Carotenoids in Staple Foods

and their

Interaction with other Essential Nutrients

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"Make food thy medicine,"

(Hippocrates, circa 400 BC)

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Abstract

Carotenoids are responsible for a large proportion of the red, orange and yellow pigments found in nature. Their value in natural systems extends beyond that of colour, as they are precursors to vitamin A, antioxidants and important in eye health. The aim of the studies presented here was to identify useful sources of carotenoids in staple foods and their interactions with other nutrients.

The potential for improving carotenoid nutrition in wheat is the initial focus of the presented work. Colorimetry using the CIE L* a* b* method was identified as a useful tool for fast identification of lutein concentration in durum wheat and both lutein and provitamin A concentration in bread wheat. A further study on the effect of storage conditions on colour and carotenoid concentration showed that in wheat stored as grain an increase in temperature from 5 °C to 35 °C resulted in a large decrease in b* value, lutein and β-carotene concentration. However, in wheat stored as flour, a greater decrease in b* value and carotenoid concentration was caused by storage for 6 months, regardless of temperature.

In addition to grains, fruit and vegetables are important in diets of developed populations. Commonly consumed fruit and vegetables were analysed to identify useful sources of lutein and zeaxanthin, involved in the prevention of age related degeneration of sight. The leafy green vegetables parsley, kale and spinach were the best source of lutein isomers, with total lutein concentrations of 110, 95 and 90 mg/kg respectively. The greatest concentration of zeaxanthin isomers was found in spinach and parsley with total zeaxanthin concentrations of 3.9 mg/kg and 3.7 mg/kg respectively. Zeaxanthin concentration was not related to food type nor was the zeaxanthin to lutein isomer ratio.

In the final study the effect of lutein on iron uptake and metabolism in chickens was investigated. Four treatment diets were compared for the response in haemoglobin concentration including high concentrations of supplemental β-carotene and lutein and low concentrations of lutein from a supplement and wheat. Birds on the high supplemental lutein diet had haemoglobin concentrations equal to those on the β-carotene diet, and above that of birds on the other diets. Hepatic iron stores were not significantly different between birds on the different diets indicating that remobilization of hepatic stores was not the cause of the increase in haemoglobin concentrations. The increase in haemoglobin concentration resulting from an interaction between dietary iron and lutein determined here, has the potential to decrease the incidence of iron deficiency anaemia world-wide. Further

studies are necessary however to endorse the results of this preliminary study, determine the optimal time period and the dose response effect, and to confirm that the interaction is present in humans.

This thesis represents a valuable contribution to the understanding of the relationships between colour and carotenoid concentration in wheat. Furthermore, this study has investigated for the first time the interaction between lutein and iron bioavailability in an animal model. Iron deficiency affects populations world wide and an increase in the bioavailability of this essential nutrient due to an interaction with one of the most abundant carotenoids has the potential to dramatically reduce this insidious problem.

Declaration

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person except where reference has been made in the text.

I consent to a copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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List of Publications

Rosser, J.M. Khachik F. & Graham R.D. (1999) Distribution of lutein and zeaxanthin and related geometrical isomers in fruit, vegetables, wheat and pasta products. 12th International Carotenoid Symposium, Cairns, 19th - 23rd July 1999 (poster).

Khachik, F., Bernstein, P.S., Squires, A. and Rosser, J.M. (1999) Identification of carotenoids and related metabolites in frog retina and liver: a useful non-primate model for studying the physiological role of macular carotenoids. 12th International Carotenoid Symposium, Cairns, 19th - 23rd July 1999.

Sperazzo, P., Gellenbeck, K., Khachik, F., Bubrick P.& Rosser, J.M. (1999) Differential cellular distribution of carotenoid pigments in 3 strains of the alga *Dunaliella salina.* 12th International Carotenoid Symposium, Cairns, 19th - 23rd July 1999 (poster).

Graham, R.D. and Rosser, J.M. (1999) Carotenoids in staple foods: their potential to improve human nutrition. International Rice Research Institute workshop October 5 -7 1999, Los Banos, Philippines, organised by the International Food Policy Research Institute (IFPRI).

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Humprhies, J.M., Graham, R.D., McIntosh, G., Worsley, T. and Khachik. F. (2000) Increasing the concentration of carotenoids in wheat to improve nutrition in impoverished countries. First South East Asia and Pacific Regional Meeting on Carotenoids, Bangkok, Thailand August 2nd - 5th 2000.

Humphries J.M. and Graham R.D. (2000) Nutritionally enhanced grains for a balanced diet. Dietitians Association of Australia- South Australian Branch State Conference, Adelaide, Australia, 3rd-4th November 2000.

Graham, R.D. and Humphries, J.M. (2000) Carotenoids in staple foods: Their potential to improve human nutrition. Food Nutr.Bull. 21(4):404-409

CHAPTER 1

Literature Review

1.1 Introduction

Carotenoids are responsible for a large proportion of the red, orange and yellow pigments found in nature. They are present in all photosynthetic and some non-photosynthetic organisms and their functions include absorption of light and the quenching of singlet oxygen radicals. The main group of carotenoids can be split in two: carotenes which are hydrocarbons and have provitamin A activity, and xanthophylls which are the oxygenated derivatives of carotenes and are considered to have no vitamin A activity.

Humans are not able to synthesise carotenoids, or produce vitamin A (retinol) without the necessary precursors and so must obtain these by ingestion of plant material containing carotenoids, or by consumption of animal products containing pre-formed retinol. Due to the variation in type and level of carotenoids in plants, a wide range of fruit and vegetables must be consumed to obtain the recommended daily allowance (RDA) for retinol. The RDA ranges from 350 to 1000 μg of retinol activity equivalents (RAE) /day depending on age and gender (Olson, 1987; FAO/WHO, 1988; NRC, 1989).

Carotenoids are important as antioxidants in the prevention of atherosclerosis (Block, 1994; Iribarren, 1997; Panasenko, 1997; Dugas, 1998) and diseases related to oxidative damage of cellular DNA/RNA (Jacques, 1991; Palozza, 1992; Stahl, 1998). Provitamin A carotenoids as precursors to vitamin A are essential for sight in the absence of preformed retinol, the first physical sign of deficiency manifesting itself as night blindness. The xanthophylls are also important in human health and have been linked to maintenance of healthy sight and cancer prevention and treatment.

Vitamin A and iron are two of the most common deficient nutrients in the world (FAO/WHO, 1991; WHO, 1992), and an interaction seems inevitable given that these deficiencies occur almost without exception in the same populations. Several studies have shown positive interactions between vitamin A and iron resulting in improved concentrations of both these nutrients (Mejia, 1985; Bloem, 1995) an interaction

between iron and β-carotene has also been reported (Garcia-Casal *et al.*, 1998). A positive interaction between xanthophylls and iron may also be present depending on the nature of as yet undetermined mechanisms involved in this synergy.

At present global nutrition policies rely on supplementation and fortification programs which in turn rely heavily on governmental support for continued operation. The development of more nutritious foods that can be grown by the people who would consume them will reduce reliance on the extremely expensive and often unevenly distributed food aid (Welch *et al.*, 1997). This could be achieved by breeding crops that have higher micronutrient content and reducing the losses of nutrients that occur with the current harvesting and food production methods. Additionally, increasing the diversity of food crops, and changing the mix of foods eaten during meals to promote better absorption of nutrients by the body can help improve nutrient uptake from foods (Welch *et al.*, 1997) and thus decrease the need for supplementation programs.

This review covers the history and basic chemistry of carotenoids and their role in natural systems including human health and nutrition. Additionally, analytical techniques for the extraction, identification and quantification of carotenoids from various biological materials are covered. A review of suitable animal models for the final study presented is also given, as are reasons for the selection of the particular model. Finally the aims of the studies undertaken in this thesis are outlined.

1.2 History

Carotenoids first attracted scientists to their bright colours early in the 19th century and this was followed by investigation into the presence of these compounds in various biological substances by Branconnot (1817) and Aschoff (1818) (in Isler, 1971). Carotene was first isolated by Wackenroder (1831) (in Isler, 1971), and this was followed by the naming of the yellow colour in autumn leaves as xanthophyll by Berzelius (1837) (in Isler, 1971). Early in the 20th century Tswett and Willstatter (in Isler, 1971) identified methods for separation and identification of several carotenoids, followed by development of column chromatography specifically for carotenoid and chlorophyll separation. Considerable advances were made in the following years, in the identification of the carotenoid chemical structures leading to the recognition that vitamin A was closely related to half a molecule of β-carotene by Karrer (1931); and in

the same year McCollum and Davis (1931) (in Isler, 1971) discovered "fat soluble A". In 1957 the ultimate proof that carotene was indeed a precursor to vitamin A was demonstrated by Moore (1957) (in Isler, 1971) in rats. Thus the importance of carotenoids beyond their colours was established.

1.3 Characteristics

1.3.1 Structure

The 600 plus carotenoids identified in nature are grouped by their common eight isoprenoid units, joined so that the arrangement of the units is reversed at the centre of the structure (**Figure 1.1**). Identifying features of this group include a long chain of conjugated double bonds in the centre of the molecule and near symmetry around the central double bond (Britton, 1995). This long chain of double bonds, while giving the carotenoids their characteristic colours with spectral absorbance between 400-500 nm (Armstrong and Hearst, 1996), renders the molecules extremely susceptible to oxidative degradation (Britton, 1991). Carotenoids also differ in polarity, which determines their solubility, with the (non-polar) carotenes being more soluble in non-polar solvents, and (polar) xanthophylls, with their hydroxyl groups more soluble in polar solvents. This difference in polarity determines their interaction with the stationary phase of packed chromatographic columns, the basis of their separation by elution.

Only 38 of the 600 carotenoids identified are precursors to vitamin A, and of these few have sufficient vitamin A activity to be of importance as a source of vitamin A (Britton, 1983). Beta-carotene is the most abundant of the provitamin A carotenoids and also has the highest provitamin A activity (Scita, 1992). The provitamin A activity of a compound is defined as its ability to act as a precursor of vitamin A and is determined in part by the number of β rings present. Beta-carotene has two β-rings, and all other provitmain A carotenoids including α-carotene have one β-ring, whereas totally inactive compounds such as lycopene have none, as shown in **Figure 1.1**.

Free radical and singlet oxygen quenching is a feature of carotenoids with more than seven conjugated double bonds (Di Mascio *et al.*, 1989), and is independent of the provitamin A activity of the compound (Di Mascio *et al.*, 1989). Carotenoids are hydrophobic, and are therefore found in the hydrophobic areas of the cell (e.g.

membranes such as the plasmalemma, tonoplast, chloroplast-amyloplast; fat droplets; aleurone layer of cereals; cuticles and cuticular layers (O' Brien, 1979). Carotenoids in plants are located in plastids called chromoplasts, and are found in fruit and petals (Bramley, 1997). Organic solvents are required for extraction of carotenoids from biological materials due to their hydrophobicity; this also affects the method of analysis used.

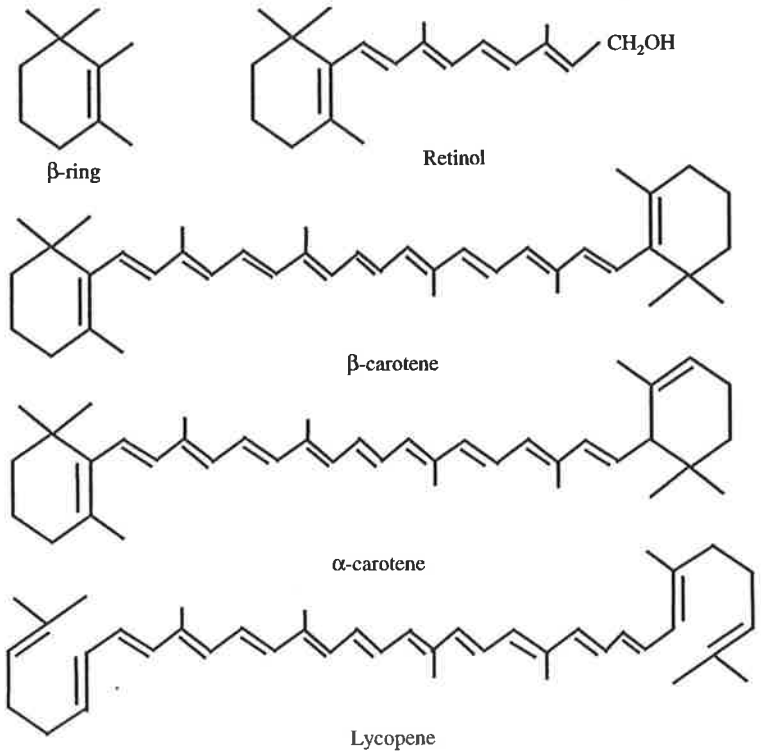


Figure 1.1 Structures of retinol, α- and β-carotene showing changes in β-ring bulk and rotational symmetry of the units at the centre of the structure (Britton, 1995).

