

The medical importance of vitamin A and carotenoids (with particular reference to developing countries) and their determination

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ABSTRACT

Vitamin A, or retinol, is an essential nutrient for man and all mammalian species since it cannot be synthesised within the body. Deficiency of the vitamin results in adverse effects on growth, reproduction and resistance to infection. The most important manifestation of severe vitamin A deficiency (VAD) is xerophthalmia, and irreversible blindness may eventually occur in one or both eyes. VAD is still an important micronutrient deficiency problem in many developing countries, afflicting large numbers of pre-school children. It is often associated with protein-energy malnutrition, parasitic infestation and diarrhoeal disease. For many communities in developing countries, the major source of vitamin A in the diet is carotenoids. These compounds are synthesised only by photosynthetic microorganisms and by members of the plant kingdom where they serve important functions in metabolism, including participating in the photosynthetic process. These pigments also provide aesthetic qualities as colourants in the plant and animal kingdoms. Most importantly, the carotenoids serve the animal kingdom as sources of vitamin A activity. Major advances have occurred in understanding the role and mechanisms of action of carotenoids. They are now thought to play specific roles in mammalian tissues related to their function in plants. Carotenoids, with their highly reactive conjugated double bonds, act as free radical traps or antioxidants and may play an important role in the prevention of cancers. In view of the wide medical importance of carotenoids, much attention has been given to the determination of these pigments in foods as well as blood. Carotenoids in foods have conventionally been analysed using the open-column chromatography technique, but the high-pressure liquid chromatographic (HPLC) method is now gaining in importance as well. The classical method for the determination of carotenoids in blood is by the spectrophotometric method while the HPLC method is also recommended for use. An example of an HPLC method developed for the simultaneous determination of retinol and carotenoids in food and blood is given. The determination of retinol and carotenoids should be further developed in view of the wide importance of carotenoids in health and disease.

INTRODUCTION

Some 75 years ago, vitamin A was discovered as a fat-soluble growth factor present in liver, and recognized as an essential biochemical for normal vision in man and animals. It is, in fact, the first vitamin to be discovered. Today, after several decades, very little is known about the vitamin's biochemical mechanisms of action in the different functions except for the process of vision (Wolf, 1980). Vitamin A deficiency remains one of the major public health nutritional problems in many developing countries, and is an important cause of preventable blindness. Afflicting large number of pre-school children in South-East Asia, vitamin A deficiency is often

associated with protein-energy malnutrition, parasitic infestation, and diarrhoeal disease. The interaction between vitamin A and infection have recently been given a great deal of attention. The synergistic effect between vitamin A deficiency and infection may be responsible for excessive childhood morbidity and mortality in many developing regions of the world. Recent studies on vitamin A supplementation and child survival and mortality have created a great deal of interest and debate in the international health and nutrition community.

The major source of vitamin A in the diet of most communities in developing countries is carotenoids. These compounds are synthesized exclusively by photosynthetic microorganisms and by members of the plant kingdom where they play a fundamental role in metabolism. Aside from providing aesthetic qualities as colourants in the plant and the animal kingdoms, these pigments also take part in the photosynthetic process. Most importantly, the carotenoids serve the animal kingdom as sources of vitamin A activity.

The carotenoids, believed to have derived their name from the fact that they constitute the major pigment in the carrot root, *Daucus carota*, are undoubtedly among the most widespread and important pigments in living organisms. This group of pigments is found throughout the plant kingdom (although their presence is often masked by chlorophyll) and in insects, birds, and other animals. These pigments provide a whole range of light yellow to dark red colourings, and when complexed with proteins, green and blue colourations are achieved. Thus, a wide variety of foods and feeds - yellow vegetables, tomatoes, apricots, oranges, egg yolk, chicken, butter, shrimp, lobsters, salmon, trout, yellow corn, etc. - owe their colour principally to carotenoids, as do certain food colour extracts from natural sources such as palm oil, paprika, annatto, and saffron (Borenstein and Bunnell, 1966; Weedon, 1971; Bauernfeind, 1972).

This review discusses the medical importance of carotenoids and vitamin A, two large groups of interrelated compounds, which are still being actively studied all over the world. Many gaps in knowledge exist, and new frontiers are being pursued. A new frontier has been the examination of a possible association between carotenoids and retinoids and the development and prevention of cancer. Recent developments in studies into these possible roles of carotenoids and retinoids beyond their classical functions have created a great deal of excitement in the biomedical community. In keeping with these developments, there has been increasing interest in the types and concentrations of various carotenoids in foods and blood. The analysis of these compounds is also discussed in this review, with particular emphasis on a method developed in this laboratory for the simultaneous determination of retinol and several carotenoids in foods and blood.

FUNCTIONS AND USES

Carotenoids as Precursors of Vitamin A

Although it is incorrect to consider vitamin A activity as a general function of carotenoids, but the provitamin A activity of the carotenoids is nevertheless given emphasis in this discussion because of the importance of vitamin A in human nutrition.

Animals are not capable of *de novo* synthesis of vitamin A-active substances, neither pre-formed retinol and its derivatives nor the carotenoid precursor forms (Underwood, 1984). Thus, apart from pre-formed vitamin A contained in foods such as milk, eggs, liver, and fish liver oils, and their derivatives (and of course synthetic vitamin A), the main source of supply of this vitamin for man are the carotenes and related carotenoids. These pigments possess vitamin A activity and are the so-called provitamin A. Indeed, β -carotene is the most important vitamin A precursor in human nutrition, since its concentration in food and feed ingredients, particularly of leaf origin, greatly exceeds that of the other vitamin A active compounds (Bauernfeid *et al*, 1971).

The diets of population groups in the tropical world rarely contain milk, eggs or liver, which are the rich sources of pre-formed vitamin A. There has thus been a great deal of emphasis on carotenoids, particularly from leafy vegetables, as the sources of vitamin A to these communities. Vitamin A deficiency is prevalent, and the tragic irony is that there is an abundance of vegetation in these countries (Oomen & Grubben, 1981).

In the USA, the average diet of the usually available food is estimated to provide about 7500 IU (2250 retinol equivalent, R.E.) of vitamin A per day. About 3500 IU (1050 R.E.) is derived from vegetables and fruits, 2000 IU (600 R.E.) from fats and oils, and dairy products, and 2000 IU (600 R.E.) from meat, fish, and eggs (Bauernfeid, 1972). Various estimates of percentage contribution of provitamin A to the human diet place the figure at around 50% (Thompson, 1965; Witschi *et al.*, 1970; NAS, 1980). Amongst various underprivileged communities, the proportion must be even higher.

Provitamin A activity 'may be derived from various carotenoids. The above estimates of contributions of carotenoids to total vitamin A activity of diets must be assumed as tentative, until food tables become more complete in listing both β -carotene and other provitamins A.

Carotenoids in Photosynthetic Tissues

The other established functions of carotenoids in plants are related to their ability to absorb visible light. In the case of photosynthetic tissues, they appear to have two well defined functions: (a) in photosynthesis itself, and (b) in protection of the photosynthetic tissue against photosensitized oxidation.

Carotenoids could act as accessory light-absorbing pigments in the photosynthesis process. By their absorption at wavelengths lower than that absorbed by chlorophyll, they extend the wavelength of light that can be used in photosynthesis (Mathews-Roth, 1981).

It has been observed that carotenoid pigments in all photosynthetic organisms, bacteria, algae, and higher plants, play an important role in protecting these organisms against the seriously damaging effects of photooxidation by their own endogenous photo-sensitizer, chlorophyll. Experiments made in photosynthetic organisms which lacked coloured carotenoid pigments have supported these observations. Extending these data to disease treatment in human, carotenoids have been used for the treatment of patients with photosensitivity disease. In this disease, known as light-sensitive porphyria, the porphyrins produced resemble the porphyrin ring of chlorophyll and act as photo-sensitizer in the patient.

Antioxidant Functions of Carotenoids

Various proposals have been put forth to explain the protective function of carotenoids against harmful photosensitized oxidations discussed in the above section. Krinsky (1979) discussed the major mechanisms whereby these pigments exert this function: (1) quenching of triplet sensitizers; (2) quenching of singlet oxygen ($^1\text{O}_2$); (3) inhibition of free radical reactions. The photochemical reactions that can induce photodamage were reviewed, and the possible mechanism of action of carotenoids on the reactive chemical species produced was discussed. In photochemically induced oxidations, carotenoid pigments have been shown to have the capacity to quench the first potentially harmful intermediate, the triplet sensitizer, at a significant rate. The remaining triplet sensitizer species could then continue to initiate a series of reactions, depending on the availability of oxygen and the nature of other potentially reactive species in the environment, with the production of singlet oxygen and free radicals.

The ability of carotenoids to deactivate reactive chemical species such as singlet oxygen, triplet photochemical sensitizers and free radicals have been actively studied in recent years, with the main focus on β -carotene. Some insight into the antioxidant activities of other naturally occurring carotenoids have also been reported (Terao, 1989; Di Mascio *et al.*, 1989).

It has been suggested that reactive oxygen species and free radicals may play an important role in cancer development. These species are continually being formed in human tissues and their safe sequestration is an important part of antioxidant defence. Thus, the protective effects of carotenoids against the harmful effects of oxidation would be expected to have a protective effect against cancer.

Carotenoids as Food Colours

There is much to the saying: "Man eats with his eyes as well". Thus, colour of food is a significant factor in determining its acceptability. Man associates a particular food with its specific 'natural' colour. He becomes cautious when a food shows an unexpected colour, interpreting it as a possible sign of spoilage, poor processing, or adulteration. An important use of carotenoids (especially carotene) is in food colouring.

Natural extracts containing carotenoids have been used for colouring food for centuries: annatto with bixin as the main colouring component, saffron with derivatives of crocetin and other carotenoids, paprika containing the two pigments capsanthin and capsorubin, xanthophyll extracts from leaves, carrot extracts of varying purity, and red palm oil (Borenstein and Bunnell, 1966; Bauernfeind *et al.*, 1971).

Several synthetic carotenoids are presently available, making it possible for them to be used widely in colouring processed and fabricated foods (Bauernfeind, 1972). β -Carotene was the first synthetic carotenoid to be marketed in 1954 (Bauernfeind *et al.*, 1971), and is now probably the most widely used carotenoid for colouring foods. With the different commercial forms of this carotene, it is technically and economically feasible to colour a wide variety of fat or water-based foods including butter, margarine, cheese, ice cream, wheat products, vegetable oils, cake mixes, candy, soups, desserts, fruit juices, and beverages.

Bauernfeind (1972) has surveyed the worldwide legal status of β -carotene, β -apo-8'-carotenal, canthaxanthin, and β -apo-8'-carotenoic acid ethyl ester as food colours. β -Carotene is permitted in some 40 countries, whereas over 20 countries allow the use of the other three carotenoids.

Role of Vitamin A

The different categories of function of vitamin A in mammal can be broadly grouped under five headings: (1) vision, (2) bone growth, (3) reproduction, (4) maintenance of epithelia, and (5) overall growth (Wolf, 1980). The following discussion concentrates on two of these functions, that is, its role in vision and in the maintenance of epithelia.

The only established function of retinol in the retina is to serve as the precursor of 11-cis-retinal-dehyde, the chromophore of all known visual pigments. The need for retinol to supply the chromophores of the visual pigments is the reason why in vitamin A deficiency, the first symptom is a fall in sensitivity of both rod and cone vision, a condition known as "night blindness" or "nyctalopia" (Wald & Hubbard, 1970).

The most striking and extensive lesions caused by vitamin A deficiency are those affecting epithelial growth and differentiation. They are all defects of the outer and inner linings of the body, and consequently, invite invasion by microorganisms. There is generally an increase in the proportion of squamous keratinizing cells (cornification), accompanied by a decrease in the proportion of columnar, mucus-secreting cells. It is, however, known that different epithelia are affected differently. In epithelial tissues such as intestinal mucosa, where there are normally no keratinizing cells, there is simply a decline in mucus-secreting cells. In other tissues such as the cornea and epidermis, where there are normally no mucus-secreting cells, hyperkeratosis results from the deficiency (Wolf, 1980, De Luca *et al.*, 1979).

In the eye, apart from the defects in the retina caused by disappearance of rhodopsin, many epithelial lesions occur in vitamin A deficiency. These lesions are manifest in both the conjunctiva and cornea as a result of lack of vitamin A for maintaining the normal differentiation of epithelial tissue. The earliest change is in the conjunctiva tissue, resulting in one or more patches of dry, nonwetable conjunctiva, a condition termed conjunctival xerosis. This is sometimes accompanied by the appearance of small plaques of a silvery grey hue, usually with a foamy surface, called bitot's spots. In more severe vitamin A deficiency, the structural changes go on further to involve the cornea. In the beginning, corneal xerosis takes place, giving the cornea a hazy appearance. This progresses to corneal ulceration, frequently referred to as keratomalacia, and may eventually end in impairment of vision in varying degree.

MEDICAL IMPORTANCE OF VITAMIN A AND CAROTENOIDS

Recommended Levels of Vitamin A Intake

As with other nutrients, recommended levels of intake of vitamin A varies from country to country. This is clearly shown in a study of the recommended dietary allowances (RDA) of 17 countries for nine nutrients by the Committee on Recommended Dietary Allowances of the

International Union of Nutritional Sciences (IUNS, 1982). For vitamin A, the recommended level for adult men and women ranged from 600 to 1500 μg RE, with a mean value of 910 μg RE. The additional amount for pregnancy ranged from 0 to 1000 μg RE, and for lactation, from 200 to 1920 μg RE.

