonenzymatic sources.[140,143-145]

Carotenoids are also being shown to be very effective quencher of peroxyl radicals,[146,147] but the mechanism of their antioxidant action has not yet been defined. A hypothesis has been presented by Burton and Ingold[148] and expanded by Burtons.[149] Unlike antioxidants that prevent the initiation of lipid peroxidation, β-carotene stops the chain reaction by trapping free radicals, and β-carotene is found to be most effective at the low oxygen concentrations found in capillary beds in tissues for removal from direct exposure to oxygen.[146]

1.5.2 Photofunctions in Plants

Photofunctions of carotenoids in plants have been reviewed comprehensively by Krinsky[150] and updated by Mathis and Schenck.[151] From the variety of functions attributed to these pigments, two principal functions were identified as essential for the plant's survival. The first is that they act as protective agents to prevent cells from undergoing damage due to a

photodynamic action. The second is that they can act as accessory pigments in photosynthetic organisms, transferring radiant energy to the actual pigments involved in photosynthesis.

1.5.3 Physiological Functions of Carotenoids

In animals, carotenoids are not synthesised by the animal itself but have to be ingested with feed directly or in the form of precursors. Some of the established functions of carotenoids are their provitamin A and antioxidant activities, their abilities to quench singlet oxygen and inhibit the growth of certain tumours.

The hypothesis that carotenoids provide an apparent chemopreventive action is supported by strong scientific rationale and animal experiments. Carotenoids may be protective against cancer through its antioxidant function since oxidative products may cause genetic damage.

(a) Pro-Vitamin A Activity

One of the most important and established physiological functions of carotenoids is to act as vitamin A precursors for the organism. Almost all animal species are able to enzymatically convert plant carotenoids of a suitable structure into vitamin A.[152] The precursors of vitamin A are those which contain at least one β-ionone ring at one end of the molecule. Although more than 600 carotenoids occur in nature, only about 50 are precursors of vitamin A. Of these, only a few occur in sufficient concentration to play a significant role in the human diet. Of all the known carotenoids, β-carotene, with two β-ionone units, possesses the highest pro-vitamin A activity; all other provitamin A carotenoids have the intact β-ionone ring at only one end of the molecule.[153] The vitamin A activity of some carotenoids found in vegetables is listed in Table 1.11.

The transformation of β-carotene to vitamin A (retinol) in the animal has been proposed to occur in the intestinal mucosa by cleavage of the carotenoid molecule at the central double bond[154] or by the cleavage of the conjugated chain adjacent to one β-ionone ring followed by sequential oxidative removal of fragments containing 2 to 5 carbon atoms through the intermediate apo-carotenal homologous.[155-157] The weight of the evidence available so far overwhelmingly favours random cleavage.[158] The main point of conversion of ingested carotenoids is the intestinal mucosa by the carotene enzyme, dioxygenase, although other organs could also convert β-carotene into vitamin A but much less effectively.[159] The retinal (vitamin A-aldehyde) form from the central cleavage of β-carotene will undergo reduction to retinol as shown in Figure 1.6. Retinol is then esterified mainly with long chain fatty acids. Other derivatives such as retinoic acid (vitamin A-acid), 11-*cis* vitamin A-aldehyde were also formed and then absorbed for various purposes.[160]

Table 1.11 Relative Vitamin A Activities of Some Carotenoids

Carotenoid	Activity (%)
all-*trans*-β-Carotene	100
9-*cis*-β-Carotene	38
13-*cis*-β-Carotene	53
all-*trans*-α-Carotene	53
9-*cis*-α-Carotene	13
13-*cis*-α-Carotene	16
all-*trans*-Cryptoxanthin	57
9-*cis*-Cryptoxanthin	27
15-*cis*-Cryptoxanthin	42
β-Carotene 5,6-epoxide	21
β-Carotene 5,8-epoxide (mutatochrome)	50
γ-Carotene	42-50
β-Zeacarotene	20-40

Sources: From Zechmeister[39] and Bauernfeind.[153]

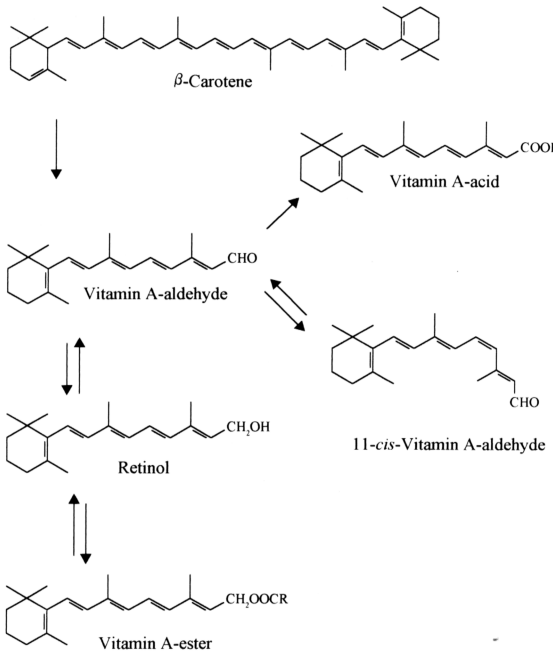


Figure 1.7 Retinol and its derivatives from β-carotene

(b) Photoprotection

The photoprotective effect of carotenoids in a wide variety of living systems[161] led to the use of β-carotene in the successful treatment of patients with inherited light-sensitive skin disorder such as erythropoietic protoporphyria.[162,163] It was also shown to have some value in patients with congenital porphyria.[164,165]