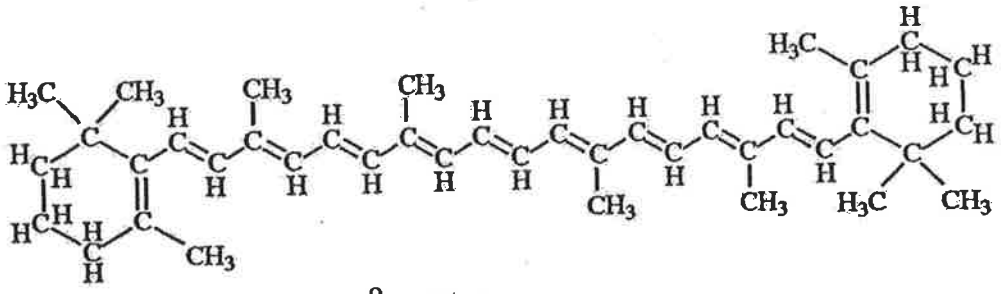
1.3.2 Nomenclature

More than 40 C-H groups are found in the backbone of the carotenoid structure and the complete structural formulae (**Figure 1.2 a**) are infrequently given in full. The skeletal

formula normally used is also shown for β-carotene in **Figure 1.2 b**. The numbering of the position of the carbons is also shown in **Figure 1.2 b**. Usually only the relevant numbers are shown for the identification of isomers.

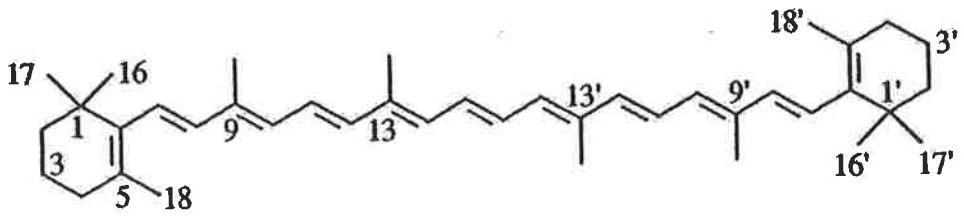
The replacement of *trans* and *cis* with *E* and *Z* respectively has been used throughout in accordance with international convention on the nomenclature of chemical isomers and are shown in **Figure 1.2c**.

a)



β-carotene

b)



β-carotene

c)

Trans (E) *Cis (Z)*

Figure 1.2 Complete structural formula **(a)**, skeletal formula and numbering of the position of carbon atoms **(b)** for β-carotene, and **(c)** *cis/trans* or *E/Z* alternatives. (Weedon and Moss, 1995)

Structurally different spatial configurations are indicated by a numerical prefix to the absolute carotenoid structure indicating the site of transformation. For example, 9Z-lutein indicates a *Z* transformation at the 9th carbon of the polyene chain of lutein.

The prefix "all" indicates that all of the double bonds in the specific carotenoid structure are in either the *E* or *Z* formation depending on the proceeding letter. For example, all *E*-lutein refers to the whole of the lutein chemical structure in the *E* configuration. Carotenoids are generally found in the all-*E* state, and unless otherwise stated this is the assumed configuration. Therefore, as a result, only all-*Z*-carotenoids will be specified.

The used of the prefix "*epi*" refers to a type of isomerisation whereby the isomeric compounds contain several asymmetric centres but differs in the configuration of only one of these centres (Weedon and Moss, 1995). In the context of **Chapter 3** 3'-epi-lutein refers to a difference in the configuration (ie. *E*) at the 3'carbon, which is otherwise in the *Z* configuration.

1.3.3 Biosynthetic Pathway

The key features of the primary biosynthetic pathway of carotenoid formation is shown in **Figure 1.3**. The initial step in carotenoid biosynthesis is the condensation of three acetyl Co-A molecules to produce 3-hydroxy-3, methylglutaryl Co-A (HMG Co-A). Acetyl Co-A is formed from either pyruvate dehydrogenation via pyruvate dehydrogenase in glycolysis or β-oxidation of fatty acids (Goodwin, 1965).

HMG Co-A is converted to mevalonic acid (MVA), which undergoes phosphorylation and decarboxylation to produce isopentenyl pyrophosphate (IPP), undergoing isomerisation to form dimethylallyl pyrophosphate (DMAPP). DMAPP acts as a starter for chain elongation for the C40 carotenoids and, combined with another IPP molecule via prenyl transferase, gives geranyl pyrophosphate. A further IPP molecule gives farnesyl pyrophosphate (FPP, C15) and addition of yet another IPP molecule results in geranyl geranyl pyrophosphate (GGPP). GGPP is the C20 precursor of phytoene (Goodwin, 1980).

Two GGPP molecules condense via a prephytoene pyrophosphate intermediate to form phytoene the first of the C40 terpenoids (Goodwin, 1980). The removal of two hydrogens at a time from alternate sides of the molecule gives phytofluene, ξ-carotene, neurosporene and lycopene. Lycopene or neurosporene undergo cyclization to produce the bicyclic α-carotene and β-carotene via the monocyclic α-zeacarotene, β-zeacarotene, δ-zeacarotene and γ-zeacarotene (Britton, 1991). Cyclization reduces the length of the

molecule and increases the bulk of the end groups. Other carotenoids, there being over 600 in nature, are mostly derived from lycopene.

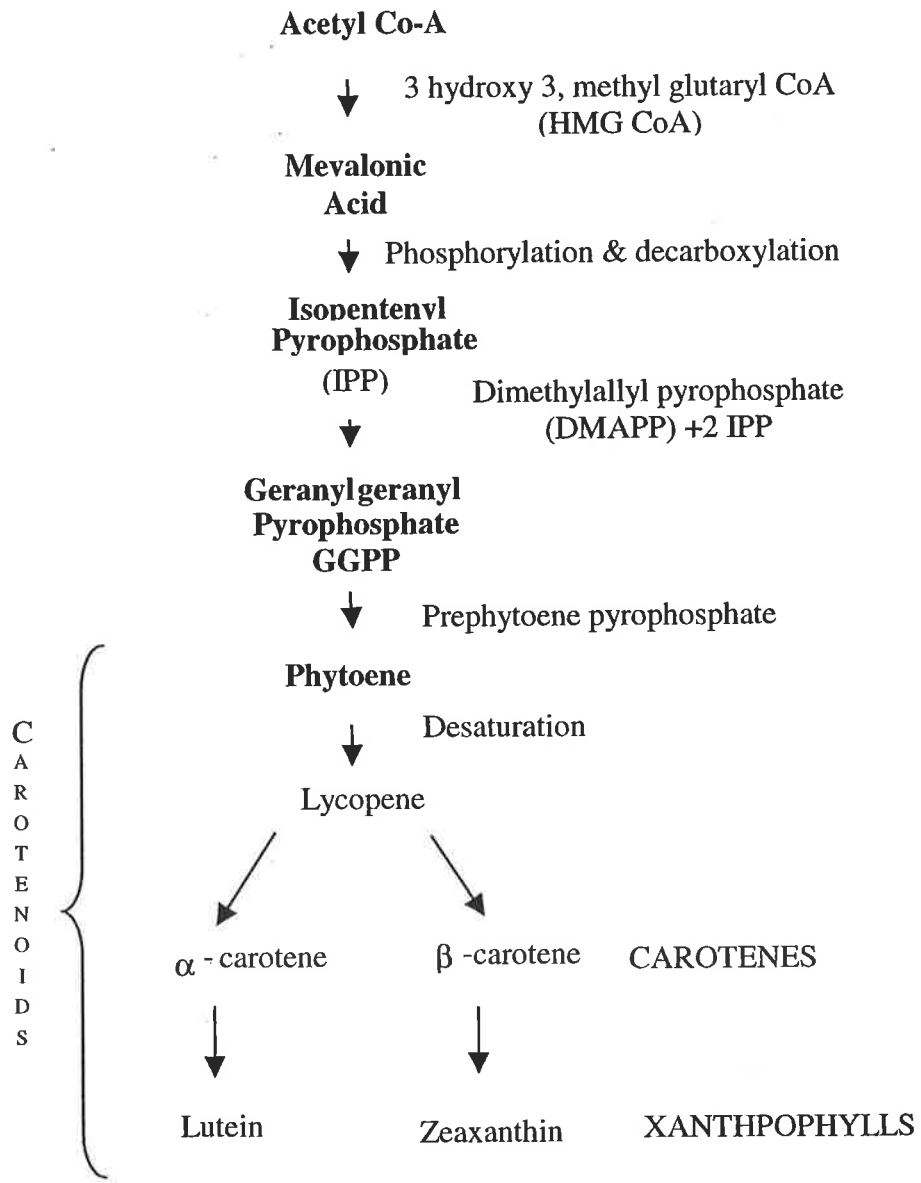


Figure 1.3 Major steps in the biosynthetic pathway of carotenoids.(Goodwin, 1993)

1.3.4 Stability

Carotenes form epoxides when in contact with oxygen, and β-carotene which has the highest provitamin A activity, is easily degraded in the presence of light and oxygen (Goodwin, 1980). Xanthophylls are also susceptible to oxidative degradation so that oxygen-free, decreased light and minimum temperature conditions are required for efficient extraction of carotenoids from biological samples.

Several studies have debated the degrading role of light, temperature and oxygen on various carotenoids (Peiser and Yang, 1979; Belitz and Grosch, 1987; Scita, 1992): and the general consensus is that minimisation of exposure to these conditions is necessary to achieve minimal degradation of carotenoids. Therefore, to ensure degradation is kept to a minimum, extractions in this project will follow a program of exclusion of all white light from the samples via the use of yellow lighting and storage under nitrogen at -20°C.

It is concluded from the literature that the addition of antioxidants during extraction, e.g. butylated hydroxytoluene (BHT) is also recommended to reduce degradation (Craft, 1992). However, complete prevention of degradation is impossible for compounds as subject to degradation as these, especially as extraction procedures require numerous steps.

1.4 Role in Nature

Carotenoids impart the red, orange and yellow colours associated with many plants and animals, and have an essential role in photoprotection (Palozza and Krinsky, 1992), and as attractants for pollinators. In all photosynthetic plants, carotenoids are associated with the grana of chloroplasts in the form of chromoproteins. They provide photo-oxidative protection against the effects of singlet oxygen and radicals, generated in the presence of light during photosynthesis, protecting photosystem II (Griffiths *et al.*, 1955; Goodwin, 1980; Armstrong and Hearst, 1996; Miller *et al.*, 1996). The carotenoids transfer absorbed radiant energy to chlorophyll molecules, dissipate excess energy and quench excited-state chlorophylls (Thommen, 1979; Palozza and Krinsky, 1992; Armstrong and Hearst, 1996). The most important carotenoids in this system are astaxanthin, canthaxanthin, lutein and zeaxanthin (Thiele and Krause, 1994).

Carotenoids are also useful in dissipating the excessive heat produced by photosynthesis in water-stressed plants(Smirnoff, 1993). The increase in heat produced when water stress is experienced is due to the limitation of CO_2 fixation, and results in exposure of chloroplasts to excessive energy (Smirnoff, 1993). The benefits of carotenoid antioxidant effects have also been observed in animals with astaxanthin, a xanthophyll, having the greatest effect (Zamora *et al.*, 1983; Miki, 1991).

1.4.1 Carotenoids in Plant Development

The levels of carotenoids in plant species vary considerably depending on the species and stage of development. An increase in the carotenoid content of tomatoes during ripening is due to an accumulation of lycopene within the plastids and loss of chlorophyll as the chloroplasts changed to chromoplasts (Fraser *et al.*, 1994). Accumulation of lycopene as the major carotenoid indicates that only a proportion of the C40 chains found are cyclised into β-carotene, resulting in accumulation of lycopene precursors in the pericarp of the ripening fruit (Laval-Martin *et al.*, 1975).

Curiously, accumulation of carotenes in ripening fruit is accompanied by or immediately preceded by a decrease in carotenogenic enzyme activity (Fraser *et al.*, 1995). This may be due to a decrease in the need for photoprotectants as the plant ripens, with a lower rate of carotenoid turnover reducing the need for enzymes (Fraser *et al.*, 1995). Fraser *et al.*, (1995) also found that concentrations of β-carotene and neaxanthin increased throughout maturation of the leaf.

Lacroix and Lier (1975) investigated changes in grain pigment concentration in durum wheats spectrophotometrically. They found that carotenoids increased in the first 10 days after anthesis, followed by a slow decrease until grain maturity. Using open column chromatography, Lacroix and Lier (1975) identified total carotenes as well as lutein, but did not separate individual carotenes; therefore the provitamin A activity cannot be calculated from their results. Additionally, this mode of chromatography exposes the carotenes to conditions likely to promote oxidation, therefore decreasing the accuracy of carotene estimation. Rosser (1997) identified and quantified carotenoids in bread wheat during grain development using high performance liquid chromatography (HPLC). A peak in carotenoid accumulation in the grain was observed 12 days after anthesis, followed by a decrease to maturity. Carotene concentrations also followed this

pattern of accumulation and degradation. This pattern of carotenoid accumulation has been confirmed in durum wheat (D. Mares *pers. comm.*, 1999).

A study of soybean varieties by Monma *et al.*, (1994) also found that β-carotene decreased as the seed matured. It may be that the decrease in carotenoids as grain matures is common to many field crops.

1.4.2 Carotenoids in Wheat

In the quest for whiter bread, the yellow pigmentation in flour has been selected against by plant breeders. The opposite is true for durum wheat cultivars where yellow colour is favoured in pasta production.