The daily intake of vitamin A recommended by FAO/WHO (1988) is tabulated in Table 1. According to these recommendations, intakes for children 1-6 years range from 350-400 μg per day, and 500-600 μg for older children and adults. There is no increase in recommended intake of vitamin A during pregnancy. However, the additional needs for lactation must be at least as great as the vitamin A secreted in the milk. This has been worked out to be 420 μg , based on an average milk yield of 850 ml per day, with a retinol content of 49 μg per 100 ml in a well-nourished population.

Table 1. Safe level of vitamin A intake

| Group | Age (years) | μg Retinol Equivalent per day |
|-----------------------------------|-------------|--|
| Infants and children (both sexes) | 0 - 1 | 350 |
| | 1 - 6 | 400 |
| | 6 - 10 | 400 |
| | 10 - 12 | 500 |
| | 12 - 15 | 600 |
| Boys | 15 - 18 | 600 |
| Girls | 15 - 18 | 500 |
| Men | 18+ | 600 |
| Women | 18+ | 500 |
| Pregnant women | | 600 |
| Lactating women | | 850 |

Source: FAO/WHO (1988)

Vitamin A Deficiency

Vitamin A malnutrition, as in other nutrient malnutrition, can be of two kinds, namely overnutrition and undernutrition. In the former, there is acute or chronic hypervitaminosis A. At the other extreme is hypovitaminosis A or vitamin A deficiency. Hypervitaminosis A is relatively rare, and could be caused by self prescription of large pharmacological doses of the vitamin A. In contrast, vitamin A deficiency is fairly common, particularly among poor children in developing countries. It has been described as among the most widespread and serious nutritional disorders to afflict mankind (WHO, 1982). The problem has been said to have remained largely unchecked and continued to be the cause of a high toll in blindness and death among young children.

The term "xerophthalmia", literally, means "dry eye", and in a restricted sense is a term used by ophthalmologists to describe the changes in the eye that occur when the secretions of the paraocular glands or of the goblet cells of the conjunctiva dry up, leading to discontinuity of the fluid films usually present over the surface of the conjunctiva and cornea. However, in a broader

sense, and in a public health context, the term has been applied to the syndrome of severe vitamin A deficiency (WHO, 1976). The term has been taken to cover all the ocular manifestations of vitamin A deficiency (including night blindness), and would thus be taken to denote an advanced degree of vitamin A deficiency, with a potential threat to sight (WHO, 1982). Particular emphasis has been given to the term because of its direct relation to the most tragic sequel of the deficiency - blindness.

“Vitamin A deficiency” refers to any state in which the vitamin A status is subnormal. It can be presumed to occur when the habitual intake of the total vitamin A is markedly below the recommended dietary intake (WHO, 1976). It therefore includes xerophthalmia.

Epidemiology of Vitamin A Deficiency

The basic underlying cause of vitamin A deficiency is a chronic inadequate dietary intake. Vitamin A deficiency is highly prevalent in communities where the dietary staple is rice, with little or no consumption of animal foods, or dark-green leafy vegetables, or of yellow/orange fruits. This is also true for communities dependant on cassava, white potato or other carbohydrate-dense foods that are virtually devoid of vitamin A and carotenoids. Vitamin A deficiency is essentially a condition of poor socioeconomic environment (Oomen, 1976). In these communities where foods from animal sources are too expensive, carotenes from plant sources are of paramount importance. However, due to ignorance and neglect, even these cheaper sources of vitamin A are very often not given to the children. Thus, it is common to find “poverty in the midst of plenty”, and destruction of eyes by xerophthalmia in environments where carotene-rich green leaves are abundant (WHO, 1976). It is therefore ironical that vitamin A deficiency should be prevalent in Southeast Asia, amidst plenty of greens and a variety of coloured fruits.

Fats are important in the absorption and metabolism of carotenoids and vitamin A. Where diets are unusually low in fat, inefficient absorption of dietary carotenoids has been implicated as contributing to development of xerophthalmia. Improved absorption of carotenoids following improved fat intakes has been reported in Ruanda (Roels *et al.*, 1958) and in Indonesia (Roels *et al.*, 1963).

Unsatisfactory early childhood feeding practices have an important bearing on the development of vitamin A deficiency. Xerophthalmia is rarely reported among breast-fed infants and seldom reported in children who continue to be breast-fed in the second year (Underwood, 1984). However, this is not to be so in many communities since the mothers themselves tend to be undernourished, with a very low vitamin A status and consequently the milk produced has a low concentration of the vitamin. Early weaning from the breast would worsen the situation. This is further aggravated when the child is weaned to inadequate diet of rice and other cereals or tubers, rather devoid of vitamin A.

Vitamin A Deficiency, Infection, and Child Survival

The interactions between vitamin A and infection have recently been given a great deal of attention. Various evidences, including laboratory experiments, clinical and epidemiologic

evidences, have shown that vitamin A deficiency plays an important role in resistance to infection, most apparent for respiratory infection. The synergistic effect between vitamin A deficiency and infection may be responsible for excessive childhood morbidity and mortality in many developing regions of the world (West *et al.*, 1989). In a group of Indonesian preschool-age rural children in West Java, Sommer *et al.* (1983) reported that mortality rate among children with mild xerophthalmia (night blindness and/or Bitot's spots) was on the average 4 times the rate, and in some age groups 8 to 12 times the rate, among children without xerophthalmia. These investigators further reported that children with mild xerophthalmia were more likely to develop respiratory disease and diarrhoea than non-xerophthalmia children, and that this increased risk was more closely associated with their vitamin A status than with their general nutritional status (Sommer *et al.*, 1984). Further studies carried out by Sommer *et al.* (1986) in Sumatra showed that vitamin A supplementation (200,000 IU vitamin A twice at six-monthly interval) was able to reduce mortality among the preschool children by as much as 34%. More recently, Rahmathullah *et al.* (1990) also reported a drastic reduction (on an average by 54%) in mortality among children in Southern India supplemented with a small (8333 IU) weekly dose of vitamin A. To avoid criticisms on the study design, a randomized, placebo-controlled, masked clinical trial among a large number of children (over 15,000) was carried out.

These studies on child survival and mortality created a great deal of interest and debate in the international health and nutrition community. These findings would also have wide policy implications (Martorell, 1989). Questions have been raised regarding the validity of the findings, including those pertaining to experimental designs and measurements, and data analysis and interpretation (Cravioto, 1990; Ramachandran, 1991). Keusch (1990), however, felt that evidences available support the implementation of vitamin A programmes in specific cases - wherever there is evidence that a population has a vitamin A deficiency, wherever protein-energy malnutrition is common, and wherever there is an excess of measles deaths. It was, however, pointed out that vitamin A supplementation should not be a substitute for immunization, primary health care and improved sanitation and water supplies. The controversy continues and the Subcommittee on Vitamin A Deficiency Prevention and Control of the US National Academy of Sciences has considered studies into vitamin A and child mortality of significant research priority (NAS, 1989). In the mean time, the International Vitamin A Consultative Group (IVACG) has issued an interim statement that evidence is accumulating that vitamin A does reduce mortality, by a mechanism(s) which is still unclear (IVACG, 1990). The statement also made it clear that the impact of improved vitamin A nutrition will vary with the severity of vitamin A deficiency and the contributions of other ecological factors.

Assessment of Vitamin A Status

Regional or nationwide surveys should be carried out to determine the frequency (prevalence) and severity of vitamin A deficiency in the population. Before any intervention programme is launched, a careful assessment of the situation should be made. Such surveys should be aimed at establishing the nature, magnitude, severity and geographical distribution of this deficiency provide a baseline for evaluating the effectiveness of future intervention.

Assessment of vitamin A status should include, whenever possible, clinical examination, biochemical determinations and dietary assessment. Sommer *et al.* (1977), WHO (1982) and,

more recently, Underwood (1990) have provided some general guidelines for the conduct and analysis of such surveys. The following paragraphs outline important aspects of each of the assessment methodologies mentioned.

a. Clinical Assessment

Vitamin A deficiency is a systemic disease affecting epithelial structures in a variety of organs, the eye being the most obvious and dramatic example. Clinical signs and symptoms occurring in the eye are specific to vitamin A deficiency. Thus, these have been widely used in clinical assessment of vitamin A status.

The ocular signs of vitamin A deficiency have been grouped under the term xerophthalmia, which literally means “dry eye”. The major xerophthalmia signs have been classified at the WHO/USAID meeting on Vitamin A Deficiency and Xerophthalmia (1976). This was subsequently modified in another WHO meeting (WHO, 1982). The classification is reproduced in Table 2. Details have been published in various monographs and reviews (WHO, 1976, 1982; McLaren *et al*, 1981; McLaren, 1981, 1984; Sommer, 1982; and Underwood, 1984). A field guide for the detection and control of xerophthalmia was also published by WHO in 1978 and recently revised (Sommer, 1995). Most of these publications include coloured photographs of the ocular signs.

Table 2. Xerophthalmia classification by ocular signs

| | |
|---|-----|
| Night blindness | XN |
| Conjunctival xerosis | X1A |
| Bitot's spot | X1B |
| Corneal xerosis | X2 |
| Corneal ulceration/keratomalacia <1/3 corneal surface | X3A |
| Corneal ulceration/keratomalacia ≥ 1/3 corneal surface | X3B |
| Corneal scar | XS |
| Xerophthalmic fundus | XF |

Source: WHO (1982)

With the exception of the last two, the ocular signs are arranged in increasing order of severity of active vitamin A deficiency. However, this does not mean that all the earlier stages necessarily occur when a later stage is detected. The earliest ocular symptom is that of night blindness (XN), or impaired dark adaptation, and is the result of too little available retinol for the rapid regeneration of rhodopsin in the eye. The symptom is said to be quite specific for vitamin A deficiency among young children, but is less so in older children and adults (Underwood, 1984). WHO (1982) considered it a useful screening tool in that it correlated closely with other evidence of vitamin A deficiency, such as serum retinol levels. However, its use requires a careful detailed history-taking, and is particularly useful in communities where the phenomenon of night blindness is recognized by the community.

The earliest structural change in xerophthalmia is conjunctival xerosis (X1A), consisting of one or more patches of dry, nonwetable conjunctiva, with loss of transparency. This extends to the formation of Bitot's spots (X1B) which are small plaques of a silvery grey hue, usually with a foamy surface, and consist of keratinized, desquamated conjunctival epithelial cells. The concurrent appearance of the two signs, conjunctival xerosis and Bitot's spot, is usually consistent with hypovitaminosis A (Underwood, 1984).

The more serious, blinding effects of severe vitamin A deficiency, involving the cornea, consist in the early stages of xerosis, loss of transparency, and nonwettability, as in the conjunctiva, giving the cornea a hazy appearance (X2). Subsequent to this corneal xerosis, there is loss of continuity of the epithelium with formation of inflammatory "ulcer". Further progression of this corneal ulceration, frequently referred to as keratomalacia (X3A and X3B), may eventually end in impairment of vision of varying degree.

Patients with prolonged and severe vitamin A deficiency may have, in addition to the eye lesions, a widespread dryness, wrinkling, slate-gray discolouration, and hyperkeratosis of the skin. It is, however, difficult to separate the role of vitamin A deficiency in causing these signs from the part played by other nutrient deficiencies. Even more controversial is the aetiology of follicular hyperkeratosis or phrynoderma, ascribed by some workers to be due to vitamin A deficiency (McLaren, 1967; WHO, 1982).

b. Biochemical Examination

Measurement of the serum or plasma vitamin A level remains the most practical available biochemical means of assessing the status of nutriture in spite of limitations in sensitivity and specificity (Underwood, 1984). Since vitamin A is stored in high concentrations in the body, almost entirely in the liver, plasma level does not closely reflect the level in the body as a whole. The plasma concentration reflects the body status only under two very different circumstances (WHO, 1976): when the body stores have been critically depleted, and when the liver has become saturated with vitamin A. The United States Interdepartmental Committee on Nutrition for National Defense (ICNND, 1963) recommended the following interpretation of plasma vitamin A levels (Table 3):

Table 3. Interpretation of plasma vitamin A and carotene levels*

| Level | μg of vitamin A/100 ml | μg of "carotene" / 100 ml |
|------------|-----------------------------------|--------------------------------------|
| high | >50 | >100 |
| acceptable | 20-49 | 40-99 |
| low | 10-19 | 20-39 |
| deficient | <10 | <20 |

*Adapted from ICNND (1963)

Of these categories, only the "deficiency" state, that is, less than 10 μg / 100 ml is recognized to be universally associated with both low liver reserves of vitamin A and an increased prevalence of clinical signs of deficiency. The so-called "low" category (10-20 μg /100 ml) should be

interpreted with caution since low plasma values may not be associated with vitamin A deficiency, but instead with other conditions such as inadequate protein intake, parasitic infestation, and liver diseases (WHO, 1976). Tee *et al.* (1994) had suggested revising the cut-off levels upwards.