(c) Immunoenhancement

Carotenoids were also shown to be able to enhance some aspects of immune function in several animal studies,[166-168] including improved tumour resistance.[169,170] β-Carotene has been shown to enhance both specific and non-specific immune responses in experimental animals and *in vitro* models.[171] A human trial on short-term high-dose β-carotene supplementation on immune system response have shown that it is able to increase the number of T4 lymphocytes (helper cells) and did not affect T8 lymphocytes (suppressor cells).[172]

(d) Lung Cancer

Recently, there has been an increase in activity for various aspects of carotenoid research, in an attempt to establish the effectiveness of these natural compounds as antitumour agents. This research was stimulated by a publication,[58] about a decade ago, which suggested that dietary β-carotene might act as an anticarcinogenic agent.

More than 50 epidemiological studies conducted during the last decade in different parts of the world have consistently demonstrated that a high intake of food rich in β-carotene is associated with reduced risk of certain cancers.[173-175] The most significant pattern that emerges is that β-carotene seems to be most protective against lung cancer.[173]

A sizeable number of studies on β-carotene intake and serum levels have shown an association between high β-carotene intake and/or status and reduced risk of lung

cancer.[176-179] In more than 25 different studies groups of individuals with the highest consumption of green and yellow fruits and vegetables (or having the highest blood levels of β-carotene), generally had half the risk of developing lung cancer than those with lowest intake or blood levels of β-carotene. A large prospective dietary study carried by Shekelle *et al.*,[180] in Western Electric Co., Chicago, for 19 years, have shown that there was a sevenfold increase in the risk of lung cancer in smokers whose carotene index fell in the lowest quartile, as compared to those in the highest quartile of intake. The intake of retinol rich foods was not related to the risk of lung cancer.

Several studies have shown that lower levels of plasma carotene/β-carotene was found in smokers and smokers are known to be approximately 15 times more likely to develop lung cancer than nonsmokers.[181-183] Smokers ingesting the same amount of β-carotene as nonsmokers have shown to achieve lower serum levels of β-carotene.[184] However, there are also contradictions; for example a recent Finland study has thrown doubt in β-carotene as it was found to increase the incidence of lung cancer in heavy smokers.[185]

(e) Other Cancers

A strong association between low levels of carotene and greater cancer risk has also been reported for cervical[186-188] and endometrical cancers.[187,189] Again, there were no significant differences in plasma retinol levels.[187]

Relatively high intakes of β-carotene rich foods or higher blood levels of β-carotene has also been associated with significantly lower risk of other cancers such as esophageal, stomach and breast cancers.[173,190,191] Following the β-carotene supplementation, an increase in the β-carotene concentration in oral mucosal cells in most subjects was accompanied by lower numbers of micronucleated cells (MNC) at the side in the mouths, in this case vitamin A treatment also significantly lowered the incidence of MNC.[192,193]

A study carried out in Massachusetts[194] showed that a highest intake of carotene-containing vegetables had a threefold lower risk of all the cancer mortality than those with the lowest intake. Similar result was also shown by Wald, 1988, England, where serum β-carotene levels were found to be significantly lower for subjects who developed any type of cancer.[177]

(f) Cataract

Cataract is caused by the changes in the structure of the lens of the eye and have been linked to oxidative stress and radical damage.[195] To investigate the relationship between nutritional factors and antioxidant defence of the lens of the eye, Jacques *et al* conducted a retro-spective study of persons with cataracts and matched controls.[196] It was found that low levels of serum carotenoid group had over 5½ times the risk of developing cataracts than the group with high levels of serum carotenoids.

1.5.4 Safety

β-carotene is absorbed from the intestine with less efficiency as the dietary intake of β-carotene intake increases. The conversion of β-carotene to vitamin A also declines with increasing β-carotene intake.[197] The consequence is an increase in circulating β-carotene levels with no significant increase in circulating levels of vitamin A. Therefore, a high intake of β-carotene does not lead to abnormally elevated levels of vitamin A.

Studies have also shown that β-carotene is not mutagenic, carcinogenic or embryotoxic.[198] The safety of β-carotene supplementation at doses of 15-60 mg/day has been demonstrated in numerous clinical trials.[199,200]