In mature wheat grains, a greater concentration of the carotenes are found in the embryo (0.72 mg/kg), than in the starchy endosperm (0.09 mg/kg) (Kruger and Reed, 1988). This was confirmed by Rosser (1997) by analysis of flour fractions obtained from a commercial wheat quality mill. However, the lower concentration in the starchy endosperm is negated to some extent by the greater proportion of this fraction consumed. The concentration of provitamin A carotenoids in wheat is therefore potentially of use in increasing the vitamin A nutrition of populations. Durum wheat cultivars, with their distinct yellow flour, have also been analysed for carotenoid content (Lacroix and Lier, 1975), and lutein was determined to be the major carotenoid present.

Many of the published studies into the carotenoid content of wheat were performed prior to the routine use of high performance liquid chromatography (HPLC), and therefore do not have the desired separation and quantification of carotenes needed to enable accurate determination of provitamin A activity. However, several bread wheat varieties have been evaluated for their provitamin A activity using HPLC techniques (Rosser, 1997). Carotenes, appear to make up a small proportion of the whole carotenoid profile of wheat, with lutein the dominant carotenoid.

Colour measurement in wheat

Scoring methods for flour colour of bread wheat have been extensively reported in literature (Lepage and Sims, 1968; Sims and Lepage, 1968; Bhatt and Mc Master, 1976; Shuey, 1976; Shuey *et al.*, 1977; Watson and Shuey, 1977; Miskelly, 1984; Robinson *et*

al., 1984). However these studies relied on extraction of the carotenoids from the flour, and colour determination by various chromatographic procedures that are inaccurate (Robinson, 1984). .

When comparing flour colour, the inaccuracies associated with visual observations can be avoided by the use of an arbitrary colour measure such as the Minolta chroma-meter, which gives a detailed colour analysis (in the form of L*, a* and b* values) that is independent of the operator.

Wheat lines can be analysed for colour using a Minolta Chroma Meter (model C-100) that incorporates a handheld device linked to a processing unit with an in-built printer. The Minolta Chroma Meter, using the L*, a*, b* system, which is most suitable for yellow colour measurements, allocates each sample a colour reference on a theoretical colour wheel. L* representing the brightness of the colour on a 0-100 unit scale, a* ranging from -60 (pure green) to +60 (pure red) and b* ranging from -60 (pure blue) to +60 (pure yellow) (**Figure 1.4**).

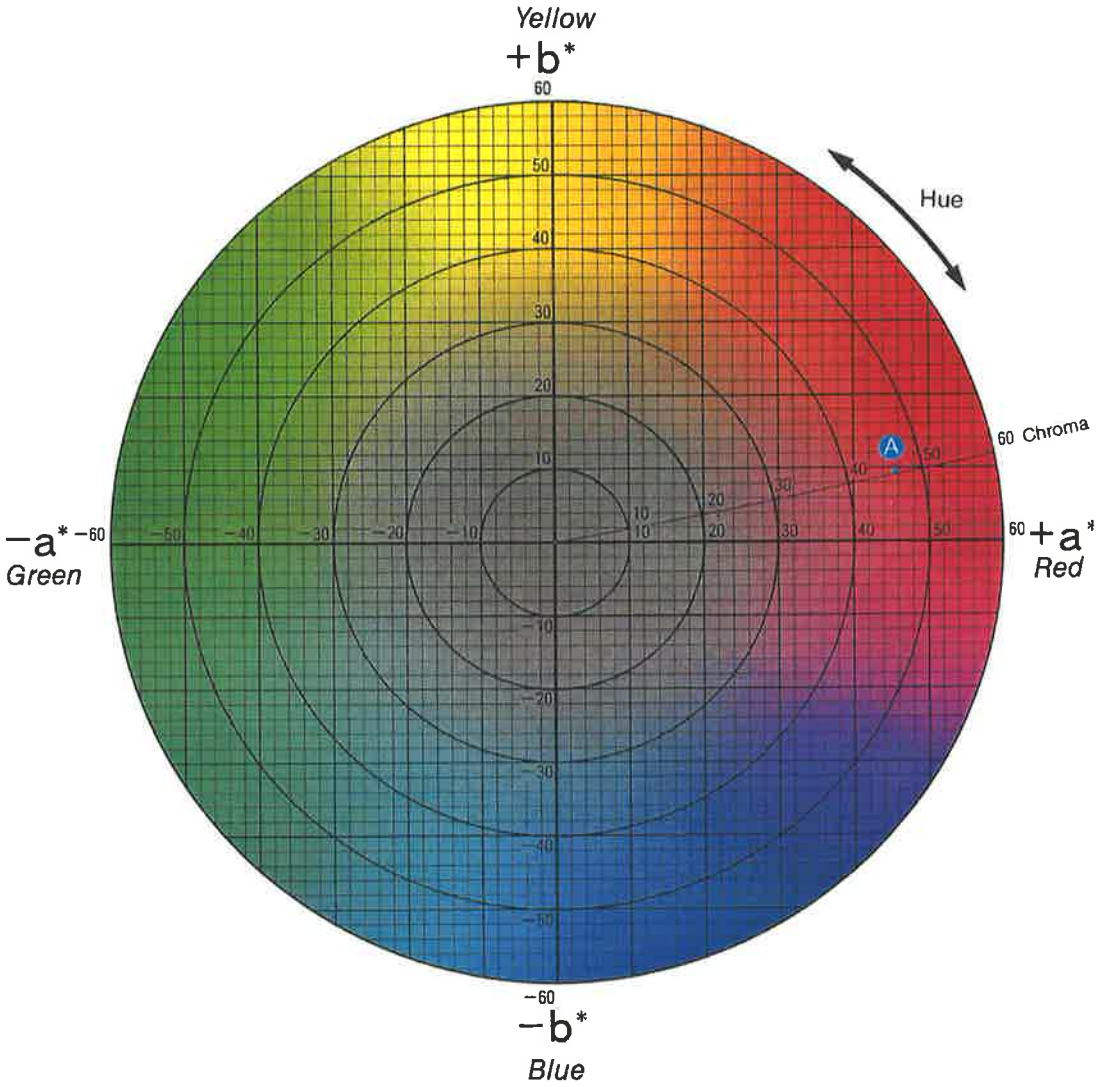


Figure 1.4 Visual representation of Minolta Colour Meter values b* and a* (McGuire, 1992).

1.5 Human Health

In humans, carotenoids exhibit a range of biological activities. They act as vitamin A precursors, and as antioxidants (Burton, 1989) that modulate cell growth, gene expression (King *et al.*, 1997) and immune response (Krishnan *et al.*, 1974) and are essential for sight (Rock, 1997). The xanthophylls, lutein and zeaxanthin are also involved in the maintenance of visual acuity (Seddon *et al.*, 1994). Additionally, the antioxidant ability of carotenoids has been investigated in cancer prevention and treatment (van Poppel, 1993). The amount of a nutrient present in a diet does not necessarily indicate the amount available for physiological functions. An understanding of the factors involved in the bioconversion and bioavailability of carotenoids is essential to determine the value of foods containing high carotenoid concentrations.

1.5.1 Metabolism of Carotenoids

Absorption

Carotenoids are absorbed by humans in a non-specific way that allows both carotenes and xanthophylls to be absorbed, leading to a complex mixture of carotenoids in human plasma (Parker, 1989). Carotenoids are absorbed from the intestine with the aid of dietary fat, and carotenes are cleaved to retinaldehyde and further reduced to form retinal. Retinol from this source and preformed vitamin A are incorporated into chylomicrons (triglyceride-rich absorptive lipoproteins) for transport in the serum (Erdman *et al.*, 1993). Distribution of the various carotenoids in organs and tissues is related to their structure, as well as their biological activity, provitamin A activity and *in vivo* conversion to vitamin A (Parker, 1989).

Transport

In plasma, carotenoids along with α- tocopherol (vitamin E), are bound to lipoproteins while retinol itself is specifically bound to retinol-binding protein (Ringer *et al.*, 1991). Carotenoids are transported in human plasma exclusively by lipoproteins. Seventy five percent of hydrocarbon carotenoids (those that have only hydrogen and carbon atoms) including β-carotene, α-carotene and lycopene, are associated with the low-density lipoproteins (LDL) and the remaining 25% with the high-density lipopoteins (HDL). Lutein, a non-hydrocarbon carotenoid, and vitamin E are equally distributed between the

LDL and the HDL fraction (Parker, 1989). Beta-carotene is carried in the blood by low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL) and high density lipoproteins (HDL) (Krinski *et al.*, 1958; Marenah *et al.*, 1983).

Large amounts of carotenoids that are more polar than lutein have not been found in human plasma, even though they are common in fruit and vegetables (Parker, 1989). While this could be due to poor absorption or inefficient metabolism, it is probably due to degradation in the acids of the stomach, which is known to be the case with xanthophylls such as violaxanthin. Some of the carotenoids found in blood and tissues may actually be stable degradation products of those carotenoids affected by stomach acid. For example Jensen *et al.*, (1986) reported no increase in blood 9-Z-β-carotene concentration following supplementation, which suggests that it might be converted to the all-*trans* isomer in the gastrointestinal tract.

Storage

Provitamin A carotenoids are generally cleaved into retinaldehyde in the intestinal mucosa. However, in the human system some of the carotenoids pass through the mucosal cells uncleaved. These are incorporated into the chylomicra and stored in the tissues of the body, predominantly the adipose tissues. Small amounts of carotenoids are taken up by the liver (Olson, 1984), while 90% of vitamin A is stored in the liver. Due to their hydrophobic character carotenoids are associated with the lipid portions of human tissues including lipid rich aggregates and membranes, as well as proteins and enzymes (Parker, 1989). Adrenal tissues which have a high rate of LDL uptake contain the highest concentration of carotenoids (Racia *et al.*, 1972; Spady *et al.*, 1985). Additionally, Ringer *et al.* (1991), found a positive correlation between β-carotene concentration and body mass index and a similar tendency with percent body fat that may indicate a deposition of β-carotene in the adipose tissue.

Excretion

Of the ingested vitamin A, 20% is not absorbed but is excreted via faeces. Of the remaining 80%, 20-50% is conjugated or oxidised to products that are excreted within one week via urine or faeces and the remaining 30-60% is stored. Therefore 1/3-2/3 of vitamin A is excreted within one week and the remainder is stored (Olson, 1984).

1.5.2 Antioxidants / Free Radical scavengers / Singlet Oxygen Quenchers

A free radical is an atom or molecule that has one or more unpaired electrons. It is therefore highly energized, and damages what it strikes. Such damage to DNA may lead to new cells that can become benign tumours or malignancies (Olson, 1984). The formation of free radicals is part of normal cellular processes (eg. reduction of molecular oxygen to water) and to counteract this there are substances that deactivate the free radicals (Niki *et al.*, 1991). These are called antioxidants or free radical scavengers (Olson, 1984).

Compounds such as vitamins E and C, lycopene and other carotenoids couple with or donate an electron to the free radical to deactivate it. The reactivity of the molecule is passed from one to another until it is nullified or passed from the body as a water-soluble compound (Olson, 1984). For example, when vitamin E scavenges a free radical it becomes a free radical itself; carotenoids deactivate this radical becoming a free radical compound. Then vitamin C donates an electron, but this molecule can be flushed from the body as it is a water-soluble compound (Olson, 1996).

The importance of β-carotene as a precursor to vitamin A has resulted in extensive investigation into its properties. Some of the more researched functions are shown in **Figure 1.5**. Beta-carotene can be grouped with the antioxidant vitamins C and E (Byers and Peery, 1992; Olson and Krinsky, 1995) as it functions as a singlet oxygen quencher, and as an antioxidant preventing free radical damage to cellular components (Peto *et al.*, 1981). Photosensitivity diseases such as the genetic disease of porphyrin metabolism, erythropoietic protoporphyria (EPP) have also been treated successfully for many years with β-carotene (Laval-Martin *et al.*, 1975; Mathews-Roth, 1991; Olson and Krinsky, 1995).

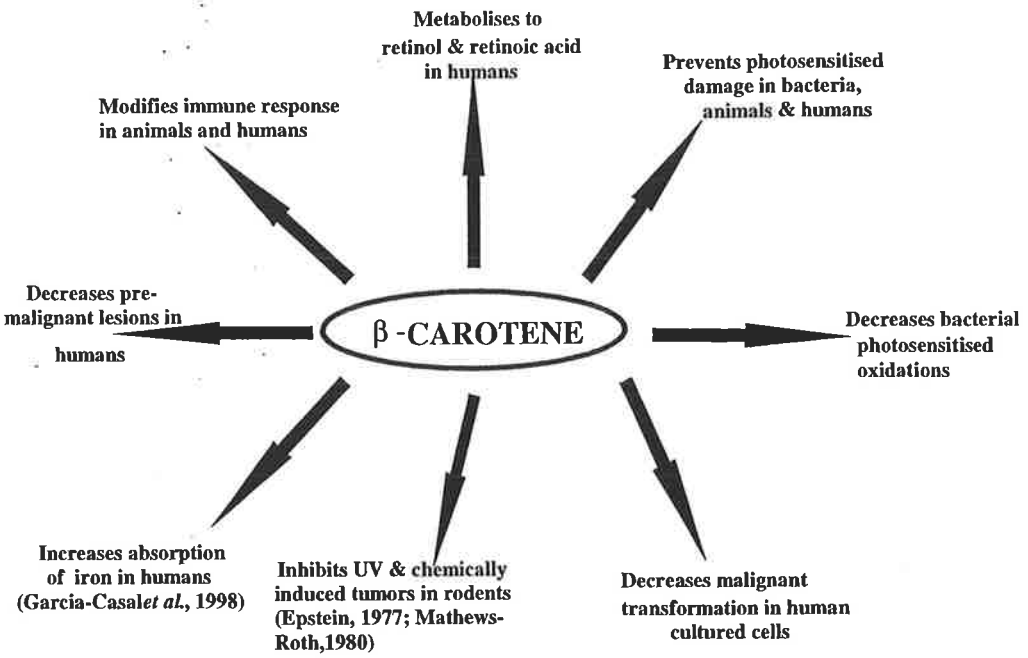


Figure 1.5 Biological functions of β-carotene (modified from (Krinsky, 1991).