Plasma carotene concentration is generally considered not to be a reliable indicator of vitamin A status because it reflects the level of immediate dietary intake (WHO, 1976). Furthermore, depending on the dietary composition, a considerable porportion of the plasma carotenoids may be non-vitamin A compounds. However, when considered with plasma retinol levels, carotenoid analysis can be useful (Sommer *et al.*, 1977; Underwood, 1984). The ICNND (1963) used the levels given in Table 3 for the interpretation of serum “carotene” levels. It would be more appropriate to refer to these levels as “total carotenoid”, since the colorimetric method used is not able to determine individual carotenes (Tee & Lim, 1991a).

c. Dietary Assessment

The difficulty of obtaining food consumption data is well documented. Many factors are difficult to control, making the results obtainable by even the most conscientious workers approximations only. Dedicated workers are definitely a must.

For the calculation of nutrient intake from the food consumption data collected, a good food composition database is required. It would be quite pointless in taking great pains to carry out the data collection, but lack good data on nutrient composition of foods for calculations. For vitamin A, obtaining good nutrient intake data is particularly difficult. This is related to the non-availability of good food composition databases with accurate vitamin A and carotene values. Vitamin A activity of foods in many food tables may be unreliable, due to the lack of precision of methodologies for the analysis of vitamin A and carotenoid content of foods. In recent years, there has been particular emphasis on obtaining more accurate data on the types and concentrations of various carotenoids and retinoids in foods (Tee & Lim, 1991a).

Various methods have been used to assess dietary intake of nutrients and each has its strengths and weaknesses. The method of choice would have to depend on the objectives of the study. Dietary history to quantitate the amounts of foods eaten, for example, using the 3 days 24-hour recall method, can provide fairly good data. The method is, however, time-consuming especially when used for large population groups. In addition, memory recalls are difficult to administer to young children. Household food consumption data can be obtained using the frequency of food consumption and purchase method. Such data, however, cannot be relied upon to provide an indication of the intake of individual members of the household. A compromise would be to collect family-based qualitative data to determine the frequency with which different foods are eaten. A comparison of three dietary methods for estimating vitamin A intake has been reported by Russell-Briefel *et al.* (1985).

The IVACG recently introduced guidelines for the development of a simplified dietary assessment to identify groups “at risk” for inadequate intake of vitamin A. (Underwood *et al.*, 1989). The semi quantitative method is particularly suitable for communities where almost all the vitamin A intake is from carotenoids contained in a limited number of food groups. Based on

food composition tables, these foods are classified into groups of high moderate or low levels of vitamin A activity. It has been emphasized that the simplified method will not provide precise quantitative information on levels of intake and alone will not be adequate for a quantitative assessment of vitamin A status of either communities or individuals.

Prevalence of Vitamin A Deficiency

In most reviews on the prevalence of vitamin A deficiency, the global survey by Oomen *et al* (1964) has been quoted. Drs. Oomen, McLaren and Escapini visited some 36 countries and compiled data based on hospital records, government statistics, questionnaire studies and personal observations. Based on this WHO sponsored global survey and a detailed notification system in Jordan, the annual incidence of xerophthalmia in the world prior to 1970 was estimated to be 100,000 (WHO, 1982). The areas found to be most affected were overpopulated, rice-dependent countries of Asia. Other regions that were affected to a lesser extent include Africa, Latin America, and the Middle East.

Subsequently, surveys have been conducted in several countries, most notably the nationwide prevalence survey in populous Indonesia. The incidence (appearance of new cases) of active corneal disease in Indonesia was estimated to be around 2.7 per 1000 preschool children per year (Sommer, 1982). Given an incidence of this order, the Administrative Committee on Coordination-Subcommittee on Nutrition of the United Nations (ACC-SCN, 1985) has estimated that some 400,000 to 500,000 preschool children in Bangladesh, India, Indonesia, and the Philippines combined will develop active corneal lesions resulting in partial or total blindness. It has been further pointed out that these are conservative estimates, since the assumption that identical incidence rates prevail for all the four countries is not correct; higher rates are known to occur in Bangladesh and India. The incidence of non-corneal xerophthalmia (mild, and generally reversible, forms of the disease) in these countries has been estimated to be in the order of 5 million preschool children per year.

Applying these estimates from the four Asian countries mentioned above, a worldwide projection of some 700,000 cases of new active corneal lesions occur annually among pre-school children has been made. The total incidence of noncorneal xerophthalmia among preschool children has been similarly estimated to be 6-7 million new cases per year and some 20-40 million suffering from at least mild deficiency at any one time, of which nearly half are in India (ACC-SCN, 1987).

WHO (1976) had suggested that with respect to prevalence of vitamin A deficiency, the world may be divided into three regions:

1. technologically developed countries where there is no xerophthalmia problem but some hypovitaminosis A may exist;
2. some rice-dependent developing countries of Asia where there is a problem of public health magnitude; and

3. the rest of the world including Africa, Latin America, and the Middle East, where the problem is not extensive but is intermittently intensive and highly sensitive to changing social and economic conditions.

Some detail descriptions of the problem as encountered in each of these three regions are given in several reviews, for example, WHO (1976, 1982), Tielsch and Sommer (1984), and Underwood (1984). A map showing the world geographical distribution of vitamin A deficiency and xerophthalmia in 1987 has been provided by report of the ACCSCN (1992).

Prevention and Control of Vitamin A Deficiency

Vitamin A deficiency has been considered as one of the “big four” nutritional deficiencies in developing countries; the other three are protein-energy malnutrition, iron deficiency anaemia, and endemic goitre due to iodine deficiency. It is the most important cause of preventable childhood blindness in these countries. In recent years, there has been an increasing appreciation by governments and international agencies of the magnitude of the problem and of the means available for dealing with xerophthalmia (Latham, 1984). Various intervention strategies have been implemented to combat the problem, and volumes have been written on these programmes (WHO, 1976, 1982; Arroyave et al., 1977; Bauernfeind, 1980; West and Sommer, 1984, 1987). Intervention programmes to prevent vitamin A deficiency are in operation nationally in at least 8 of the 34 countries with known vitamin A deficiency (ACC-SCN, 1987). Coverage of the population at risk in some of these countries has reached sufficiently high levels, whilst in others coverage is still unsatisfactory. The Administrative Committee on Coordination of the United Nations has emphasized that if intervention programmes are energetically implemented, vitamin A deficiency could be reduced to a level that it no longer poses a public health problem (ACC-SCN, 1987).

The objectives of an intervention programme must necessarily depend on many factors, such as the severity of the problem. Inadequate vitamin A status varies from a marginal condition of inadequate body reserves of the vitamin without clinical signs through the presence of early and reversible clinical signs, to a severely depleted state mainly characterised by advanced corneal changes and the high probability of blindness (WHO, 1982). Control programmes should therefore be directed towards the particular problem at hand.

Control of vitamin A deficiency may be approached through one of three major intervention strategies: a. improvement of dietary vitamin A intake; b. vitamin A fortification of foods; and c. periodic administration of a massive dose of vitamin A.

a. Improvement of Dietary Vitamin A Intake

Dietary improvement has been recognized as the major long term solution to controlling vitamin A deficiency in a community. Through a combination of horticultural and related activities, educational and socio-economic inputs, the habitual intake of food sources of vitamin A is gradually increased and maintained at a level which minimizes the risk of developing vitamin A deficiency among the vulnerable segments of a population (West and Sommer, 1984). Even

when other intervention strategies have been implemented, these measures should be carried out as on-going long term strategies.

In those parts of the world where vitamin A deficiency is prevalent, vegetable products are the main source of dietary vitamin A, in the form of carotenes. Thus horticultural and related activities to increase the availability of carotene-rich fruits and vegetables have been emphasized (WHO, 1982). These would include not only matters related to production (varieties for promotion, pest and disease control), but also problems of transport, marketing, storage, and preservation. These activities should be coordinated with the aspects of nutrition education programmes, since availability does not necessarily mean consumption of these foods by the vulnerable segments of the population, especially young children. The reasons why these foods are not consumed in significant amounts, even in areas where they are abundant should be understood.

b. Vitamin A Fortification of Foods

Fortification or nutrification of a widely consumed food with vitamin A offers a major technically feasible intervention which can be relatively inexpensive and effective (Latham, 1984; West and Sommer, 1984). Various considerations would have to be given before fortification can be considered a viable intervention. Firstly, with regards to a suitable food vehicle for vitamin A fortification, it should have the following characteristics:

1. the food should be technically fortifiable;
2. it should be widely or universally consumed by the population of the target area, in quantities that will make a significant contribution to the diet;
3. there should be little variation in per capita daily consumption of the food;
4. the food item should show no appreciable changes in its organoleptic characteristics, so that its acceptability is not affected after addition of vitamin A;
5. it should be economically feasible to fortify the food on an industrial scale.

Depending upon local factors and programme costs, fortification can offer both short and long term solutions in a chronically vitamin A deficient population (West and Sommer, 1984). Since the amounts of vitamin A added are adjusted to permit the recipient to receive the approximate recommended daily allowance for vitamin A, there is no risk of potential hypervitaminosis A. To date there are no indications clinically or biochemically that hypervitaminosis A has resulted from any of the nutrification projects currently in operation (Bauernfeind, 1980)

c. Periodic Administration of a Massive Dose of Vitamin A

On the basis of available evidence from nearly two decades of experience, large-dose vitamin A distribution can generally be regarded as a safe and potentially effective intervention to prevent xerophthalmia (West and Sommer, 1987; WHO, 1988). The underlying objective is to maximise liver stores from a single dose with little if any risk of acute toxicity. A 200,000 IU dose of vitamin A (110 mg retinyl palmitate or 66 mg retinol acetate), with 40 IU vitamin E in an oil solution for oral administration given every three to six months is used in most prevention programmes described in the literature (WHO, 1976, 1982; West and Sommer, 1984, 1987). The

programme has been instituted in the early and mid-1970's in several countries with severe xerophthalmia problem (WHO, 1982). These include India, Bangladesh, Indonesia, Haiti, Sri Lanka, and the Philippines. The successful implementation of a massive vitamin A supplementation programme covering 229 villages in Sumatra has been recently reported by Sommer *et al.* (1986)

Although the approach appears conceptually simple, the adequacy and efficiency of programmes pose major challenges and determine their success. Since the intervention involves a deliverer-recipient individual contact at specified times, a delivery system with a high requirement of personnel is needed to execute the programme. However, the personnel involved need not have a high level of technical expertise. Distribution systems should be designed and implemented with emphasis on primary level conditions, existing infrastructures, and ongoing health activities, with the involvement of local leadership. This strategy has the advantage of immediate implementation, but suffers from the disadvantage that it applies only to the isolated nutrient, vitamin A, and requires repetitive administration. In addition, complete coverage is seldom achieved. Of the total cost, the major percentage is in the mechanism for delivery and not of the nutrient per se. Education in health and nutrition should be an integral part of the periodic dosing programme. Underwood (1989) has emphasized that although these programmes have proven to be beneficial, the focus should be on approaches that foster practical solutions attainable through better utilization of available food and other resources. Although these preventive measures are more difficult to implement and take longer to bring about the needed behavioral changes, they are more permanent. More importantly, these measures address health and nutrition issues that commonly coexist with vitamin A deficiency.

Hypervitaminosis A

The intake of vitamin A above that which an individual can metabolize, either in a single excessively high intake (acute) or very high intakes for prolonged periods (chronic), causes hypervitaminosis A. It is essentially a result of abuse of this essential nutrient. Acute hypervitaminosis A has been reported to occur with ingestion of a single massive dose of 100,000 µg vitamin A in infants and young children, and 600,000 µg in adults. Chronic hypervitaminosis A may occur in infants after 4 months of receiving 10,000 µg daily, and in children and adults taking between 20,000 and 50,000 µg daily for several years (Rutishauser, 1986). Reviewing various evidences, Hathcock *et al.* (1990) concluded that exposure to high doses of vitamin A (> 30,000 µg/d) for relatively short periods (days or a few weeks) or lower doses (7,500 - 15,000 µg/d) for periods of several months or more can produce multiple adverse effects. Several predisposing conditions, such as viral hepatitis, cirrhosis, and other liver diseases, may greatly increase susceptibility and thus lower the amount of vitamin A necessary to produce adverse effects (Hathcock *et al.*, 1990). Blood levels of vitamin A become very high in hypervitaminosis A, well in excess of 100 µg/dl (Underwood, 1984). Various clinical symptoms have been recorded, including nausea, headaches, vomiting, diarrhoea, irritability, drowsiness, fatigue, and abnormal responses of the blood, skin, hair, and bone (Bauernfeind, 1980; Barker, 1982; Bendich and Langseth, 1989; Olson, 1990). These adverse symptoms are expected to vary with the dose and the duration of exposure, as well as with the age of the individual exposed. A rapid recovery usually results when excessive intakes are discontinued, and no permanent health effects are known to occur (Underwood, 1984). Bendich and Langseth (1989) and Olson (1990)

have, however, emphasized the danger of possible teratogenic and embryotoxic effects of excess vitamin A intake in women of childbearing age. Hathcock *et al.* (1990) have given a detailed review of the animal studies carried out on toxicity and overdose of vitamin A.