Terao *et al.* (1990) reported that astaxanthin and canthaxanthin showed more efficient scavenging of free radicals than β-carotene and zeaxanthin. This led to the hypothesis that carotenoids possessing oxygen side-groups were more efficient scavengers than those without the oxygen side-groups. This was reinforced by Di Mascio *et al.* (1991) who suggested that the quenching properties of carotenoids were related not only to the triplet energy state (ie. length of the conjugated double bond system) but also to the functional groups.

Lycopene is the most active biological free radical fighter and efficient oxygen quencher *in vivo*, being twice as effective as β-carotene (Di Mascio *et al.*, 1991; Mackerras, 1995). However, lycopene, lutein and zeaxanthin were all reported to react rapidly with oxidising agents in a study by Woodall *et al.* (1997a).

Thiele and Krause (1994) determined that only carotenes were directly involved in singlet oxygen quenching while xanthophylls acted more as quenching amplifiers, which are not directly involved in the quenching process. Vitamin A itself has been determined to be a poor antioxidant compared with β-carotene, lacking the ability to quench singlet oxygen (Passwater, 1993).

1.5.3 Heart disease and cancer

A wide range of carotenoids both alone and in combination have been found to have a protective effect against stroke and coronary heart disease (Morris and Kritchevesky, 1994; Mosca *et al.*, 1997; Clinton, 1998).

The chemopreventive effect of carotenoids was originally related to vitamin A activity (Malone, 1991). However, it is now known that the cancer protection provided by carotenoids has no relationship to their ability to form vitamin A (Ziegler, 1991), but the protection is related to its properties as a singlet oxygen scavenger and antioxidant. Epidemiological evidence indicates that diets rich in total carotenoids (not just carotenes) are protective against cancer (Murakoshi *et al.*, 1989). There have been extensive studies conducted on the association between increased cancer risk and carotene status (Colditz *et al.*, 1985; Pagnini-Hill *et al.*, 1987; Connett *et al.*, 1989; Stähelin *et al.*, 1991; Ziegler, 1991) all concluding that there is a definite inverse relationship.

Many carotenoids have been investigated in relation to the treatment and reduction of cancer and related tumours, including skin, oral, gastrointestinal, prostate, breast and cervical cancers. An extensive review of the literature on carotenoids and cancer presented by van Poppel (1993), concluding that there is a strong relationship between a high intake of carotenoid-rich fruit and vegetables and a decreased incidence of cancer. However, further studies are necessary to identify specific carotenoids effectiveness on individual cancers and the focus until now has been on β-carotene.

1.5.4 Maintenance of sight

Retinal is crucial in sight, and a source such as β-carotene or vitamin A, (all-*trans*-retinol) which can be converted into all-*trans*-retinal by NAD^+ oxidation and cleavage, followed by isomerisation to 11-*cis*-retinal, is essential (Mathews and van-Holde, 1990). Lutein and zeaxanthin are essential carotenoids that function critically as visual and accessory photopigments to protect the eye from free radical damage, and thus are found in high concentrations in the retina. These xanthophylls protect the synapses that support the neurons and photoreceptors from oxidative destruction (Mathews and van-

Holde, 1990). Additionally, lutein and zeaxanthin are important in the maintenance of visual acuity.

At the centre of the retina directly behind the lens is the macular region which receives the greatest concentration of light of all areas of the eye. Zeaxanthin is concentrated in this region, while lutein is dispersed throughout the whole retina. An increase in the consumption of leafy green vegetables leading to a high serum level of carotenoids is thought to reduce the incidence of a degenerative disease of this region, termed age related macular degeneration (AMD) (Brown *et al.*, 1998; Hammond *et al.*, 1997a). The protective effect is attributed to lutein and zeaxanthin filtering out the blue-light that makes the retina and lens vulnerable to degeneration (Bruno and Medeiros, 2000).

An inverse relationship between plasma β-carotene and the incidence of cataract has been identified (Jacques and Chylack, 1991). Additionally, since β-carotene can trap radicals and is able to function at low partial pressures of oxygen (Burton, 1989; Burton and Ingold, 1984), it is critical in the lens (Kwan *et al.*, 1972; Varma, 1991).

1.5.2 Precursors to vitamin A

The role of vitamin A in health and nutrition is discussed here as the provitamin A carotenoids have a major role in reducing the diseases associated with deficiency of vitamin A. Vitamin A is required for the maintenance of healthy epithelial tissues, mucous membrane production and adequate immune response as well as for sight (Mc Laren, 1986).

Carotenoid Conversion to Vitamin A

Provitamin A carotenoids cannot be utilised as vitamin A precursors by humans without conversion to retinaldehyde (**Figure 1.6**). This conversion involves cleavage of the central double bond (central cleavage) by an enzyme carotenoid-15,15' dioxygenase, which is found in the cytoplasm of liver cells and the intestine, to give retinal (one molecule from α-carotene, two from β-carotene). Carotenoid -15,15' dioxygenase requires oxygen as a direct reactant which adds to the -15,15' double bond of the carotenoid, which is then cleaved into retinaldehyde (one from α-carotene, two from β-carotene). The carotenoid-15,15' dioxygenase cleavage of β-and α-carotene, and many apocarotenals is central to synthesis of retinaldehyde (Mathews and van-Holde, 1990).

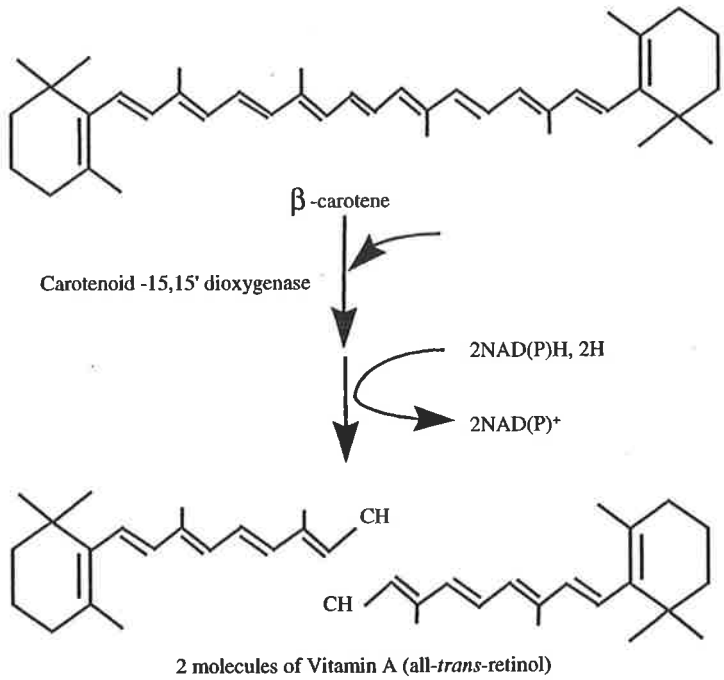


Figure 1.6 Synthesis of vitamin A from β-carotene.
(Mathews and van-Holde, 1990)

Early reports (Grangaud *et al.*, 1962, In Matsuno, 1991) that animals from the lower orders such as fish and crustacea had the ability to reduce xanthophylls to retinol were not confirmed until a study by Gross and Budowski (1966) identified that astaxanthin, canthaxanthin and isozeaxanthin (β,β-carotene-4,4'-diol) were converted into vitamin A in fish. It was also confirmed that zeaxanthin was converted to vitamin A as it crossed the intestinal wall in goldfish (Austern and Gawienowski, 1969). A more recent study determined that the reduction of hydroxy-carotenoids to retinol and 3-dehydroretinol was also present in a mammal (rat) (Matsuno, 1991). The mechanism is reported to be reliant on negative feed-back with vitamin A replete individuals not expressing this mechanism (Scheidt *et al.*, 1985). To my knowledge this mechanism has not been investigated in birds, however, this mechanism may be contributing to the apparent effect of lutein on iron absorption observed in **Chapter 4**.

Bioavailability and Bioconversion

Bioavailability is defined as the fraction of an ingested nutrient available for utilisation in normal physiological functions and for storage (West and Eilander, 2001), or more

briefly, the proportion of a nutrient ingested that can be used (Castenmiller and West, 1997). Bioconversion is defined as the fraction of a bioavailable nutrient (in this case an absorbed provitamin A carotenoid) converted to the active form of the nutrient (retinol) (West and Eilander, 2001). Combined, bioavailability and bioconversion are termed bioefficacy, the efficiency that an ingested nutrient is absorbed and converted to the active form of the nutrient (West and Eilander, 2001).

In light of a study on pregnant women in Indonesia (de Pee *et al.*, 1995), the conversion estimate of 6 μg of β-carotene to produce 1 μg of retinol (FAO/WHO, 1967) has recently been reviewed (US Institute of Medicine, 2000). Signs of vitamin A deficiency were observed despite adequate intake of vitamin A precursors from fruit and leafy-green vegetables. 12 μg of β-carotene is now regarded as 1 μg retinol activity equivalent (RAE), replacing the FAO/WHO principle of 6 μg β-carotene regarded as 1 μg retinol equivalent (RE). Beta-carotene has the highest RAE of all the carotenes, double that of α-carotene, and oxygenated carotenoids such as lutein are generally though to have no provitamin A activity. However, xanthophylls such as canthaxanthin, zeaxanthin and tunaxanthin have been found to be precursors to retinoids in both fish and rats (Schiedt *et al.*, 1985; Goodwin, 1986; Matsuno, 1991).

Retinol Activity Equivalents (RAE) can be calculated by the following internationally accepted formula:

$$\textbf{1 μg RAE} = \frac{\textbf{(μg β-carotene / 6) + (μg α-carotene + μg cryptoxanthin) / 12)}}{\textbf{2}}$$

(US Institute of Medicine, 2000)

This is a general formula for the determination of the retinol value of a food. However, to obtain a more accurate idea of the retinol value of a whole diet, separate equations need to be used for each type of food. For example, van Lieshout *et al.* (2001), determined that the bioefficacy of β-carotene from pumpkin was 2.1 times that of this carotenoid in spinach. Other investigations into the bioefficacy of carotenoids from different food sources include studies of dark green leafy vegetables and yellow/orange fruit (de Pee *et al.*, 1998; Khan *et al.*, 1998; Tang *et al.*, 1999; van het Hof *et al.*, 1999;

van het Hof *et al.*, 2000). From the limited data available, it seems that the xanthophylls may be absorbed more efficiently than the carotenes in humans (Furr and Clark, 1997). From these reports it is clear that the bioavailability of carotenoids from foods must be taken into account when recommending foods to combat carotenoid deficiencies. It is also apparent that single integer can be used to determine bioavailability as this varies between and also within foods. Therefore total dietary intake of carotenoids is dependent on both the concentration present in the food, the amount of the food consumed and the bioavailability of the carotenoid.

Vitamin A deficiency

Vitamin A deficiency usually manifests itself in the form of night blindness followed by permanent blindness if not treated (Solon, 1986). Worldwide over 500,000 new cases of xerophthalmia (nutritional blindness) occur every year with a disproportionate number in developing countries. This is directly related to a deficiency of vitamin A precursors (carotenes) in the diet. (Solon, 1986).

Diets deficient in vitamin A account for 1-2 million deaths each year in children aged 1 to 4 years (UNICEF, 2000). Additionally, deficiency results in more than 250 million children annually being unable to maintain their immune systems or the lining of their respiratory tracts thereby reducing resistance to disease and infection (Bloch, 1928; Boynton and Bradford, 1931; Beisel, 1982). Every year 250,000 children go blind and another 250 000 have their sight impaired. Of these, at least 100, 000 die within a few weeks, directly as a result of vitamin A deficiency (Grant, 1991).

Fortification Programs

Vitamin A deficiency is such an enormous problem that the World Health Organisation has urged all member states to give high priority to the prevention and control of vitamin A deficiency and xerophthalmia. Fortification programs have been carried out in many areas and are generally quite successful (Emodi and Scialpi, 1980). Unfortunately the products fortified are inaccessible to people most in need and the programs are costly to implement and require continued governmental funding.

An increase in the content of vitamin A precursors in staple foods, such as wheat flour, could help alleviate this problem, but little information has been published on

carotenoid content of grain using the modern analytical capabilities now available. Unfortunately, many foods high in starch are low in carotene content (Mc Laren, 1986). Such foods include the staples rice, wheat and white maize, leading to deficiencies in populations that can least afford to combat the problem. However yellow maize, sorghum and sweet potato have high concentrations of carotenes (Duffus and Slaughter, 1980; Ali *et al.*, 1994; Mwanri *et al.*, 2000) so these staple are therefore valuable in the prevention of vitamin A deficiency.

1.6 Vitamin and Mineral interactions

1.6.1 Vitamin A and Zinc

The interaction between vitamin A (retinol) and zinc is synergistic and has been well documented (Smith, 1980). Zinc is involved in the synthesis of retinol binding protein (RBP) which in turn releases retinol from the liver, while retinol enhances absorption and transport of zinc by promoting the synthesis of a related protein (Bliss, 1951; Vallee and Hoch, 1957). Udomkesmalee *et al.* (1992) demonstrated the strong synergistic effects of supplementing with these nutrients together leading to a rapid reduction in child deficiencies of these nutrients. Smith (1980), Solomons and Russell (1980) and Christian and West (1998) have presented detailed reviews on the interaction between zinc and vitamin A.

1.6.2 Vitamin A and Iron

The interaction between retinol and iron was initially determined by Hodges *et al.* (1978) in a study that showed that anaemia responded to retinol but not iron supplementation in retinol deficient individuals. This has been reaffirmed by further studies in animals (Roodenburg *et al.*, 1994), and humans in various populations (Mejia and Arroyave, 1982; Muhilal *et al.*, 1998). Garcia-Casal *et al.* (1998) found an improvement in nonheme iron absorption with supplementation of vitamin A or β-carotene added to diets of wheat, rice or corn. In addition, vitamin A deficiency has been associated with poor iron status (Mejia and Arroyave, 1982; Bloem *et al.*, 1989; Suharno *et al.*, 1993) and several studies have shown that vitamin A supplementation increases blood haemoglobin levels (Mejia and Chew, 1988; Suharno *et al.*, 1993; Muhilal *et al.*, 1998).

The mechanism behind the increased absorption of iron with supplemental β-carotene has been attributed to the ability of chemical compounds containing double bonds to react with iron (Garcia-Casal *et al.*, 1998; Elschenbroch and Salzer, 1992). Iron that is liberated during digestion forms a complex with β-carotene, and by acting as a chelating agent prevents complexes forming with phytates and polyphenols. Based on this mechanism the xanthophylls also have the potential to increase iron absorption as they too have numerous conjugated double bonds.

The potential for xanthophylls to interact with iron and enhance absorption is the basis for **Chapter 4**. In order to determine if this interaction existed and to relate results to human nutrition it was essential to identify an animal model that closely resembled the absorption and metabolism of both iron and xanthophylls in humans. The following section presents a comparison of animal models used previously in iron and carotenoid studies, identifying the model that will be used in **Chapter 4**.

1.7 Animal Models

1.7.1 Carotenoid Research

Progress in research into the bioavailability and metabolism of carotenoids has been restricted due to the lack of an animal model that reliably replicates human carotenoid metabolism (Furr and Clark, 1997; Lee *et al.*, 1999). Many animal models have been proposed and used in various areas of carotenoid research; however the model needs to be selected specifically for the research purpose to ensure suitability, and no single model is suitable for all carotenoid research (Lee *et al.*, 1999). Animals used for carotenoid research include: the Mongolian gerbil (*Meriones unguicullatus*) (Lee *et al.*, 1998), domestic ferrets (Boileau *et al.*, 1999), pre-ruminant calves (Bierer *et al.*, 1995), rats and mice (Sato *et al.*, 1997), non-human primates (Slifka *et al.*, 1999), pigs (Chew *et al.*, 1991), hamsters (Schwartz and Shklar, 1987; Erdman *et al.*, 1986), dogs (Chew *et al.*, 1998), rabbits (Yap *et al.*, 1997; Dorey *et al.*, 1998), frogs (Khachik *et al.*, 1999d) and chickens (Ganguly *et al.*, 1959; Dua *et al.*, 1967; Erdman *et al.*, 1986; Tyczkowski and Hamilton, 1986). The diversity of this list is an indication of the restrictions pertaining to, and the specificity of each model, making selection difficult for more than a limited range of analyses from one experimental model. Availability of the animal

selected for this study also had to be considered, ruling out what is considered one of the most useful models, the Mongolian gerbil, due to quarantine restrictions in Australia.

There are differing opinions on the usefulness of one of the most readily available animal models, the rat. Clark *et al.* (1998) showed the rat to be a suitable model for studying xanthophylls. However, a review of animal models, (Lee *et al.*, 1999) stated that the rat absorbed carotenoids differently to humans and therefore extrapolation of data to humans must be treated with care. Increasing evidence has shown that the rat is not suitable to extrapolate to humans for carotenoid uptake and metabolism (G. Britton *pers.com.*, 2000). However, they are a suitable model for studying the effects of vitamin A deficiency (Lee *et al.*, 1999), and the effects of carotenoids on cancer development (Moon, 1989).

Chickens have been evaluated as a bioavailability model and have been used for numerous absorption studies involving carotenoids. Erdman *et al.* (1986) used chickens to study fibre and β-carotene utilisation determining that dietary fibre decreased β-carotene untilisation. Dua *et al.* (1967) looked at the utilisation of xanthophylls from natural sources by the chicken and found that there was a linear relationship between increased dietary xanthophylls and skin and plasma xanthophylls. Tyczkowski and Hamilton (1986) investigated differential absorption of zeacarotene, cryptoxanthin and lutein in poultry and found differences in sites of absorption for these carotenoids in the intestine. Rats and chickens have been compared for their sites of absorption of carotenoids, vitamin A and cholesterol, (Ganguly *et al.*, 1959) and it was found that while both the rat and chicken convert β-carotene into vitamin A in the intestine, the rat does not absorb any xanthophylls while the chicken does. The differential absorption of xanthophylls between the rat and chicken has considerable relevance in selecting an animal model for the proposed study (**Chapter 4**) which aims to determine the effect of a xanthophyll on haemoglobin concentration.

1.7.2 Iron Research

The importance of iron in human nutrition has led to extensive studies in both animal and human systems. Several studies on the utilisation and metabolism of iron in chickens have been conducted (Davis *et al.*, 1968; Cao *et al.*, 1996), and Pla and Fritz (1971) compared the rat and chicken as models of iron bioavailability in humans

determining both animals to be equally suitable. An additional study (Pla *et al.*, 1973) showed that the chicken utilised large particles of iron more efficiently than the rat and human due to the action of the crop on the large particles. Particle size was not an issue in the proposed study as the particle size was the same as that used in the AOAC rat haemoglobin repletion test. Chicks have also been found to resemble humans more closely in the effects of iron salts (sodium iron pyrophosphate and ferric orthophosphate) (Pennell *et al.*, 1976). Chickens were selected for iron studies based on the ease of achieving anaemia and the absence of coprophagy (consumption of faeces) which can be a confounding factor in rat studies, depending on the iron source (Ranger and Neale, 1984; Zhang *et al.*, 1992). A close correlation between dietary iron and hepatic and renal iron concentrations has been determined (Cao *et al.*, 1996), though this was determined under supplementation in otherwise replete individuals.

Rats are favoured as the model for iron repletion studies although several differences between humans and rats have been noticed. Rats absorb ferrous and ferric iron while humans preferentially absorb the ferrous form, and the bioavailability of haem and non-haem iron to rats is similar while humans preferentially absorb haem iron. Additionally rats are able to synthesise ascorbic acid and possess intestinal phytase activity while humans do not (Reddy and Cook, 1991).

Of all the animal models available, the chicken most closely resembles the human for absorption of the xanthophyll, lutein. The most commonly used animal model for iron analysis, the rat, is unsuitable here as it does not absorb xanthophylls. Therefore the chicken was selected as the best model.

1.8 Analytical Techniques

Chromatographic Methods

Separation of carotenoids initially involved a two-step chromatographic method involving open-column and thin-layer chromatography (TLC). These two methods have been combined in high performance liquid chromatography (HPLC), which is now the preferred method for carotenoid analysis. HPLC is preferred due to fast elution times and decreased exposure to agents that can degrade carotenes, leading to inaccurate estimates of provitamin A activity (Hsieh and Karel, 1983). The non-destructive nature

of HPLC allows for collection of separated compounds for further analysis (Taylor and Little, 1983).

Gas chromatography is not suitable for thermally labile substances such as carotenoids (Raymundo *et al.*, 1967; Davies, 1976). While spectrometric methods analyse overall carotenoid content (Simpson *et al.*, 1979; Broich *et al.*, 1983) without quantifying specific isomers.

Extracts from complex biological samples can contain compounds that block the column and other parts of the system leading to an increase in back pressure. To avoid damaging the HPLC column, a guard column, which is made of similar material to that of the HPLC column, is used to collect these compounds and is replaced periodically (Chen and Bailey, 1987).

The temperature of the HPLC column is controlled to ensure fluctuations are not large enough to cause differences in elution times, as the identification of peaks depends on the comparison of elution times of the unknown sample and the standards. Any factor that changes the elution times prevents strict comparisons, and therefore, identification of peaks.

High Performance Liquid Chromatography (HPLC)

Normal and Reverse Phase Liquid Chromatography

Both normal phase and reverse phase HPLC can be used for carotenoid separation (Craft, 1992). Normal phase columns have polar bonded phases such as alkylamine, alkylnitrile and alkylglyco, and a non-polar mobile phase. The least polar carotenoids (hydrocarbons such as carotenes) elute first and the oxygenated compounds such as the xanthophylls elute later (Craft, 1992). Normal phase HPLC is used here for separation of lutein and zeaxanthin as shown in **Figure 1.7**.

Reverse phase HPLC (RP-HPLC) incorporates a non-polar bonded phase (e.g. octyl C8, octydecyl C_{18} residues bonded onto silica gel) and a polar mobile phase (Taylor and Little, 1983; Craft, 1992). Elution is opposite to that of normal phase (**Figure 1.8**) with the xanthophylls partitioning into the mobile phase and eluting first. The carotenes separate into the stationary phase and elute later (Craft, 1992). Reverse phase is

preferable to normal phase due to the long retention time and lack of separation of α- and β-carotene in normal phase systems (Broich *et al.*, 1983). The packing used for reverse phase does not promote the degradation of the sample and is unaffected by the presence of water or changes in the mobile phase (Zakaria and Simpson, 1979).

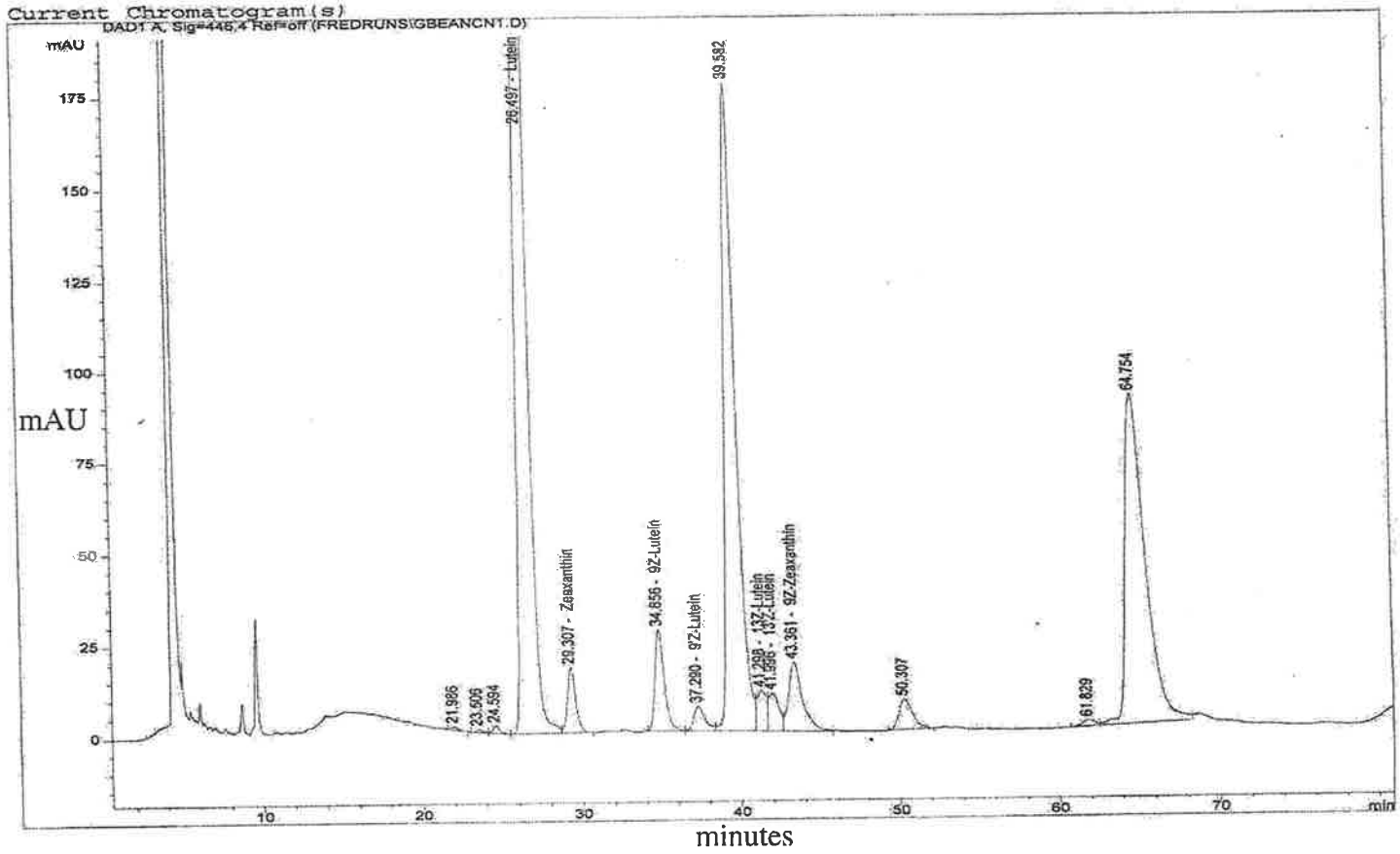


Figure 1.7 Normal phase HPLC chromatogram of carotenoids showing separation of lutein and zeaxanthin isomers using isocratic elution.