Bauernfeind (1980), who has given a detailed account of the subject, estimated that worldwide, several hundred instances of hypervitaminosis A probably occur annually, 90 percent of which cause fleeting side effects which are self correcting within hours or a day after a single high level dosage. The remaining cases may cause sufficiently severe symptoms to require weeks or months to reverse. No deaths have been attributed solely to hypervitaminosis A. Vitamin A toxicity therefore remains a very minor clinical or nutritional problem. When hypervitaminosis A is suspected, a history of vitamin A consumption should be taken from the patient and serum retinol level determined. If overdosage is ascertained, the simple remedy is to stop the excessive vitamin A intake. However, there is growing concern, especially in developed countries, that prevalence of chronic hypervitaminosis A may increase in future years (Underwood, 1984; Olson, 1990). There is fear that excessive intake could result from misuse or over-consumption, either out of ignorance or carelessness or out of the misconception among food faddists that continued excessively high intakes will provide some unusual health benefit. Vitamin A preparations are available commercially without prescription in concentrations of up to 7,500 retinol equivalents (RE), and there are those who advocate routine megavitamin ingestion. Furthermore, for young children who are given concentrated vitamin supplements as well as fortified foods, they could be on the verge of toxicity. Such fears are real, and even developing countries should be wary of the situation. Underwood (1984) had also pointed out that in view of the linkage of retinoids in animal studies to cancer prevention, and the epidemiologic associations in humans of dietary and serum levels of vitamin A to cancer risk, there is the danger that this will lead the uninformed public to increased consumption of mega-doses. The Committee on Dietary Allowances of the United States National Research Council has recommended that regular ingestion of supplements of retinol exceeding 3,000 RE by infants and children be undertaken only under the direction of a physician, and that for adults, regular ingestion of more than 7,500 RE daily is not prudent (NAS, 1980). Hathcock *et al.* (1990) have emphasized that educational efforts should point out clearly that there are no well-established therapeutic or prophylactic benefits to intakes above the usual RDA, and that toxicity is possible from excessive intakes.

Excessive consumption of carotenoids does not generate a correspondingly high vitamin A tissue content and does not cause hypervitaminosis A, although there is usually a raise in serum retinol level (Leung, 1987). This has been attributed to the sharp decline in efficiency of intestinal absorption and rate of conversion of carotenoids to vitamin A in the body (Bauernfeind, 1980). Individuals who consume high levels of carotenoids in pharmacological dosage forms, or as large quantities of carotene-rich fruits, will develop a yellow or orange pigmentation of skin, especially the palms of the hands and soles of the feet. Hypercarotenemia results, and serum levels of carotenoids may be in excess of 300 µg/dl (Underwood, 1984). These conditions have no effect on health and will slowly disappear following elimination of the excessive dietary carotenoids. Reviewing various evidences, Bendich (1988) and Hathcock *et al.* (1990) concluded that daily supplementation with high doses of β-carotene for extended periods of time is not associated with other side effects.

Hypercarotenemia, also known by other terms such as “carotenemia”, “hyperlipochromia”, “xanthemia”, and “carotenosis cutis”, may also be associated with various conditions other than excessive dietary intake (Leung, 1987). These include diabetes mellitus, hypothyroidism, hypothalamic amenorrhea, anorexia nervosa, liver disease, and certain inborn error of metabolism. Although the mechanisms of hypercarotenemia in these conditions have not been fully understood, it is known that the decreased rate of conversion of carotene to vitamin A is an important factor.

Carotenoids, Retinoids and Cancer

Cancer is a condition of unrestrained cellular growth, commonly associated with poorly differentiated cells. The condition arises from a myriad of causes, may affect cells from essentially all tissues (Olson, 1986). There appears to be three distinct stages in tumour development (Wolf, 1980). The first is the initiation stage, when a carcinogen causes a permanent change to a few cells of the affected tissue. In the preneoplastic or latent stage, the affected cells gradually develop into cancer cells. A characteristic feature of this phase is that the preneoplastic cells can sometimes remain transformed but not multiply for a long time. These cells can sometimes revert to normal or be prevented in their progression toward the tumour phase. Finally, in the third stage, the transformed cells are permitted or encouraged to multiply rapidly, transforming the tissue into a tumour. It is now known that cancer incidence is influenced by various environmental factors, aside from genetic factors. The environmental factors include the air, water, and food (NRC, 1982). The food we eat daily can participate in tumour development in that certain food components can act as initiators (carcinogens) and/or promoters (cocarcinogens), thereby increasing the incidence or speed of development of particular tumours.

On the other hand, the foods we eat may contain components (anti-carcinogens) that can act to block initiation, to enhance the immune system’s ability to identify and destroy transformed cells, or to inhibit development to promotion stage, thereby lowering incidence or slowing tumour development. Epidemiologic studies, supported by experimental observations in laboratory animals have suggested that dietary practices are a promising area to explore in the search for preventive measures against cancer (Greenwald et al., 1986). As Peto *et al.* (1981) put it, it would be more attractive to discover anticancer substances in certain foods that can be prescribed rather than carcinogens that must be proscribed, since people are more willing to accept prescription rather than proscription.

Thus, the role of diet in cancer development has been actively studied in recent years. Various epidemiologic and laboratory studies have been carried out. In the past twenty years or so, attention has been focused on carotenoids and pre-formed vitamin A. The possibility that carotenoids and retinoids may play a role in cancer development has added a whole new, exciting dimension to the studies of these compounds in human nutrition.

Several lines of evidence suggest that important relations exist between carotenoids, retinoids and cancer (Mathews-Roth, 1985; Krinsky, 1988; Ziegler, 1989; Williams and Dickerson, 1990). These are based on experimental studies of retinoids and carotenoids in cancer; epidemiologic

evidence suggesting associations between reduced risk of cancer and vitamin A status; and findings from clinical trials and studies in the field of oncology (Tee, 1992).

DETERMINATION OF VITAMIN A AND CAROTENOIDS

A basic tool in carotenoid and retinoid research and development activities is the content of these two groups of compounds in foods. In recent years, there has been particular emphasis on understanding the types and concentrations of various carotenoids in foods. It is thought that previously reported values of vitamin A activity in food composition tables may have been unreliable since methodologies used were not sufficiently discriminative and thus had included carotenoids that do not possess vitamin A activity. Advances in studies into the structure and properties of various carotenoids have shown that only a handful of the hundreds of carotenoids occurring in nature possess vitamin A activity. Some of these may occur in higher concentrations than β -carotene, the most potent precursor of vitamin A (Borenstein and Bunnell, 1966; Simpson and Chichester, 1981; Bauernfeind, 1972).

Even epidemiologic studies in the area of cancer prevention would require more accurate data on retinol and carotenoid content of foods. Currently, association is on a broad basis, linking vegetable consumption or "carotene" content of foods to risk of developing cancer. Better methods of determination would help narrow down the compound(s) of interest, not only in foods but in blood serum as well. Furthermore, there is also the possibility that carotenoids not possessing vitamin A activity may be associated with lower cancer risk.

This part of the review discusses developments in the methodologies for the determination of carotenoids and retinol in foods and serum. Experiences in developing an HPLC method for the simultaneous determination of retinol and several carotenoids in a variety of food and serum in this laboratory are briefly described.

Preliminary Sample Treatment

A pre-requisite in the analysis of carotenoids and retinoids is preliminary sample treatment in order to release, isolate and extract compounds of interest from the food matrix. A search through the literature indicates numerous procedures for the preliminary treatment of these compounds. In general, however, the procedures follow a few basic steps, upon which various modifications have been made. The procedures of the Association of Official Analytical Chemists methods (Williams, 1984) are first described to illustrate the basic steps involved. Major modifications made by various investigators are next discussed. For clarity, treatment procedures for plant materials, animal foods, and blood samples are separately discussed. A short discussion on the precautions to be taken during handling of samples will also be given in view of the unstable nature of these compounds.

Plant Materials

For the analysis of carotenes in plant materials, in the AOAC procedure (Williams, 1984), the well-blended material is extracted with a mixture of acetone-hexane in the presence of

magnesium carbonate. The extraction mixture is filtered, and the residue extracted with aliquots of acetone and hexane. Acetone is then removed from the pooled extract using water. The carotenoids are made up in a solution of acetone-hexane mixture for chromatography in a column of magnesia and Hyflo Super Cel mixture.

For the determination of carotenes and xanthophylls, especially in dried plant materials, the AOAC method includes a saponification step (Williams, 1984). Two procedures have been suggested, namely hot and cold (overnight) saponification. After extracting with an extractant consisting of hexane-acetone-absolute alcohol-toluene, the mixture is saponified using methanolic potassium hydroxide at 56°C for 20 minutes for hot saponification or left to stand in the dark for 16 hours if cold saponification is preferred. The former is for rapid extraction and for samples containing xanthophyll esters. For samples containing xanthophylls rather than xanthophyll esters, cold saponification is suggested since xanthophylls are particularly sensitive to heat. Hexane is then added to the saponification mixture which is then made up to volume with sodium sulphate solution. The upper phase of this mixture is used for column chromatography.

Most investigators employing open column chromatography for the analysis of carotenoids in plant materials have used extraction procedures similar to those outlined above. Slight modifications to the extracting solvent have been used, for example using petroleum ether or diethyl ether in place of hexane.

Similar extraction procedures were also used by investigators employing HPLC as the separation process, for example early reports on HPLC by Sweeney & Marsh (1970), Van de Weerdhof *et al.* (1973), and Reeder & Park (1975). The hexane or petroleum ether extracts were injected directly into magnesia or alumina columns for normal-phase HPLC.

In the method developed by this laboratory (Tee & Lim 1991b), fresh plant and fruit materials were first saponified using alcoholic potassium hydroxide by boiling for 30 minutes. The saponified mixture was then extracted with several aliquotes of hexane till the extract was colourless. The hexane extracts were pooled, washed until free of alkali, dried over sodium sulphate and reduced to a small volume by heating over a water-bath with the aid of a stream of oxygen-free nitrogen. The resulting solution was made up immediately to a suitable volume with hexane. The hexane was then evaporated off on a waterbath with the aid of nitrogen gas. The residue was immediately redissolved in a suitable volume of the mobile phase and ready for HPLC.

Foods of Animal Origin

For foods of animal origin, mixed foods and processed foods, the AOAC method requires an initial alkaline hydrolysis to be carried out (Williams, 1984). After grinding the food, the sample is saponified using ethanolic potassium hydroxide. Retinoids (and carotenoids, if present) in the unsaponifiable fraction are then extracted several times into hexane. The hexane extract, after diluting to a suitable volume, is then chromatographed on a column of alumina.

Similar preliminary treatment procedures were used by investigators employing HPLC for the separation of carotenoids and retinoids. Most investigators carried out initial saponification of the sample using ethanolic potassium hydroxide, carrying out the refluxing at about 80°C. In the series of foods of animal origin studied by this laboratory (Tee & Lim, 1992), foods were saponified and treated as described for the AOAC method.

Stancher & Zonta (1982, 1984a, 1984b), however, preferred cold alkaline digestion overnight in their studies of retinol in cheese, fish and fish oils. In a number of studies of various foods including oil and margarine, breakfast cereals, and infant formula, Landen & coworkers did not saponify the samples studied (Landen & Eltenmiller, 1979; Landen, 1980; Granade, 1982). In a study of various fortified foods such as dairy products, breakfast cereal and vitamin supplement, Ashoor & Knox (1987) also avoided saponification in order to limit loss of retinol. Barnett *et al.* (1980), on the other hand, incubated the infant formula and dairy products with lipase for 1 hour at 37°C.

Serum and Plasma

Saponification is usually not required for blood samples. Over 95% of the vitamin A normally found in fasting blood is bound to its carrier, a retinol-binding protein. Only after ingestion of a meal rich in vitamin A, after the administration of a high dose of vitamin A, or in certain disease states (e.g. vitamin A toxicity, severe liver disease, or severe protein-energy malnutrition) are lipoprotein-bound retinyl esters found in circulation (Arroyave *et al.*, 1982).

Samples are usually first treated with ethanol (or methanol) to precipitate the protein. The sample is next extracted with an organic solvent, e.g. petroleum ether or hexane. After separation from the protein, the absorbance of the extract may be read in a spectrophotometer to determine carotene and an aliquot reacted with antimony trichloride or trifluoroacetic acid to quantitate retinol. The organic extract may also be separated by high-pressure liquid chromatography (HPLC). If reverse-phase HPLC is used, the organic extract injected has to be compatible with the generally polar mobile phase used. Hence, if petroleum ether or hexane is used for extracting carotenoids and retinoids, the solvent has to be evaporated and the residue redissolved in a suitable solvent such as isopropanol (Puglisi & de Silva, 1976; Katrangi *et al.*, 1984) prior to separation by HPLC. On the other hand, solvents such as a mixture of tetrahydrofuran and ethyl acetate (Peng *et al.*, 1983; Nierenberg, 1985) and chloroform (Broich *et al.*, 1983) may be used for extracting carotenoids and retinoids, and the extract used directly for HPLC.

In the method developed by this laboratory (Tee *et al.*, 1994) protein in serum was first precipitated using ethanol. After separation from the protein, the solution was extracted with petroleum ether. An aliquot of the extractant was evaporated to dryness with the aid of a stream of nitrogen. The residues were immediately redissolved in the HPLC mobile phase. Using the mobile phase as the extractant, it was found that retinol values were similar to those obtained using petroleum ether as the extractant. However, total carotenoid concentrations as well as the levels of the three major carotenoids were much lower when the mobile phase was used as the extractant.

Precautions in Handling of Samples

The main problems associated with work on retinoids and carotenoids arise from the inherent instability of these compounds, especially towards light, heat, oxygen and acids. To prevent oxidation of the vitamins during saponification, extraction, and chromatography some investigators have reported the use of various antioxidants during these processes. These include the use of ethoxyquin, pyrogallol, ascorbic acid, sodium ascorbate, and hydroquinone. Butylated hydroxytoluene (BHT) appears to be one of the most commonly used antioxidant, used by various investigators over the years (Tee & Lim, 1991a).

The antioxidants have been used in several ways; for example, they are added to the sample during grinding, or saponification, or added to solvents such as THF and diisopropylether, or to the standard carotenoids and retinol. The advantages of using antioxidants has not been clearly shown. In many cases, it has not been shown that adding antioxidants improved results, although they did not seem to have adverse effects either (Parrish, 1977). The AOAC procedures have not suggested the use of these antioxidants in the analysis of retinoids and carotenoids (Williams, 1984).

Besides the use of antioxidants, other measures have also been taken to prevent oxidation of these compounds. Oxygen-free nitrogen has often been used to create an inert atmosphere. This is frequently used when evaporating off solvents, usually with elevated temperatures, from test solutions to reduce the volume or to enable the use of another solvent. The use of a stream of nitrogen not only excludes oxygen from the warm solution, but also speeds up the evaporation process. Other precautions that need to be taken include protection of samples from acids and metal ions which may be present in the solvents used. Particular care should therefore be given to the use of suitable solvents, or carry out purification procedures of the solvents. In using HPLC as the separation procedure, another factor to be remembered is the possibility of interference from impurities, particularly when monitoring at low wavelength.

The need for protection of analytical samples from direct exposure to light, particularly sunlight, has often been emphasized. Carotenoids and retinoids undergo structural photo-transformations, particularly when exposed to light in the ultra-violet region (below 350 nm). Various approaches can be taken to minimize this destruction of the compounds. Sunlight into the laboratory can be greatly reduced by using suitable blinds or tinted windows and glass panes. Amber (low-actinic) glassware may be used, although it is rather expensive and difficult to obtain. Aluminium foil may also be used to wrap containers or chromatography columns.

Proper storage of solutions in carotenoid and retinoid analysis has often been emphasized. Solutions should be stored in the dark (e.g. in amber containers), at about -20°C , and, if possible, under nitrogen. Containers should be scrupulously cleaned, with well-sealed containers. Standard solutions are not stable for a long time, and their concentration and purity checked before use. Routine UV-vis spectral analysis of solutions would be an easy way to detect gross changes to standard or sample solutions.

Separation and Quantitation Procedures

General Trend in Procedures

β -Carotene and several carotenoids are coloured and absorb maximally at about 450 nm. These compounds can be easily detected and quantitated in a spectrophotometer in the visible range. Similarly, retinol may be detected and quantitated by measurements at 325 nm. It is thus possible to quantitate these compounds without any prior separation procedure. For the determination of β -carotene and retinol in serum, hexane extracts may be read at the two wavelengths mentioned (Bessey *et al.*, 1946; Underwood & Stekel, 1984). Several reports were also encountered in the literature where food extracts were read directly in a spectrophotometer at wavelengths around 450 nm (Thomas, 1975; Mudambi & Rajagopal, 1977; Maeda & Salunkhe, 1981; and Picha, 1985). The most important limitation of such procedures is that because no prior separation process is carried out, absorbance readings taken at selected wavelengths are not specific for the compounds of interest. Thus, for carotene estimation at 450 nm, the results so obtained would, at best, be referred to as total carotenoid concentrations. Absorbance at 325 nm should be related to retinoids and other compounds rather than retinol.

Another approach that was adopted for the quantitation of retinol was to react the vitamin with various reagents to produce a coloured compound which was then read in a spectrophotometer. The search for a colorimetric method to replace the more tedious biological test based on growth promotion of experimental animals for quantitating vitamin A was initiated even in the early part of the century. Several chemicals have been studied, including sulphuric acid, arsenic chloride and trichloroacetic acid.

Subsequently, efforts were made to further improve the colorimetric method for the quantitation of vitamin A. The search was for similar reagents that could give more quantitative and reproducible results. Carr & Price (1926) proposed the use of a solution of antimony trichloride in chloroform as the colour reagent. The blue colour produced was said to be more intense and more permanent than other reagents tried, with more reproducible results being obtainable. The Carr-Price reaction became well received by many analysts and was widely used in various analytical procedures. Adapted for use for serum samples, hexane extracts were evaporated and the residue reacted with antimony trichloride reagent for the quantitation of retinol. The procedure has been used widely for the assessment of the vitamin A status of communities (ICNND, 1957). Some investigators subjected the serum extract to an initial alumina column chromatography procedure, but a separation step was often not included, especially when a large number of samples needed to be analysed.

Several problems have, however, been encountered in the use of the Carr-Price reaction. It has been established that even small amounts of moisture will result in turbidity of the antimony trichloride reagent. The evanescent nature of the blue colour obtained presents considerable practical difficulty in taking readings in a colorimeter.

The search for an even more suitable colour reagent thus continued. Trifluoroacetic acid was subsequently offered as an alternative. The reagent has been said to retain the sensitivity and specificity of the antimony trichloride reaction, but does not exhibit the turbidity property of the antimony trichloride reagent (Neeld & Pearson, 1963). It is also less toxic and does not form a

tenacious film on the cuvette. The blue colour formed with trifluoroacetic acid, however, is also transitory.

Retinol and its esters fluoresce in ultra-violet light, and this property has been used for quantitating the vitamin. Fluorescence may be determined using excitation wavelength at 330 nm and emission at 480 nm. Fluorometric measurements of serum extracts have been described by Thompson *et al.* (1971) and Wu *et al.* (1981). It has been suggested that corrections be made for fluorescence by interfering compounds. The carotenoid phytofluene was felt to be the only significant fluorometrically active contaminant in human serum (Thompson *et al.*, 1971). Fluorescence detection has also been used for the analysis of vitamin A in foods. The technique has been used after initial fractionation of food samples using high-pressure liquid chromatography (HPLC). Several studies employing this approach will be cited in the section dealing with HPLC.

Most of the studies encountered in this review generally involved a separation prior to the quantitation of carotenoids and retinoids. As can be expected, this results in better quantitation of the compounds of interest. This review is thus mainly concerned with procedures involving various separation procedures.

A wide variety of separation and detection and quantitation procedures have been used in studies of carotenoids and retinoids. A general trend in the change of these methodologies is evident over the last two decades. An early technique used for the separation of carotenoids in plant materials, mainly in the 1960's, was countercurrent distribution. A few early studies using paper chromatography were also reported. In the 1970's, a few studies of carotenoids and retinoids using gas-liquid chromatography and gel-permeation chromatography were encountered. A number of studies employing thin-layer chromatography as a single technique for the separation of carotenoids in plant materials were encountered throughout the 1970's and the early 80's. TLC was also used by several investigators in combination with other separation techniques. The procedure was less widely used in studies of retinoids. Adsorption (open) column chromatography, utilising primarily descending, gravity-flow columns, was widely used for the study of carotenoids and retinoids in foods, even in the 1960's. The procedure remained very much in use in the 70's and 80's. Commencing from the late 1970's, a new column chromatography procedure became more prominent in the literature. High-pressure liquid chromatography has become a widely used procedure for the separation of carotenoids and retinoids in various materials (Tee & Lim, 1991a).

This review discusses the use of the two most common procedures for the separation and quantitation of carotenoids and retinoids in food and blood samples, namely the adsorption column chromatography and the high-pressure liquid chromatography methods.

Adsorption Column Chromatography

Adsorption column chromatography has been relatively widely used in the study of carotenoids and retinoids in foods. The technique has also been referred to as open column chromatography. Basically it utilizes descending, gravity-flow columns, as distinguished from columns running under pressure, such as those in high-pressure liquid chromatography (HPLC). In the following

discussion, its use in the analysis of carotenoids in plant materials will first be discussed, followed by the use of this technique for the separation of retinoids.

Adsorption column chromatography has been widely used for the fractionation of carotenoids in plant materials, even in the 1960's. Most of these studies have employed procedures very similar to those described in the AOAC manual, which described methods for two groups of plant materials, namely carotenes in fresh plant materials and silages, and carotenes and xanthophylls in dried plant materials and mixed feeds. The procedures described in the latest edition of the manual (Williams, 1984) have not changed very much from those prescribed in the earlier edition. The only major change was the inclusion of the alternative of hot saponification in the later edition. The technique has developed over at least a couple of decades. Davies (1976) and Taylor (1983) have discussed in some detail several practical considerations in the use of the technique, including choice of adsorbent and solvent, and preparation of column for chromatography.

In the AOAC method for determination of carotenes in fresh plant materials, chromatography is carried out in a 22 mm (o.d.) x 175 mm glass column, packed with a mixture of activated magnesia (Sea Sorb 43) and diatomaceous earth (Hyflo Super Cel). The carotene band, visible on the column, is eluted using a solvent mixture of acetone-hexane. The carotenes pass rapidly through the column, leaving behind bands of xanthophylls, carotene oxidation products and chlorophylls on the column.

After the eluate is made up to a suitable volume in a mixture of acetone-hexane, the absorbance of the solution is read in a spectrophotometer at 436 nm. Quantitation of the sample is carried out by comparing absorbance reading with a standard curve prepared from (-carotene. Various workers have used procedures very similar to those described above for the study of carotenoids in plant materials.

For the determination of carotenes and xanthophylls in dried plant materials and mixed feeds, AOAC has designated a different chromatographic procedure (Williams, 1984). The adsorbent used in a 12.5 mm (i.d.) x 30 cm glass column is a mixture of silica gel G and diatomaceous earth (Hyflo Super Cel). Carotenes are eluted from the column using a mixture of hexane-acetone (96+4), leaving behind the xanthophylls. Monohydroxy pigments (zeinoxanthin and cryptoxanthin) are eluted from the column using a mixture of hexaneacetone (90+10), whilst the dihydroxy pigments (lutein, zeaxanthin and their isomers) are eluted using hexane-acetone (80+20). The polyhydroxy pigments are said to remain on the column. Absorbance of solutions of the eluted carotenes, and mono- and dihydroxy pigments are obtained at 436 nm for the former and 474 nm for the latter two. Quantitation of these pigments is by comparing absorbance readings with that obtained using C.I. Solvent Yellow 14 dye.

If the concentration of total xanthophylls is required for these dried plant materials and mixed feeds, chromatography has to be performed on a mixture of activated magnesia and diatomaceous earth as adsorbent. Carotenes are eluted from the column with hexaneacetone (90+10), whilst total xanthophylls are eluted using hexane-acetone-methanol (80+10+10). Quantitation of these two fractions is then carried out as described in the previous paragraph.

Adsorption column chromatography has thus been widely used by various investigators for the separation of carotenoids. Various plant materials have been studied, including some fruits, vegetables, cereals, and feed materials. The method has been used by many laboratories for the generation of data for food composition tables, eg the Malaysian Food Composition Table (Tee *et al.*, 1988).

The AOAC procedures, and other adsorption column chromatographic techniques summarised above have been noted to have various disadvantages. Firstly, these procedures are long and laborious, and only a few samples may be handled at a time. Other potential sources of errors in these procedures include incomplete solvent extraction, destruction and stereochemical changes during extraction and chromatography (Beecher & Khachik, 1984).

The major problem in adsorption column chromatography procedures is related to the quantitation of vitamin A activity of the sample, based on the separation obtained. For fresh plant materials, carotenes are separated from xanthophylls, but the method does not separate individual carotenes, their *cis* isomers, or carotenoid esters. The carotenes eluted are assumed to be total β -carotene and quantitated as such. This assumption gives rise to considerable error in the calculated vitamin A activities, since the carotenes possess widely differing biological activity. In the method for dried plant materials, individual carotenes are again not separated, whilst xanthophylls may be further separated as mono- di- and polyhydroxy pigments using other adsorbents and eluting solvent mixtures. This non-separation of the various carotenes and xanthophylls is a major drawback of these procedures and constitutes a major source of analytical errors in the vitamin A values obtained.

Throughout the 1970's and early 80's, several studies on the use of adsorption column chromatography for the study of retinoids were reported. These were, however, much fewer in number compared with those dealing with separation of carotenoids in plant materials. One of the earliest studies using this procedure was reported by Erdman *et al.* (1973), where the investigators studied a variety of foods including cereals, meat, margarine and butter. The methods reported in the literature are essentially those in the AOAC manual (Horwitz, 1970). After saponification of the food to remove fat-soluble interfering substances, vitamin A and carotenoids remaining in the unsaponifiable fraction were extracted into hexane. The extract prepared was next chromatographed on a column of deactivated alumina to further purify the extract. Carotenes in the extract were separated in the process and eluted using 4% acetone in hexane. Retinol remaining on the column, visible by brief inspection with UV light, was eluted using 15% acetone in hexane. The volume of the eluates was suitably adjusted and read in a spectrophotometer. The carotene eluates was read at 450 nm, while retinol was reacted with antimony trichloride (Carr-Price reaction) and read at 620 nm.