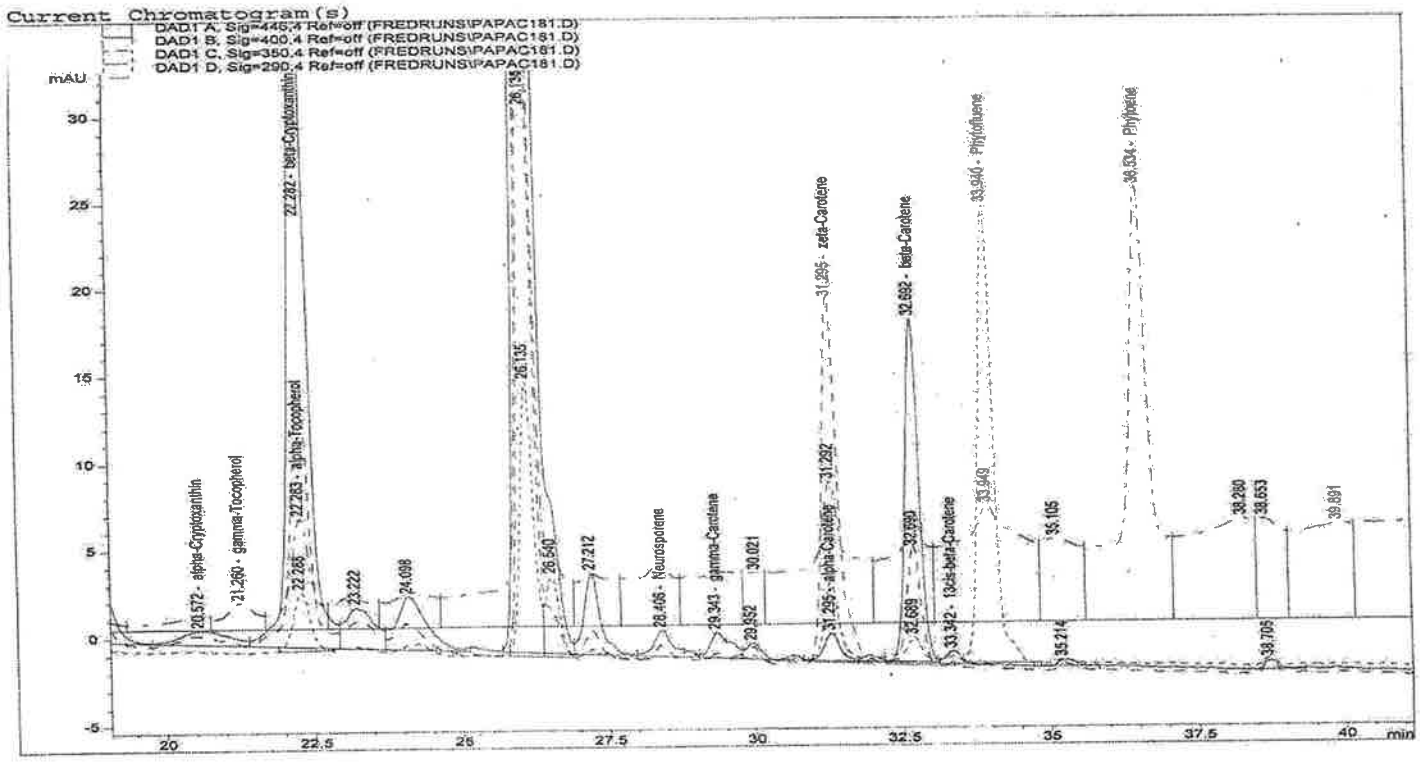


Figure 1.8 Reverse phase HPLC chromatogram of carotenoids showing separation of all carotenoids with the exception of lutein and zeaxanthin isomers using gradient elution. Absorbance at 446nm (α-cryptoxanthin, β-cryptoxanthin, neurosporene, γ-carotene, α-carotene, β-carotene, 13 *Z*-β-carotene), 400nm (ζ-carotene), 350nm (phytofluene) and 290 nm (α-tocopherol, γ-tocopherol, phytoene). Different lines indicate different wavelength absorbance.

Isocratic and Gradient Elution

Reverse phase liquid chromatography can be used with either an isocratic (constant solvent) or a gradient (changing solvent composition) elution system (Craft, 1992). Gradient elution has improved sensitivity and selectivity over isocratic elution for complex extracts that contain many carotenoids (Britton, 1991). Gradient elution elutes the strongly retained compounds at the end of the gradient, (Craft *et al.*, 1989) and will be used in combination with RP-HPLC here. The mobile phase of carotenoid reverse phase HPLC is usually a weak organic solvent with low viscosity that has adequate carotenoid solubility and low back-pressure (Craft, 1992). Solvents that fall into this category include acetonitrile, dichloromethane (methylene chloride), hexane and methanol, which have low viscosity and separate xanthophylls well when combined with C_{18} column (Khachik *et al.*, 1992a).

Disadvantages of gradient elution include the requirement for the column to be returned to the original solvent between samples and to have good solvent miscibility to prevent base line disturbance. Another problem outlined by Craft (1992) is the appearance of unwanted peaks due to contaminants detectable in the ultra violet region in weaker solvents. However, this is not a problem with carotenoids as they are detected within visible wavelengths.

Isocratic elution offers some advantages in that analysis can be achieved using single high-pressure pumps and premixed solvents resulting in stable base lines and reproducible retention times. However, mixing must be accurate and consistent, and the solvents cannot be volatile as this would result in changes in concentrations, due to evaporation over time (Craft, 1992). To combat the volatility of the solvents and the associated change in solvent concentrations used in HPLC of carotenoids, solvents solutions were mixed daily and contained in lidded bottles

In order to be able to identify and quantify carotenoids precisely enough to determine the provitamin A activity of a sample, the HPLC equipment must have certain minimum requirements. These requirements include a high-pressure pump capable of achieving 4000 psi, gradient elution capacity and a detector sensitive in the visible range. The availability of a photodiode array detector allows simultaneous detection and monitoring

at any chosen wavelength and continuous determination of absorption spectra during chromatography (Britton *et al.*, 1975; De Leenheer and Neils, 1992). This enables positive identification of carotenoids without the need for mass spectrometry. These options are available to this project.

Identification of Carotenoid Peaks

The identification of carotenoids from HPLC peaks mainly depends on the elution time, which is compared with standards (Nam *et al.*, 1988). An increase in the solvent flow rate will lead to poorly separated peaks while a decrease in the flow rate will lead to greater peak separation (Sander and Craft, 1990; Sander and Wise, 1989). Such variation leads to incorrect identification of peaks, and incorrect calculation of the provitamin A content of the samples.

Standards are not always available for all of the possible peaks for carotenoids in biological samples. Moreover, unequivocal identification can only be achieved by collection and analysis of the eluted fraction. Poor resolution and incomplete separation of peaks reduces the reliability of the method (De Leenheer and Neils, 1992).

The presence of a photodiode array detector (DAD) allows the recording of the complete absorption spectrum of a peak in less than one second. The distinctive shape associated with carotenoids allows immediate identification as a carotenoid (**Figure 1.8**). The absence of a DAD may lead to the dismissal of peaks thought not to be carotenoids, and the quantification of peaks that are not carotenoids (De Leenheer and Neils, 1992). Photodiode array detectors also ensure that co-eluting compounds are detected (De Leenheer and Neils, 1992). Comparison of the maximum wavelength at the highest point of the absorption spectrum, with known absorption spectra for that carotenoid allows absolute confirmation of the carotenoid.

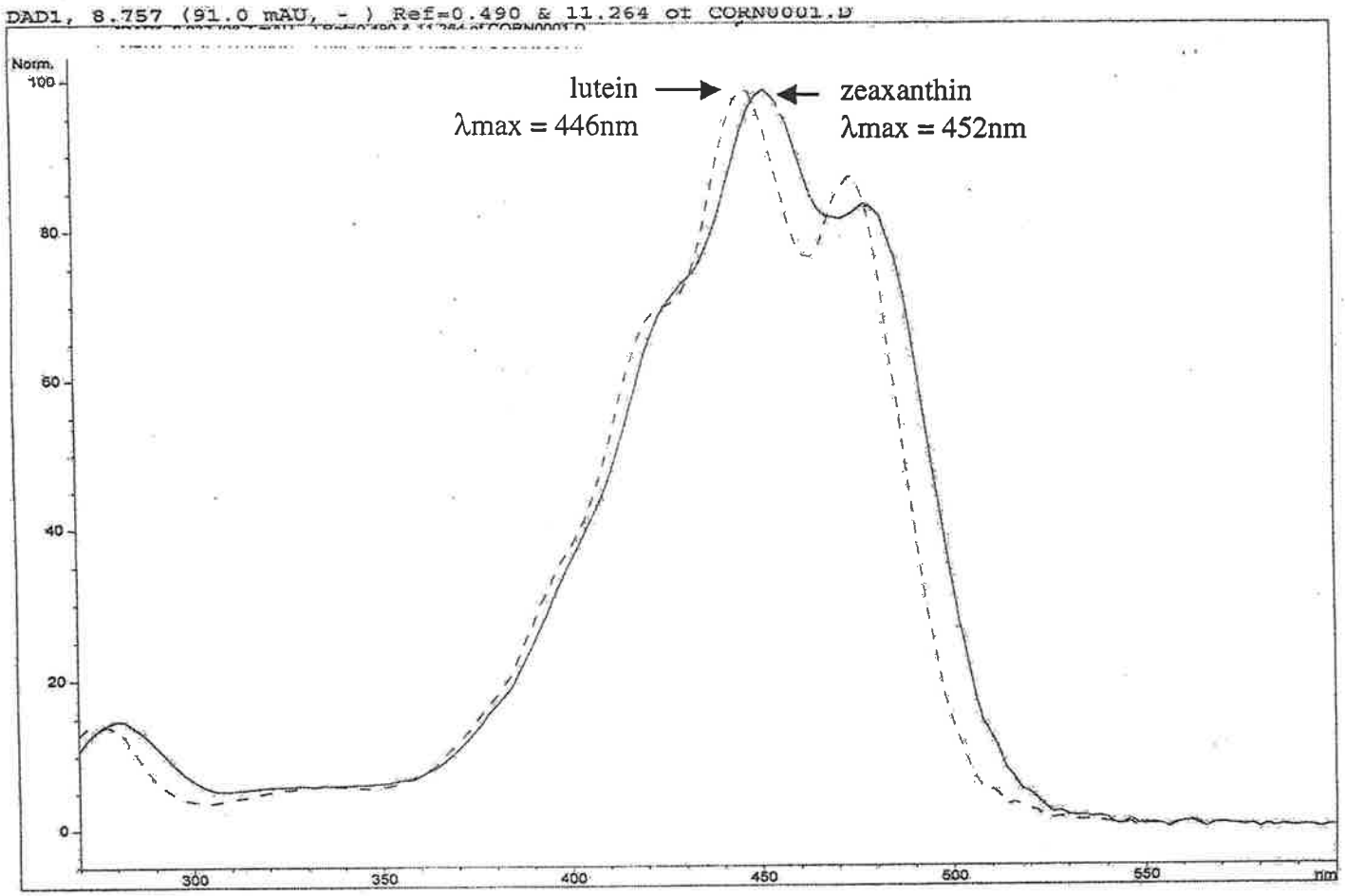


Figure 1.9 UV / Vis spectrum of lutein and zeaxanthin showing maximum wavelength. Lutein 446nm, zeaxanthin 452nm.

1.9 Hypotheses

The hypotheses proposed were:

1) *that the CIE L*a*b* colorimetry method would be a fast and accurate method to determine carotenoid concentrations in wheat*

Despite the importance of colour in wheat quality a correlation between colour and carotenoids in wheat has not been established previously. Several early studies attempted to quantify colour by the extraction of carotenoids from wheat flour and comparison with reference standards. This study analyses numerous wheat varieties for colour using near-infrared-reflectometry, and carotenoids by HPLC, and attempts to correlate these two parameters.

2) *that the CIE L*a*b* colorimetry method would remain a useful method for determining carotenoid concentrations in wheat under different storage conditions*

Follows on from the previous study.

3) *that commonly consumed foods have varying concentrations of lutein and zeaxanthin isomers*

The carotenoid profiles of commonly consumed fruit and vegetables are determined with a focus on lutein and zeaxanthin and their geometrical isomers. These carotenoids are important in the maintenance of the macula region of the eye. The ratio of these carotenoids in the macular region are not reflected in food or blood, and preferential uptake has been proposed as the mechanism behind this disparity, making food sources important.