The method has not changed very much since it was reported in the 11th edition of the AOAC manual Horwitz, 1970). The 14th edition of the publication (Williams, 1984) retains all the main features of the method. Whilst previously it was specified for the analysis of mixed feeds, the latest edition of the manual has extended it to the analysis of pre-mixes and foods as well. It has become a widely used procedure, mainly because it does not require sophisticated instrumentation. It is, however, a rather tedious method, requiring several hours for the separation. Because of this long separation time, retinoids and carotenoids in the open column

chromatography procedure are exposed to the destructive influences of light and oxygen (Taylor, 1983). In addition, resolution of the compounds to be separated is not entirely satisfactory. In the AOAC procedure, only separation of retinols from carotenes is achieved, while separation of individual retinols and carotenes is not effected. Cryptoxanthin and similar pigments are eluted in the retinol fraction. If these are present in significant amounts, a correction has to be made to the readings obtained, since they also react with antimony trichloride.

Another major problem with the method is related to quantitation of the separated retinol. Difficulties encountered in using antimony trichloride colour reagent has been previously discussed. Attempts to overcome these difficulties included finding alternatives to antimony trichloride, *e.g.* trichloroacetic acid (Kamangar & Fawzi, 1978), and trifluoroacetic acid (Wang *et al.*, 1978).

High-pressure Liquid Chromatography

From the late 1970's, numerous studies of carotenoids and retinoids using high-pressure liquid chromatography (HPLC) were found in the literature. It is quite obvious that the technique is replacing other methods, including the once widely used column chromatography procedure. The discussion below serves to provide some examples of the use of the technique in the study of various food samples and biological specimens, to illustrate the technique involved and the capabilities of the method. A more detailed review of the subject has been given by Tee and Lim (1991a).

a. Carotenoids in plant materials

One of the earlier studies on plant materials using HPLC made use of a mixture of calcium and magnesium hydroxides for the separation of carotene stereo-isomers in several vegetables (Sweeney & Marsh, 1970). Rather large diameter (15 mm i.d.) columns were used, specially designed to withstand pressures of up to 20 p.s.i. Seven isomers were eluted using *p*-methylanisole in petroleum ether and acetone in petroleum ether. Fractions were concentrated and studied in a spectrophotometer. In the following year, Stewart & Wheaton (1971) reported the use of a method that bears closer resemblance to the present-day HPLC procedure. A pump capable to operation up to 2000 p.s.i. was used, and several gradient systems were studied. Both glass and stainless steel columns were used, and a variety of adsorbents were tried. Magnesium oxide was selected for the separation of carotenes and zinc carbonate for xanthophylls. Pigments were eluted using hexane and tertiary pentyl alcohol mixtures, and effluent monitored at 440 nm in a spectrophotometer. TLC (on layers of zinc carbonate) was used to aid in the identification of carotenoids.

The stationary phases used in the studies above were all normal-phase columns, employing non-polar primary solvents such as *n*-hexane or petroleum ether. Another mode of HPLC introduced made use of reverse-phase columns in which a non-polar hydrocarbonaceous layer is chemically bonded onto the surface of the silica gel by silylation of the free hydroxyl groups with an alkyl trichlorosilane. The latter was usually octyl or octadecyltrichlorosilane, referred to as C₈ or C₁₈ (ODS) columns respectively. In addition to the main column, a precolumn (or guard column) was usually included to act as a trap for contaminants and protect the analytical column. Packing

material for the pre-column is usually the same as that in the analytical column, except that the particle size is larger. Polar solvent mixtures were usually employed in reverse-phase HPLC, e.g. water, methanol and acetonitrile. Zakaria *et al.* (1979) reported the use of four of these reverse-phase columns for the separation of α - and β -carotene and lycopene in tomato. The best separation was said to have been obtained using a 5- μ m particle size Partisil column and a mobile phase consisting of 8% chloroform in acetonitrile was used to elute the pigments isocratically. The pigments were detected at 470 nm, and identification was based on retention of standards and stopped-flow visible spectra of the peaks. These investigators pointed out that non-polar reverse-phase columns possess several advantages over the normal-phase polar adsorbents. The former are neutral to the sample and are unaffected by the presence of water or changes in the mobile phase, and are therefore more suitable for routine sample analysis.

Numerous subsequent studies had made use of reverse-phase HPLC for the study of carotenoids in plant materials. Speek *et al.* (1986, 1988) provided data on vitamin A activity for a large number of fruits and vegetables using a reverse-phase HPLC. A home-packed ODS-Hypersil column was used, while a mixture of four solvents consisting of methanol, acetonitrile, chloroform, and water was used as the mobile phase. The procedure was also different from studies previously described in that total carotenoids was also determined by absorption spectrophotometry in addition to the determination of β -carotene concentration by HPLC (Speek *et al.*, 1986). Using this procedure, Speek *et al.* (1988) reported the analysis of 55 Thai fruits and vegetables. When compared with values tabulated in the Thai Food Composition Table, the analytical values were generally lower. The effect of processing (cooking, frying, fermenting and sun-drying) on carotene content was also studied.

A reversed-phase HPLC method was also developed by this laboratory for the determination of carotenoids in vegetables and fruits (Tee and Lim, 1991b). Six carotenoids were separated isocratically on an octadecylsilane (C_{18}) column using a ternary mixture of acetonitrile, methanol and ethyl acetate (Figure 1). A total of 40 vegetables and 14 fruits were simultaneously studied using this HPLC method and the AOAC method (Tables 4, 5 and 6). Results obtained showed that the AOAC method gave falsely elevated carotene values for samples containing α -carotene, as well as those with low β -carotene concentrations. The study clearly showed that the HPLC method would give a more complete picture of the carotenoid composition as well as a more accurate quantitation of the vitamin A value of the foods. The method was subsequently used for the study of these carotenoids in several legumes, tubers and starchy roots (Tee *et al.*, 1995).

b. Carotenoids in blood

Several studies on the analysis of carotenoids in blood serum or plasma samples were reported. No studies on other biological specimens were encountered. All the studies encountered employed reverse-phase HPLC on a C_{18} column, and frequently only a few carotenoids were studied. An early report by Puglisi & de Silva (1976) studied phytoene concentration in dog blood after intravenous and oral administration of the carotenoid. A methanol-water mixture was used as the mobile phase and the carotenoid was detected at 280 nm. No other studies on the determination of this straight-chain, 40-carbon precursor in the biosynthesis of β -carotene were encountered. Peng *et al.* (1983) reported the separation and quantitation of α - and β -carotene using an Ultrasphere-ODS column and a mobile phase of 88% acetonitrile-tetrahydrofuran (3:1,

v/v) and 12% methanol- ammonium acetate (1%) (3:2, v/v), with the detector set at 436 nm. In the study reported by Nierenberg (1985), lycopene was also determined besides α - and β -carotene. The chromatographic conditions used were similar to those reported by Peng *et al.* (1983), including column, mobile phase, and detector. In both studies, β -carotene appeared to be partially separated and was not given much emphasis. Later, Sowell *et al.* (1988) reported the study of cis-isomers of carotenoids using a column that was maintained at 40°C, and a mixture of ethanol and acetonitrile as the mobile phase. Two detectors, at 450 nm and 340 nm were used to detect and monitor the carotenoids and the isomers.

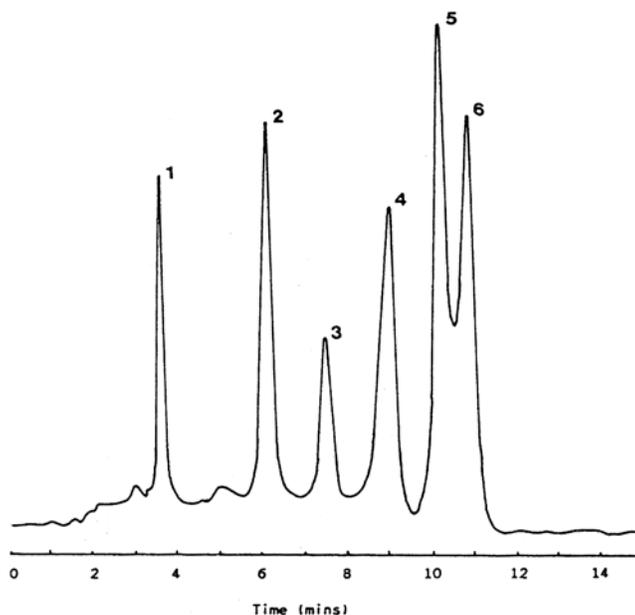


Figure 1. HPLC chromatogram of carotenoid standards. Detector 436 nm, 0.02 AUFS. Other chromatography conditions as given in text. Concentrations of lutein, cryptoxanthin and lycopene were 0.5 $\mu\text{g}/\text{ml}$, and of α -, β - and γ -carotenes were 1.0 $\mu\text{g}/\text{ml}$. 100 μl used for injection. 1 = Lutein; 2 = cryptoxanthin; 3 = lycopene; 4 = γ -carotene; 5 = α -carotene; 6 = β -carotene.

Table 4. Content¹ of major carotenoids in green, leafy vegetables

| Name of Vegetable | Lutein | Cryptoxanthin | Lycopene | γ -carotene | α -carotene | β -carotene | Others ² | Sum ³ |
|------------------------|--------|---------------|----------|--------------------|--------------------|-------------------|---------------------|------------------|
| Cashew leaves | 773 | 0 | 0 | 0 | 0 | 1340 | 0 | 2110 |
| Ceylon spinach | 1300 | 0 | 0 | 0 | 0 | 3530 | 0 | 4830 |
| <i>C. griffithii</i> | 9870 | 0 | 0 | 0 | 3680 | 32220 | 0 | 16800 |
| Chinese cabbage | 963 | 0 | 0 | 0 | 0 | 3020 | 0 | 3980 |
| Chinese chives | 1080 | 0 | 0 | 0 | 0 | 3510 | 0 | 4590 |
| Chinese kale | 1540 | 0 | 0 | 0 | 0 | 4090 | 0 | 5630 |
| Chinese mustard leaves | 1020 | 0 | 0 | 0 | 0 | 2930 | 0 | 3950 |
| Coriander leaves | 1340 | 0 | 0 | 0 | 0 | 3170 | 0 | 4510 |
| Curry leaves | 5250 | 0 | 0 | 0 | 0 | 9330 | 0 | 14600 |
| Drumstick leaves | 7130 | 0 | 0 | 0 | 0 | 7540 | 0 | 14700 |
| Fern shoots | 1000 | 0 | 0 | 0 | 0 | 1440 | 0 | 2440 |
| <i>H. javanica</i> | 1310 | 0 | 0 | 0 | 0 | 3840 | 0 | 5150 |

The medical importance of vitamin A and carotenoids

| | | | | | | | | |
|------------------|-------|---|---|---|---|-------|------|-------|
| Lettuce | 73 | 0 | 0 | 0 | 0 | 97 | 0 | 170 |
| Mint leaves | 1700 | 0 | 0 | 0 | 0 | 4840 | 0 | 6540 |
| M. citrifolia | 8840 | 0 | 0 | 0 | 0 | 3110 | 1300 | 13300 |
| N. oleracea | 6240 | 0 | 0 | 0 | 0 | 11400 | 0 | 17600 |
| Papaya shoots | 821 | 0 | 0 | 0 | 0 | 1830 | 0 | 2650 |
| Salted vegetable | 7320 | 0 | 0 | 0 | 0 | 3400 | 0 | 10700 |
| S. androgynus | 29900 | 0 | 0 | 0 | 0 | 13400 | 3290 | 46600 |
| Sesbania | 20200 | 0 | 0 | 0 | 0 | 13600 | 5890 | 39700 |
| S. nigrum | 2890 | 0 | 0 | 0 | 0 | 7050 | 0 | 9940 |
| Spinach | 4180 | 0 | 0 | 0 | 0 | 3180 | 0 | 7360 |
| Spinach, red | 2050 | 0 | 0 | 0 | 0 | 5090 | 0 | 7140 |
| Spring onion | 323 | 0 | 0 | 0 | 0 | 1280 | 0 | 1600 |
| Swamp cabbage | 335 | 0 | 0 | 0 | 0 | 1900 | 0 | 2240 |
| Topioca shoots | 1680 | 0 | 0 | 0 | 0 | 5720 | 0 | 7400 |
| Wolfberry leaves | 7590 | 0 | 0 | 0 | 0 | 5870 | 0 | 13500 |

1 mean of duplicate analysis: expressed as μg per 100 g of edible portion

2 unidentified carotenoids

3 summation of all carotenoids tabulated

Source: Tee & Lim (1991b)

Table 5. Content¹ of major carotenoids in green, non-leafy and other vegetables

| Name of Vegetable | Lutein | Cryptoxanthin | Lycopene | γ -carotene | α -carotene | β -carotene | Others ² | Sum ³ |
|-------------------------|--------|---------------|----------|--------------------|--------------------|-------------------|---------------------|------------------|
| Green, non-leafy | | | | | | | | |
| Chilli, green | 386 | 0 | 0 | 0 | 0 | 468 | 0 | 854 |
| Four-angled bean | 142 | 0 | 0 | 0 | 0 | 476 | 0 | 618 |
| French bean | 460 | 0 | 0 | 0 | 0 | 236 | 154 | 850 |
| Long bean (dark green) | 423 | 0 | 0 | 0 | 0 | 569 | 153 | 1150 |
| Long bean (light green) | 300 | 0 | 0 | 0 | 0 | 412 | 146 | 858 |
| Paprika/Bell pepper | 223 | 0 | 0 | 0 | 0 | 267 | 0 | 490 |
| Snake gourd | 225 | 0 | 0 | 0 | 0 | 148 | 0 | 373 |
| S. torvum | 154 | 0 | 0 | 0 | 0 | 74 | 0 | 228 |
| Others | | | | | | | | |
| Carrot | 0 | 0 | 0 | 0 | 3410 | 6770 | 0 | 10200 |
| Chilli, red | 941 | 1750 | 0 | 0 | 0 | 1660 | 1970 | 6320 |
| Pumpkin | 940 | 0 | 0 | 0 | 756 | 578 | 0 | 2270 |
| Tomato | 130 | 0 | 723 | 0 | 0 | 365 | 0 | 1220 |
| Yam stalks | 80 | 0 | 0 | 0 | 0 | 61 | 0 | 141 |

1 mean of duplicate analysis: expressed as μg per 100 g of edible portion

2 unidentified carotenoids

3 summation of all carotenoids tabulated

Source: Tee & Lim (1991b)

Table 6. Content¹ of major carotenoids in fruits

| Name of Vegetable | Lutein | Cryptoxanthin | Lycopene | γ - carotene | α - carotene | β - carotene | Others ² | Sum ³ |
|------------------------|--------|---------------|----------|------------------------|------------------------|-----------------------|---------------------|------------------|
| Banana (Pisang emas) | 27 | 0 | 0 | 0 | 62 | 40 | 0 | 129 |
| Banana (Pisang tanduk) | 37 | 0 | 0 | 0 | 157 | 92 | 0 | 286 |
| B. macrophylla | 457 | 155 | 0 | 52 | 0 | 301 | 514 | 1480 |
| Jackfruit | 95 | 17 | 0 | 0 | 0 | 56 | 56 | 224 |
| Mandarin orange | 113 | 688 | 0 | 59 | 0 | 81 | 151 | 1090 |
| Mango (Black Gold) | 0 | 0 | 0 | 0 | 0 | 615 | 0 | 615 |
| Musk lime | 65 | 446 | 0 | 0 | 0 | 12 | 0 | 523 |
| Orange | 30 | 332 | 0 | 0 | 0 | 25 | 218 | 605 |
| Papaya | 0 | 1480 | 2000 | 118 | 0 | 0228 | 294 | 4120 |
| Papaya (Exotica) | 0 | 615 | 2330 | 189 | 0 | 321 | 304 | 3760 |
| Plum, red | 149 | 40 | 0 | 0 | 0 | 127 | 0 | 316 |
| Starfruit/carambola | 66 | 1070 | 0 | 0 | 0 | 28 | 551 | 1720 |
| Tree tomato | 0 | 1240 | 0 | 0 | 0 | 599 | 0 | 1840 |
| Watermelon (red) | 0 | 457 | 5300 | 90 | 0 | 324 | 0 | 6170 |

1 mean of duplicate analysis: expressed as μg per 100 g of edible portion

2 unidentified carotenoids

3 summation of all carotenoids tabulated

Source: Tee & Lim (1991b)

c. Retinoids in food and feed

HPLC was also widely used in the determination of retinol in various foodstuffs. As for determination of carotenoids in plant materials, earlier studies had made use of normal-phase HPLC, whereas later studies tended to favour the use of reverse-phase HPLC. Two early studies using normal-phase HPLC were reported before 1980. Head & Gibbs (1977) reported the determination of retinol in mixed food items or composites of complete meals using a LiChrosorb Si60 column. The pigments were eluted using gradient elution by programmed mixing of hexane from one pump with methylene chloride-isopropanol from another. Retinol in the samples was monitored at 325 nm. In the same year, Dennison & Kirk (1977) reported the analysis of retinol in several cereal products. A μ Porasil column was used, and retinol eluted isocratically using a mobile phase consisting of a mixture of hexane and chloroform (containing 1% ethanol) and detected at 313 nm. In both studies, the investigators reported that the presence of carotenoids did not interfere with the determination of retinol.

d. Retinoids in blood

As for the analysis of retinoids in foods, early studies of retinoids in blood were carried out using normal-phase HPLC. De Ruyter and de Leenher (1976) reported the use of a 10- μm silica column with a mixture of petroleum ether, dichloromethane and isopropanol for the determination of serum retinol. Subsequently, these investigators employed reverse-phase HPLC

for the simultaneous determination of retinol and retinyl esters in serum (De Ruyter & de Leenher, 1978).

In general, reverse-phase HPLC applied to the determination of vitamin A in blood plasma or serum by various investigators were as described above for various foodstuffs. One early study in this area was reported by De Leenher *et al.* (1979), in which retinol and tocopherol were simultaneously determined. These two fat-soluble vitamins were also determined by Chow & Omaye (1983), Nierenberg & Lester (1985), and Sanz & Santa-Cruz (1986). In the last named study, the detector was first set at 340 nm for the first three minutes and thereafter at 280 nm. Williams (1985) employed fluorescence detection for the determination of retinol and tocopherol in serum. The detector was programmed for excitation and emission at two wavelengths. Huang *et al.* (1986) introduced further sophistication in the detection procedure in their study. Retinol was detected at 313 nm, whereas tocopherols were monitored using electrochemical detection.

Besides retinol, other retinoids in serum or blood samples were also investigated. Besner & Leclaire (1980) reported the determination of retinol and retinoic acid. In the study by Annesley *et al.* (1984), programmed gradient elution was used to effect the separation of various retinoids, namely retinol, retinyl acetate, trans- and cis-retinoic acid and etretinate. For the separation of isotretinoin, tretinoin and their 4-oxo metabolites, Wyss & Bucheli (1988) used two reverse-phase columns coupled in series. A fully automated gradient system and the column-switching technique was employed.

e. Carotenoids and retinoids in food samples

Several studies were encountered that determined both carotenes and retinol in various food samples. Since β -carotene has an absorption maximum at 450 nm but does not absorb at 325 nm, and the converse is true for retinol, both compounds could not be simultaneously detected at one wavelength. Thus, in the studies cited above, samples had to be injected twice in order to determine both compounds. However, simultaneous determinations could be achieved if two detectors set at 450 and 325 nm could be connected in series for continuous monitoring of the eluate. Alternatively, if a single detector was used, the wavelength could be changed in the middle of a run. The switch could be done manually, which would require close monitoring of the run, and could be rather tedious. More recently, programmable detectors have become available, in which wavelength change can be programmed at a pre-determined time. Several studies are cited below to illustrate the approaches taken to achieve simultaneous detection of carotene and retinol.

Landen & Eitenmiller (1979) reported the continuous monitoring of β -carotene at 436 nm and retinol and retinyl esters at 313 nm eluted from a μ Bondapak C₁₈ column using a mixture of methylene chloride and acetonitrile. Stancher & Zonta (1982) described their experiences in the simultaneous determination of retinol and its isomer, and α - and β -carotene in cheese. Normal-phase HPLC was carried out using a LiChrosorb Si60 column, an isocratic mobile phase of methylethylketone-hexane. To enable the detection of retinol and carotene in a single injection, the detector was first set at 450 nm detection of the latter. After the elution of carotene, the detector was quickly switched to 340 nm for retinol and its isomers. The investigators reported that the system was not able to separate α - and β -carotene.

Tee & Lim (1992) reported the simultaneous determination of retinol and several carotenoids in an HPLC system developed for the study of foods of animal origin. The carotenoids and retinol were separated isocratically on an octadecylsilane (C₁₈) column using a ternary mixture of acetonitrile, methanol and ethyl acetate. This is the same system as that described earlier for the determination of several carotenoids in fruits and vegetables. Two detectors connected in series were used to detect and quantify carotenoids simultaneously at 436 nm and retinol at 313 nm in a single chromatographic run (Figure 2). All the samples were also simultaneously determined using the AOAC open-column (alumina) chromatographic method. The AOAC method was found to give significantly higher retinol contents in the foods studied, due to the presence of other pigments that gave falsely elevated absorbance readings (Table 7). Although there was no statistically significant difference in β -carotene contents given by the HPLC and AOAC methods, there were more foods with higher results given by the latter method (Table 8).

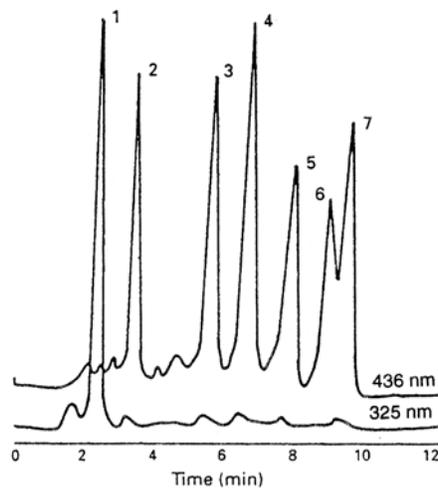


Figure 2. HPLC chromatogram of a mixture of retinol and carotenid standards. Retinol and carotenoids were detected at 325 nm and 436 nm respectively, using two detectors connected in series. Other chromatography conditions are as given in the text. A volume of 50 μ l of the standard mixture was injected. 1 = retinol; 2 = lutein; 3 = cryptoxanthin; 4 = lycopene; 5 = γ -carotene; 6 = α -carotene; 7 = β -carotene.

Source: Tee *et al.*, (1994)

g. Carotenoids and retinoids in blood

Several studies on the determination of both carotenes and retinol in blood or serum samples have also been made. As described for food samples above, earlier studies made use of two separate sample injections to enable detection of carotene and retinol in the same sample. Broich *et al.* (1983) reported the determination of lycopene, α - and β -carotene and retinyl esters using a Supelco C₁₈ column and a mobile phase of methanol, acetonitrile and chloroform. The carotenoids were monitored at 446 nm, and retinyl esters at 325 nm using two separate sample injections. In another study, Katrangi *et al.* (1984) reported the separation of lycopene, α -, β -, δ - and γ -carotene using a μ Bondapak C₁₈ column and acetonitrile-chloroform as the mobile phase. The carotenes were monitored at 462 nm. Delta- and γ -carotenes were not separated and usually comprised a small proportion of total carotenoids. α -Carotene was also not separated from β -

carotene, appearing only as a shoulder on the latter peak. Total carotenoids in the serum samples were also determined by obtaining absorbance reading of extracts at 450 nm. Retinol was also determined in the study, but using a separate chromatographic system. A 10- μ m Radial Pak C₈ reverse-phase column and a mobile phase consisting of methanol-water were used for this determination.

Table 7. Mean retinol content of selected foods of animal origin and processed foods

| Name of food | μ g per 100g of edible portion | | |
|--|------------------------------------|-------------|------------|
| | AOAC method | HPLC method | AOAC: HPLC |
| Eggs | | | |
| Century egg | 118 | 96.0 | 1.2 |
| Duck egg ¹ | 196 | 141 | 1.4 |
| Hen egg | 140 | 90.5 | 1.6 |
| Quail egg | 209 | 97.0 | 2.2 |
| Salted egg | 136 | 54.0 | 2.5 |
| Fish and Seafoods | | | |
| Anchovies | 50.5 | 14.5 | 3.5 |
| Black pomfret | 52.0 | 62.5 | 0.8 |
| Canned sardine | 76.5 | 13.5 | 5.7 |
| Cockles ¹ | 69.3 | 8.3 | 8.3 |
| Crab meat | 7.5 | 2.0 | 3.8 |
| Cuttlefish | 16.5 | 9.5 | 1.7 |
| Dried cuttlefish | 36.0 | 12.0 | 3.0 |
| Dried oyster | 132 | 32.5 | 4.1 |
| Dried prawn | 28.0 | 1.0 | 28.0 |
| Fermented shrimp | 143 | 98.5 | 1.5 |
| Indian mackerel | 25.5 | 8.0 | 3.2 |
| Lobster | 9.5 | 2.5 | 3.8 |
| Pink prawn | 8.5 | 3.0 | 2.8 |
| Scallop | 74.0 | 16.0 | 4.6 |
| Shrimp paste | 203 | 52.5 | 3.9 |
| Spanish mackerel | 34.0 | 7.5 | 4.5 |
| Meat and Meat Products | | | |
| Beef, local | 12.0 | 1.0 | 12.0 |
| Chicken burger | 79.5 | 15.5 | 5.1 |
| Chicken frankfurter | 46.5 | 11.5 | 4.0 |
| Chicken heart | 45.0 | 15.5 | 2.9 |
| Chicken liver ¹ | 6260 | 8240 | 0.8 |
| Chicken thigh | 24.5 | 16.5 | 1.5 |
| Mutton, local | 23.5 | 4.5 | 5.2 |
| Milk and Milk Products | | | |
| Butter | 176 | 140 | 1.3 |
| Cheese ² | 138 | 129 | 1.1 |
| Malted milk powder ¹ | 586 | 552 | 1.1 |
| Full-cream milk powder ¹ | 661 | 672 | 1.0 |
| Sweetened condensed filled milk ¹ | 265 | 281 | 0.9 |
| Sweetened condensed milk | 274 | 257 | 1.1 |
| Ultra-high temperature (UHT) milk | 38.5 | 20.5 | 1.9 |
| Oils | | | |
| Ghee | 169 | 151 | 1.1 |
| Margarine | 246 | 243 | 1.0 |
| Other Processed Foods | | | |

| | | | |
|---------------------|------|-----|------|
| Canned baked bean | 28.0 | 1.0 | 28.0 |
| Cornflakes | 764 | 803 | 1.0 |
| Wheat infant cereal | 363 | 363 | 1.0 |