4) *that lutein can increase haemoglobin concentrations, as has been found for β-carotens and vitamin A, in chickens,*

The final aim of this study is to investigate the effect of lutein on haemoglobin levels in chickens. A diet and time period for achieving depletion is established, followed by a comparison of the response in haemoglobin concentrations to 5 diets: 1) a commercial diet replete in all nutrients, 2) a diet high in supplemental lutein, 3) a diet based on wheat with a high concentration of lutein, 4) a diet with supplemental lutein at a concentration equal to that found in the high lutein wheat diet, and 5) a diet with β-carotene as the only source of vitamin A.

CHAPTER 2

General Introduction to Carotenoids in Wheat

Wheat (*Triticum sp.*) is a staple food for billions of people worldwide, however it is a relatively poor source of some essential nutrients. In some countries, a lack of dietary diversity has meant that wheat is relied upon to supply the bulk of a population's energy and nutritional needs. This has resulted in an increase in the number of people suffering from nutrient deficiencies, including those of iron, zinc and provitamin A. Currently, nutritional deficiencies are addressed by supplementation programs, which rely heavily on continued intervention, and are limited in the number of people they can help by the need for direct contact (Gibson, 1994). This reliance on external assistance can be reduced significantly by the introduction of foods with enhanced levels of nutrients. As wheat is a staple food for so many people, nutritionally enhanced varieties would have the potential to substantially reduce deficiencies and associated illnesses.

Nutritionally enhanced wheat varieties can be developed in breeding programs by crossing locally adapted cultivars with genotypes that have high concentrations of the desired nutrients. Development of breeding programs in the country where the food will be consumed can vastly improve the adoption and availability of these foods by allowing local input into the programs. This will ensure that the varieties produced meet the agronomic requirements of the area where they will be grown, which is essential if the food is to be abundant and available to all of the population.

Most breeding programs are based on the identification and selection of high nutrient traits from the natural variation available, and the introduction of these traits into existing cultivars. The variation for the accumulation of carotenoids has not been comprehensively explored and so extensive analysis of available germplasm is required. As large numbers of varieties need to be analysed, fast and cost efficient methods are essential. Identification of such methods will allow countries with few resources to develop breeding programs that address their specific nutritional requirements.

One such specific nutritional requirement is for increased carotenoid content. Increased levels of provitamin A carotenoids such as α- and β-carotene are important as these carotenoids are precursors to vitamin A. Vitamin A is essential in eye function and

health, and sources of these precursors are especially important in populations where animal products which are excellent sources of preformed vitamin A are not readily available (McLaren, 2001). Non-provitamin A carotenoids, such as lutein and zeaxanthin are also valuable for human health, as they are antioxidants and also important for sight. A relationship between decreased serum concentrations of lutein and zeaxanthin and an increased incidence of degeneration of the macula region of the eye has been reported (Snodderly, 1995). However, carotenoids cannot be synthesised by animals, and consumption of foods rich in these compounds is therefore essential.

Although most wheat cultivars are not particularly rich in carotenoids, this grain does contain both provitamin A and other carotenoids, and as wheat forms the basis of diets around the world, this staple food is a potential vehicle for delivery of increased levels of specific carotenoids. The selection for and against colour in wheat, and the strong association between carotenoid content and colour that can be attributed to the chemical structure of carotenoids, is likely to have influenced carotenoid levels. The association between carotenoids and colour has led to considerable investigation into the major carotenoids in wheat. Lutein has been identified as the major carotenoid (Lepage and Sims, 1968), with β-carotene also detected at low levels (Wildfeuer and Acker, 1968; Heinonen *et al.*, 1989). The differing requirements for colour in wheat has led to divergent selection for wheat flour colour between bread wheat (*Triticum aestivum*) where little pigmentation is desired, and pasta wheat (*Triticum turgidum*), where strong pigmentation is an important quality parameter.

Early studies that attempted to correlate provitamin A carotenoids and colour in wheat were inaccurate as the misclassification of non-provitamin A carotenoids resulted in an over estimation of the provitamin A content (Pomeranz, 1960). Further investigations into colour and carotenoid relationships were conducted following the separation of the carotenes and xanthophylls and are discussed in a review by Fortmann and Joiner (1988). Many of the methods used to measure colour rely on time-consuming extraction of carotenoid pigments from flour, and subsequent spectrophotometric comparison with standard reference colours. Even though fast and reliable methods have been used to evaluate flour colour (for example the Agtron and Gardner instruments) these have only been related to total carotenoids (Skarsaune and Shuey,

1975), and in order to establish a useful relationship between colour and specific carotenoids it is necessary to revisit this area of research.

The importance of colour in wheat quality has resulted in the development of fast and reliable methods for colour evaluation. The Minolta Chroma Meter is one instrument currently used for colour evaluation of wheat (and other foods). This instrument is fast, requiring only grinding of the wheat grain before analysis, and accurate. Additionally, this instrument measures colour within an internationally recognised 3-dimensional space using CIE (Commission Internationale de l' Eclaisage) co-ordinates (L*, a*, b*) which increases the usefulness of the measurements as they can be compared with other readings internationally using the same system. This feature is especially important when analysing the large number of samples required as a base for the breeding programs mentioned previously. For these reasons the Minolta Chroma Meter was chosen as the most suitable instrument for colour determination in the following two studies into the relationship between colour and carotenoids in wheat.

CHAPTER 2.1

Correlation of Carotenoids, α- and β-carotene and Lutein, with Colour in Wheat

Aim

To determine the relationship between flour colour and specific carotenoid levels in wheat with a view to the identification of a fast and accurate method for selecting varieties high in specific carotenoids.

2.1.1 Introduction

The extraction of wheat xanthophylls has been carried out for many years, and involves water saturated butanol extraction and measurement on a spectrophotometer. Additionally, the reflectance of flour samples has been used for many years for determining colour, and a relationship between b* and extractable yellow pigments is widely accepted. However, current methods for the identification of wheat genotypes high in specific carotenoids involve high performance liquid chromatography and are slow, costly and highly labour intensive. This method is a major barrier to the extensive germplasm screening that is required for the identification of high carotenoid wheat varieties. The chemical structure of carotenoids indicates that a correlation with colour is likely and it is therefore possible that divergent selection for colour in bread and pasta wheat has influenced the carotenoid content of these species. Determination of a correlation between a fast and accurate colour measurement such as that obtained from the Minolta Chroma Meter, and carotenoid concentration determined by HPLC, would vastly increase the number of samples that can be screened in a given time period. The work reported in this chapter investigates this correlation by the comparison of over 1000 wheat accessions from eight wheat groups, from an international wheat collection (CIMMYT) for flour colour, with the carotenoid concentration of 15 samples from each of the eight wheat groups, selection was based on Chroma Meter readings.

2.1.2 Materials and Methods

2.1.2 a Wheat

Source

Bread and durum wheat varieties grown under irrigation from the 1995-1996 harvest were obtained from the International Maize and Wheat Improvement Centre (CIMMYT) collection and held in quarantine facilities at the Waite Agricultural Research Institute. The average moisture content was ~11%. The bread wheat (*Triticum aestivum*) groups were:

CBME1SY: 154 varieties

J731A: 144 semi-dwarf varieties, with exception of two old tall varieties, containing 27 of the main varieties released by CIMMYT since 1950.

J731BI: 182 varieties

J731BII: 182 varieties

Lukas: 296 varieties

ISO-Seri: 279 varieties

ISO-Heat: 72 varieties

The durum wheat (*Triticum turgidum*) group was:

29th IDSN: durum wheat (*Triticum turgidum*) 230 varieties

Milling

10 g of each wheat sample were milled on a Quadrumat Junior Mill (Brabender, Germany) located in the SARDI wheat quality laboratory, University of Adelaide, Waite Campus. This mill allows separate collection of the flour, which is mainly endosperm, and the bran that combines the bran, seed coat, aleurone, some flour and germ. When combined these fractions are referred to as "whole-meal".

2.1.2 b Colour Analysis

L*, a*, b*

Colour of flour, bran and whole-meal was analysed using a Minolta Chroma Meter C-100 (Minolta Camera Co. Ltd., Osaka, Japan) incorporating a handheld device linked to a processing unit within an in-built printer. The Minolta Chroma Meter, using the L*, a*, b* system, allocates each sample a colour reference with the CIE-Lab 3-dimensional colour space. L* values represent brightness on a 0 (pure black) -100 (pure white) unit scale, a* values range from -60 (pure green) to +60 (pure red), and b* values range from -60 (pure blue) to +60 (pure yellow). Each L*, a*, b* value printed is averaged from three readings. The Chroma Meter was calibrated with a white tile and black card initially and periodically throughout analysis, though no recalibration was necessary. Further details and a diagrammatical explanation of the classification of colour by this method can be found in **Chapter 1**. The target b* value in Australian bread wheat flour is 9-11, while >11 is unacceptable due to the strong yellow colouration that is undesirable in many bread wheat end products and gives a visible yellow tinge to the flour.

2.1.2 c Carotenoid Extraction and Analysis

Analysis of the individual wheat fractions was not possible due to the limited amount of wheat available. The whole-meal fraction only was analysed for the carotenoid profile. Random replicates were included to identify experimental error. Ethyl-β-apo-8'-carotenoate was used as an internal standard. The extraction and high performance liquid chromatographic (HPLC) methods are as reported in Khachik *et al.*, (1991b).

Extraction

Five grams of each wheat sample were ground in an IKA analytical mill for 60 seconds, weighed and extracted for 1 hour in 20 ml tetrahydrofuran (THF) and 10% of the sample weight sodium carbonate (Na_2CO_3). The extractant was partitioned with CH_2Cl_2 and water to remove water-soluble compounds, and dried under a stream of high-purity nitrogen. The sample was stored in darkened glass vials at –20 °C until required for high performance liquid chromatography (HPLC) analysis. Further details are given in **Appendix A1.1**.

Analysis

Extracted samples were removed from -20° C and allowed to come to room temperature before analysis by HPLC. Carotenoids were identified based on elution times in comparison with standard reference samples of authentic all-*E*-lutein, all-*E*-α-carotene, all-*E*-β-carotene (SIGMA), and concurrence with wavelength for standard compounds (see literature review).

Column

Analysis was conducted on a Hewlett-Packard 1090 Series II liquid chromatograph with rapid screening UV/VIS and photodiode array detector. Using a Waters Spherisorb (250mm length X 4.6mm i.d.) ODS-2 (5µm spherical particles) reverse phase column (Alltech Associates (Aust.) Pty. Ltd.), protected by a Spherisorb guard cartridge (20mm length X 4.6 mm i.d.) packed with ODS-2 (5µm particle size).

Solvents and Method

Solvent A: 90% acetonitrile and 10% methanol. Solvent B: 45% dichloromethane, 45% hexane, 10% methanol and 0.1% DIPEA.

Method: at time 0 minutes, 100% Solvent A (0% Solvent B), followed by a linear gradient beginning at 10 minutes and completed at 40 minutes resulting in 45% Solvent A (55% Solvent B).

Injection solvent: 40% acetonitrile, 20% methanol, 20% dichloromethane, 20% hexane, 0.1% DIPEA.

2.1.2 d Calculation of Retinol Activity Equivalents (RAE)

Retinol activity equivalents (RAE), defined as half the number of Retinol Equivalents (RE), were calculated using the following internationally accepted formulae (US Institute of Medicine, 2000). The terms retinol equivalents and retinol activity equivalents are explained in the literature review.

Equation 2.1.1 Calculation of Retinol Activity Equivalents (RAE)

1 Retinol Equivalent = (μg β-carotene / 6) + (μg other provitamin A carotenoids / 12)

and 1 Retinol Activity Equivalent = Retinol Equivalent / 2

therefore 1 Retinol Activity Equivalent =

$$\frac{(\mu g\ \beta\text{-carotene} / 6) + (\mu g\ \text{other provitamin A carotenoids} / 12)}{2}$$

One Retinol Activity Equivalent is therefore equivalent to 1 μg retinol, 12 μg β-carotene or 24 μg of other provitamin A carotenoids.

2.1.3 Results

2.1.3 a Colour

Overall, the L* value varied the least between wheat groups and fractions, while the b* value varied the most both between wheat groups and between fractions. Variation within and between wheat groups is shown in **Table 2.1.1**.

Table 2.1.1 Range of colour values obtained from the bran, flour and whole-meal fractions for each wheat group. Means are shown in parentheses. Whole-meal results only are available for ISO-Seri.