Mean of duplicate analyses, except items marked 1 (6 analyses) and 2 (4 analyses)
Source: Tee & Lim (1992)

Table 8. Mean β -carotene content of selected foods of animal origin and processed foods

| Name of food | $\mu\text{g per 100g of edible portion}$ | | |
|--|--|-------------|------------|
| | AOAC method | HPLC method | AOAC: HPLC |
| Eggs | | | |
| Century egg | 11.0 | 3.5 | 3.1 |
| Duck egg ¹ | 24.0 | 31.8 | 0.8 |
| Hen egg | 16.0 | 1.0 | 16.0 |
| Quail egg | 20.0 | 5.0 | 4.0 |
| Salted egg | 18.5 | 9.5 | 2.0 |
| Fish and Seafoods | | | |
| Anchovies | 7.0 | 6.0 | 1.2 |
| Black pomfret | 0.4 | 0.1 | 4.0 |
| Canned sardine | 1980 | 62.5 | 31.7 |
| Cockles ¹ | 157 | 194 | 0.8 |
| Crab meat | 33.0 | 44.0 | 0.8 |
| Cuttlefish | 0.0 | 0.0 | 1.0 |
| Dried cuttlefish | 9.5 | 1.0 | 9.5 |
| Dried oyster | 208 | 138 | 1.5 |
| Dried prawn | 33.0 | 34.5 | 1.0 |
| Fermented shrimp | 1.5 | 0.1 | 15.0 |
| Indian mackerel | 1.5 | 0.1 | 15.0 |
| Lobster | 136 | 149 | 0.9 |
| Pink prawn | 41.0 | 49.5 | 0.8 |
| Scallop | 55.0 | 39.0 | 1.4 |
| Shrimp paste | 14.5 | 7.5 | 1.9 |
| Spanish mackerel | 0.3 | 0.1 | 3.0 |
| Meat and Meat Products | | | |
| Beef, local | 6.5 | 9.5 | 0.7 |
| Chicken burger | 0.7 | 3.5 | 0.2 |
| Chicken frankfurter | 0.2 | 1.4 | 0.2 |
| Chicken heart | 1.5 | 0.1 | 15.0 |
| Chicken liver ¹ | 29.3 | 17.7 | 1.7 |
| Chicken thigh | 0.0 | 0.0 | 1.0 |
| Mutton, local | 0.0 | 0.0 | 1.0 |
| Milk and Milk Products | | | |
| Butter | 563 | 675 | 0.8 |
| Cheese ² | 173 | 221 | 0.8 |
| Malted milk powder ¹ | 14.7 | 7.5 | 2.0 |
| Full-cream milk powder ¹ | 128 | 137 | 0.9 |
| Sweetened condensed filled milk ¹ | 2.5 | 5.5 | 0.5 |
| Sweetened condensed milk | 37.2 | 52.8 | 0.7 |
| Ultra-high temperature (UHT) milk | 8.5 | 20.0 | 0.4 |
| Oils | | | |
| Ghee | 452 | 549 | 0.8 |
| Margarine | 907 | 897 | 1.0 |

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| | | | |
|-----------------------|------|------|------|
| Other Processed Foods | | | |
| Canned baked bean | 2050 | 127 | 16.2 |
| Cornflakes | 78.5 | 51.5 | 1.5 |
| Wheat infant cereal | 18.5 | 20.5 | 0.9 |

Mean of duplicate analyses, except items marked 1 (6 analyses) and 2 (4 analyses)

Source: Tee & Lim (1992)

This laboratory also reported the simultaneous determination of retinol and several carotenoids in serum using a reversed-phase HPLC method (Tee *et al.*, 1994). The method is as described above for the determination of retinol and carotenoids in food samples. Mean retinol and carotenoids for the 100 subjects studied are as given in Table 9. The most abundant carotenoids in the serum samples studied were lutein and cryptoxanthin, followed by β -carotene and lycopene (Figure 3). All the serum samples were also simultaneously determined using the direct spectrophotometric method for carotenoids and the Carr-Price colorimetric method for retinol. Compared to the conventional methods, the HPLC method was found to give significantly higher results for retinol and total carotenoid concentrations. The major advantages of the liquid chromatographic method are that it is more specific and that it overcomes the problems associated with the Carr-Price method. In addition, only the HPLC procedure could provide an account of the serum carotenoid profile.

The HPLC method was subsequently improved to enable the simultaneous determination of tocopherols in addition to retinol and several carotenoids (Tee and Khor, 1995). The addition of another detection channel enabled the simultaneous determination of these three groups of vitamins/provitamins. Further improvement is being made to the method to enable it to be used for small volume of blood samples from children collected from field surveys.

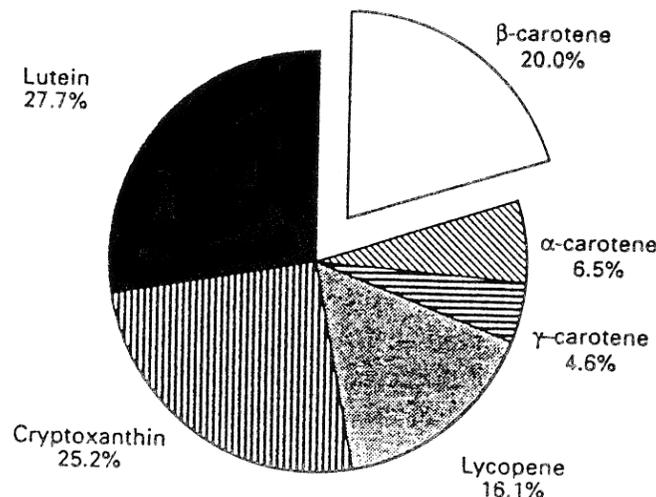


Figure 3. Proportions of major carotenoids in human sera (n = 100).

Source: Tee *et al.*, (1994)

CONCLUSIONS

Vitamin A deficiency remains a serious problem in many developing countries of the world, affecting mainly young children. An estimate is that at any one time, some 20-40 million pre-school children suffer from at least mild deficiency of the vitamin. Such numbers are mind-boggling and beyond the imagination of people living in countries relatively free of the problem. A large proportion of these affected children are from countries in the South and East Asia region. This laboratory has proposed to carry out some investigations into the extent of the problem in the country in the near future.

The main source of vitamin A in the diet of the rural communities in developing countries is carotenoids, the precursors of vitamin A, from vegetables and fruits. Pre-formed vitamin A in meat, liver, and eggs is frequently out of reach of the economically deprived. Many of these countries have abundant vegetation, rich sources of carotenoids. There should indeed be no vitamin A deficiency problem. It is however known that vegetable consumption among young children is poor. Whatever small amounts of carotenoids ingested together with a diet poor in fat and protein makes absorption of these precursors of vitamin A poorly absorbed. At the same time, it is also not clear which of the fruits and vegetables are richest in β -carotene. These are obviously important areas for studies and intervention.

Table 9. Serum retinol and carotenoid levels ($\mu\text{g}/\text{dl}$) of study subjects

| | Retinol | Lutein | Cryptoxanthi | Lycopene | γ - Carotene | α - Carotene | β - Carotene | Sum | Total |
|----------------------|---------|--------|--------------|----------|------------------------|------------------------|-----------------------|-------|-------|
| All subjects (n=100) | | | | | | | | | |
| Mean | 74.2 | 35.4 | 35.5 | 22.7 | 6.5 | 9.1 | 29.2 | 138.3 | 196.0 |
| Median | 74.0 | 31.8 | 28.9 | 18.1 | 5.4 | 7.6 | 24.1 | 128.8 | 185.4 |
| s.d | 23.0 | 13.5 | 20.5 | 13.9 | 3.8 | 6.7 | 21.3 | 61.8 | 83.2 |
| Minimum | 30.9 | 6.0 | 3.6 | 1.1 | 0.0 | 0.0 | 2.7 | 15.2 | 23.3 |
| Maximum | 167.1 | 71.2 | 105.7 | 68.3 | 19.5 | 39.6 | 116.9 | 335.8 | 448.4 |
| Male (n=58) | | | | | | | | | |
| Mean | 77.5 | 37.0 | 36.0 | 21.6 | 6.4 | 8.4 | 25.5 | 134.8 | 194.6 |
| Median | 76.4 | 31.8 | 29.4 | 16.4 | 5.7 | 7.5 | 21.3 | 128.1 | 185.4 |
| s.d | 22.8 | 14.0 | 20.6 | 13.0 | 3.7 | 6.4 | 16.4 | 55.7 | 77.0 |
| Minimum | 35.5 | 14.3 | 3.6 | 4.3 | 2.1 | 0.0 | 5.2 | 41.1 | 62.2 |
| Maximum | 167.1 | 70.8 | 98.6 | 68.3 | 19.5 | 39.6 | 80.9 | 325.3 | 443.1 |
| Female (n=42) | | | | | | | | | |
| Mean | 69.6 | 33.2 | 34.7 | 24.2 | 6.6 | 10.1 | 34.3 | 143.2 | 198.0 |
| Median | 70.5 | 32.1 | 27.9 | 21.3 | 5.3 | 8.5 | 27.9 | 134.2 | 185.9 |
| s.d. | 22.6 | 12.6 | 20.6 | 15.1 | 3.8 | 7.1 | 26.0 | 69.8 | 92.1 |
| Minimum | 30.9 | 6.0 | 4.7 | 1.1 | 0.0 | 0.0 | 2.7 | 15.2 | 23.3 |
| Maximum | 135.6 | 71.2 | 105.7 | 56.8 | 18.4 | 31.7 | 116.9 | 335.8 | 448.4 |

Source: Tee *et al.* (1994)

A new frontier in vitamin A research has been the examination of a possible association between carotenoids and retinoids and the development and prevention of cancer. Research in this area

can be likened to a huge jig-saw puzzle, with the pieces slowly being put into their places. Evidences have been accumulating from various angles to support this association. Publications in this field have been increasing at a very rapid rate. Nevertheless, it appears that it will take some time before a clear picture can be seen. It is still too early for recommendations to be made for routine retinol or carotenoid supplementation to prevent cancer.

Since the discovery of vitamin A as a fat-soluble growth factor in the early part of this century, research into carotenoids and retinoids has attracted the attention of many scientists. Volumes have been written about them, and many more will certain follow since many questions remain to be answered. Even the biochemical mechanisms of action of the vitamin and precursors remain largely unknown. There is thus a great deal of avenue for studies, for scientists looking for excitement in research.

The analysis of carotenoids and retinoids is complicated and beset with various problems. The analysis is complicated because of the large number of naturally occurring carotenoids (as many as 500 have been reported). There are much fewer retinoids, but the occurrence of cis- and trans-isomers of carotenoids and retinoids further complicates the analysis. The composition and content of carotenoids in various plant materials and retinoids in foods of animal origin vary widely. Not all of the naturally occurring carotenoids are precursors of vitamin A, and for those with provitamin A activity, the biological activity varies widely. There is also considerable variation in the biological activity of various retinoids.

The main problems associated with work on carotenoids and retinoids arise from the inherent instability of these compounds, especially towards light, heat, oxygen and acids. Various precautionary measures have to be taken during sample preparation and analysis. Thus careful and dedicated workers are required for the analysis. Another obstacle in the analysis is the difficulty in obtaining authentic reference carotenoids and retinoids as most of them are not available commercially.

It is therefore rather difficult to obtain accurate data on carotenoid and retinoid content and composition. The literature abounds with attempts at improving the analytical techniques. The procedure should be able to effectively remove interfering compounds, separate the carotenoids and retinoids and quantitate them accurately. This review has shown that there has been much work on method development and improvement in this field in the last three decades. From the use of counter-current distribution in the 1960's, thin-layer chromatography and adsorption (open) column chromatography became widely used in the 1970's and 1980's. From the late 1970's, high-pressure liquid chromatography became more prominently used for the analysis of carotenoids and retinoids, mainly because of the ability of the technique to effect rapid separation, its non-destructiveness and, more importantly, the better resolution that is achieved. The ability of HPLC to rapidly separate and quantitate various carotenoids and retinoids, at least in standard preparations, has been demonstrated. Its application to the analysis of foods, however, is still being developed and improved. As with other vitamins, the application of HPLC in the determination of these compounds will see further development and wider application in the future.

Many investigators have shown that the use of other techniques in combination with HPLC will greatly enhance the usefulness of the latter, especially so during method development. The combined use of open-column chromatography and UV-visible absorption spectra, for example, would assist in the identification and confirmation of carotenoids and retinoids.

It has often been said that many laboratories in developing countries will not be able to possess a high-pressure liquid chromatograph. Contributory factors are the high cost in the purchase as well as the maintenance of HPLC. It is also costly to run the instrument, e.g. in the purchase of solvents and columns. In addition, it requires considerable skill to operate. However, it has been shown that for the satisfactory separation of the carotenoids and retinoids, HPLC is the method of choice. It is not necessary to invest in complicated HPLC systems and look for the full range of carotenoids, many of which occur in minute amounts in foods. More accurate results and the considerably shorter time required would make a simple HPLC system a worthwhile investment.

ACKNOWLEDGEMENT

I would like to thank the Director of the Institute for Medical Research, Kuala Lumpur, for granting permission to publish this paper.

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