Bran	L*	a*	b*
29th IDSN	60.64 - 87.68 (74.79)	0.16 – 5.69 (2.6)	17.48 – 31.56 (21.6)
CBME1SY	52.18 – 80.05 (63.94)	2.28 – 6.27 (5.05)	10.94 – 23.57 (15.77)
ISO-Heat	67.64 – 77.73 (75.13)	2.53 – 3.81 (3.07)	16.563 – 20.44 (17.74)
J731A	55.09 – 70.98 (64.33)	2.93 – 6.8 (4.93)	11.2 – 21.26 (16.38)
J731BI	55.42 – 82.01 (71.85)	1.44 – 8.67 (4.4)	10.46 – 28.22 (17.5)
J731BII	55.35 – 78.61 (70.84)	2.27 – 7.31 (3.84)	11.65 – 26.44 (17.5)
Lukas	65.5 – 76.75 (72.28)	2.3 – 4.22 (3.18)	10.69 – 21.59 (12.59)

Flour	L*	a*	b*
29th IDSN	65.67 – 90.04 (77.55)	-1.48 – 3.07 (1.92)	14.76 – 24.15 (19.6)
CBME1SY	73.02 – 86.49 (80.52)	0.66 – 3.46 (1.9)	8.24 – 19.41 (13.24)
ISO-Heat	75.37 – 79.63 (77.62)	1.34 – 3.21 (2.66)	14.09 – 17.86 (16.62)
J731A	73.66 – 83.1 (78.18)	1.18 – 3.16 (2.36)	10.35 – 18.25 (14.42)
J731BI	68.9 – 88.17 (77.04)	0.96 – 5.37 (2.7)	9.77 – 25.58 (17.15)
J731BII	60.24 – 84.31 (75.35)	0.37 – 5.55 (2.83)	11.16 – 22.01 (16.7)
Lukas	74.15 – 82.94 (78.33)	1.31 – 2.96 (2.2)	9.6 – 18.9 (11.77)

Whole-meal	L*	a*	b*
29th IDSN	72.42 – 87.23 (78.21)	-1.44 – 4.37 (2.1)	16.04 – 23.48 (19.1)
CBME1SY	72.4 – 85.46 (80.32)	0.89 – 3.4 (1.9)	8.77 – 19.91 (12.81)
ISO-Heat	74.4 – 79.53 (77.44)	1 – 3.41 (2.7)	13.94 – 18.01 (16.56)
J731A	72.46 – 83.66 (78.12)	1.13 – 3.74 (2.4)	9.44 – 17.57 (14)
J731BI	68.97 – 86.7 (77.84)	1.09 – 5.37 (3)	9.94 – 25.27 (17.16)
J731BII	69.06 – 84.75 (76.38)	1.02 – 4.55 (2.9)	10.16 – 21.92 (15.9)
Lukas	71.09 – 81.17 (77.49)	1.56 – 2.98 (2.3)	9.6 – 18.46 (11.54)
ISO-Seri	72.7 – 87.4 (79.23)	1.23 – 3.07 (2.09)	7.3 – 24.06 (17.06)

L* values

Bran, flour and whole-meal fractions for each cultivar in each wheat group were analysed for L*, a* and b*. In all wheat groups the L* values of the bran fraction was lower ($p < 0.001$) than the flour and whole-meal fraction L* values. The flour and whole-meal fractions were not different (**Figure 2.1.1**). There was variation ($p < 0.001$) between wheat groups for L* value in each wheat fraction, however there was no difference in the values for wheat fractions within each wheat group. Bran L* values in wheat groups CBME1SY and J731A were lower ($p < 0.001$) than in other wheat groups. Wheat group CBME1SY had higher ($p < 0.001$) flour and whole-meal L* values than the other wheat groups, and the range of values obtained for L* are shown in **Table 2.1.1** under the appropriate wheat group.

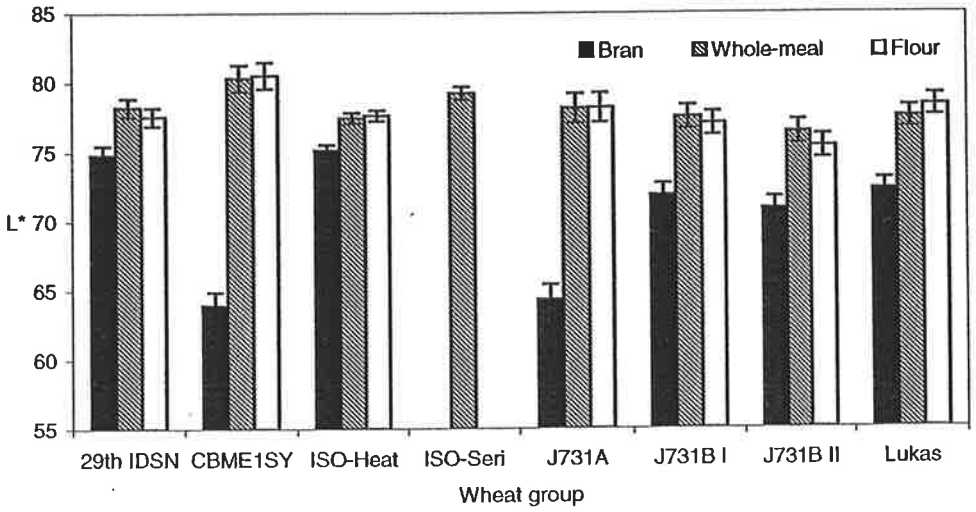


Figure 2.1.1 L* values for flour, bran and whole-meal fraction. Values shown are the mean of each group, ± standard error. Results were available for the whole-meal fraction only of ISO-Seri. J731BI, n=182; J731BII, n=182; CBME1SY, n=154; 29th IDSN, n=184; J731A, n=65; ISO-Heat, n=72; ISO-Seri, n=82; Lukas, n=60.

a* values

The mean a* value for the bran fraction was higher ($p < 0.001$) than the mean of the flour and whole-meal fractions. Additionally, the mean a* value of the flour and whole-meal fractions were not different (**Figure 2.1.2**). There were differences ($p < 0.001$) between wheat groups for a* values, and wheat groups CBME1SY and J731A had higher ($p < 0.001$) a* values than other wheat groups, while 29th IDSN had the lowest mean a* value.

Wheat groups J731BI and J731BII had higher ($p < 0.001$) whole-meal a* values than the other wheat groups. ISO-Heat, J731A, J731BI and J731BII had higher ($p < 0.001$) flour a* values than other wheat groups, and the range of values obtained for a* are shown in **Table 2.1.1**.

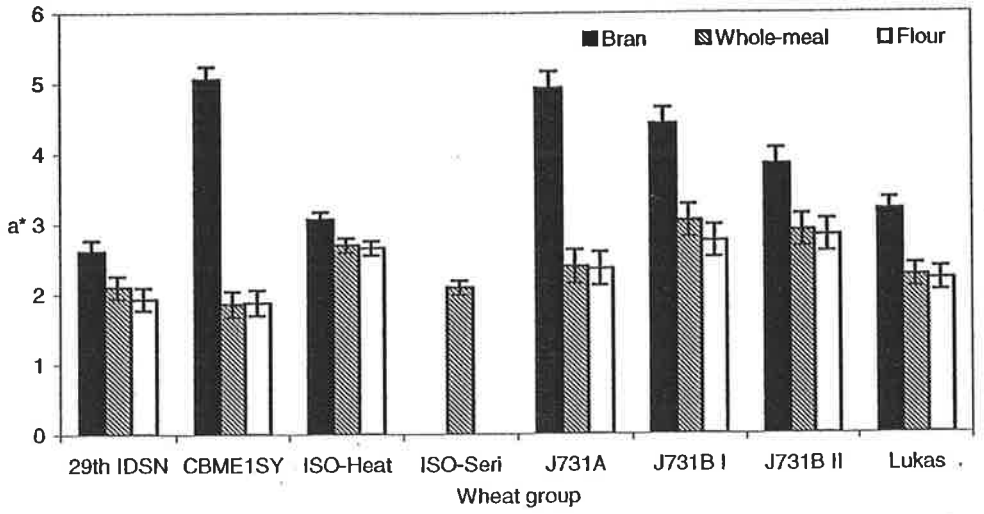


Figure 2.1.2 a* values for flour, bran and whole-meal fraction. Values shown are the mean of each group ± standard error. Results were available for the whole-meal fraction only of ISO-Seri. J731BI n=182, J731BII n=182, CBME1SY n=154, 29th IDSN n=184, J731A n=65, ISO-Heat n=72, Lukas n=60.

b* values

Wheat groups varied widely for bran b* values. However, most wheat groups had a higher ($p < 0.001$) mean bran b* value than flour and whole-meal b* value. Exceptions were the wheat group Lukas, which showed no difference in b* value between the fractions (**Figure 2.1.3**), and the wheat group J731BII, where the flour b* values and bran b* values were not different. There was no significant difference between the whole-meal and bran fraction b* values in any of the wheat groups. Wheat group Lukas had a lower ($p < 0.001$) mean b* value than all other wheat groups for all fractions. Wheat group 29th IDSN had a higher ($p < 0.001$) b* value for all fractions than the other wheat groups.

Although the flour b* value was higher than in the other fractions in all wheat groups, other than J731BI, this was not significant. In the whole-meal fraction ISO-Heat, J731BI, J731BII had higher ($p < 0.001$) b* values than wheat groups J731A and CBME1SY.

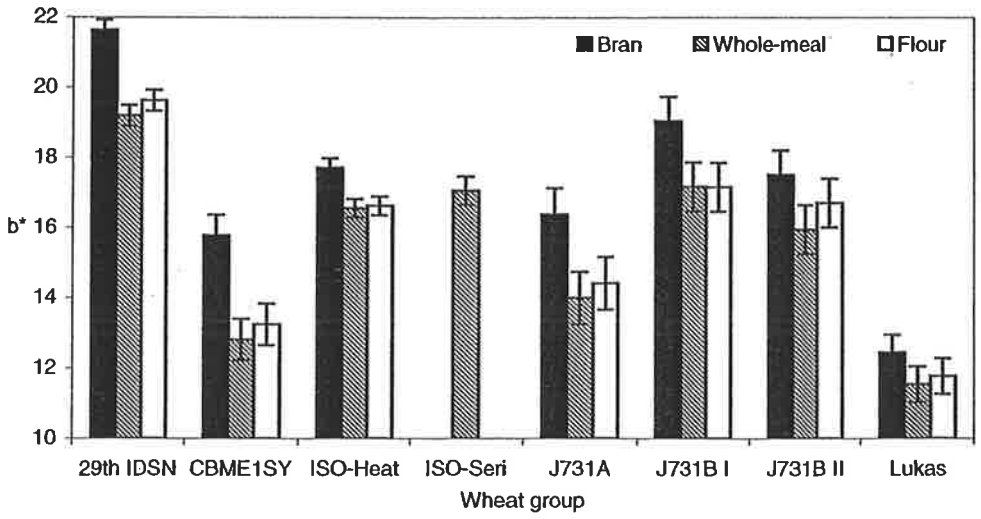


Figure 2.1.3. b* colourimetry values for flour, bran and whole-meal fraction. Values shown are the average of each group ± standard error. Results were available for the whole-meal fraction only of ISO-Seri. J731BI n=182, J731BII n=182, CBME1SY n=154, 29th IDSN n=184, J731A n=65, ISO-Heat n=72, Lukas n=60.

2.1.3 b Carotenoids

Varieties within each wheat group were classified based on their b* value as either high, low or mid range specific to that wheat group. Varieties within each range were then selected from each wheat group and analysed for specific carotenoids (**Figure 2.1.4, 2.1.5, 2.1.6**). There were differences ($p < 0.001$) between the concentrations of lutein, α- and β-carotene for each of the three b* value ranges in all wheat groups except Lukas. Although lutein, and α- and β-carotene were detected in most samples, lutein was most abundant, with some concentrations up to ten times that of β-carotene. Alpha-carotene was detected at lower concentrations than the other carotenoids, and was undetectable in some samples.

The concentration of lutein in the high b* range varieties was higher ($p < 0.001$) that in the low b* range varieties, but not significantly different between the high and mid b* range varieties. An exception was in wheat group ISO-Seri where there were significant differences between high and mid b*, and low b* values for lutein concentration.

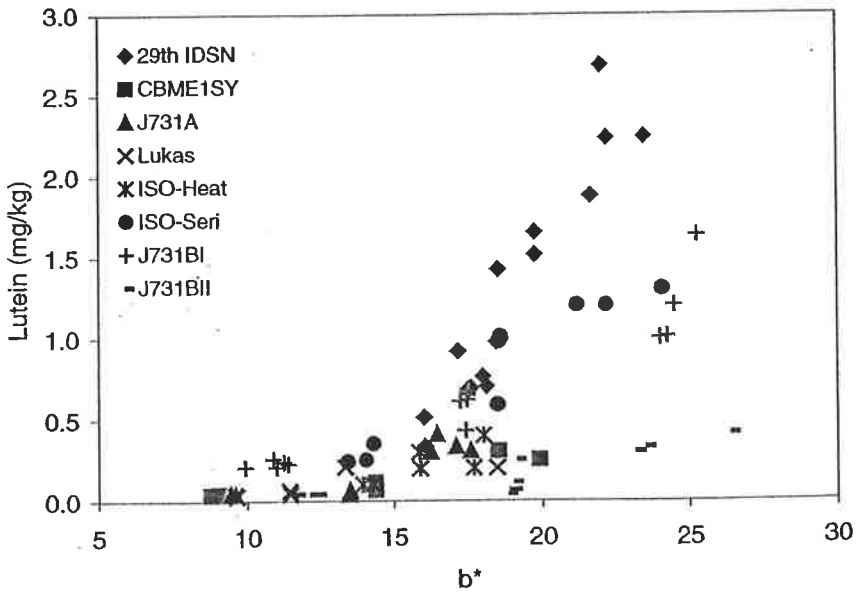


Figure 2.1.4 Lutein concentration (mg/kg) for high, mid and low range b* values of the whole-meal fraction of individual wheat groups. Error bars were removed for clarity and were within 5% of the mean. For each point n=3.

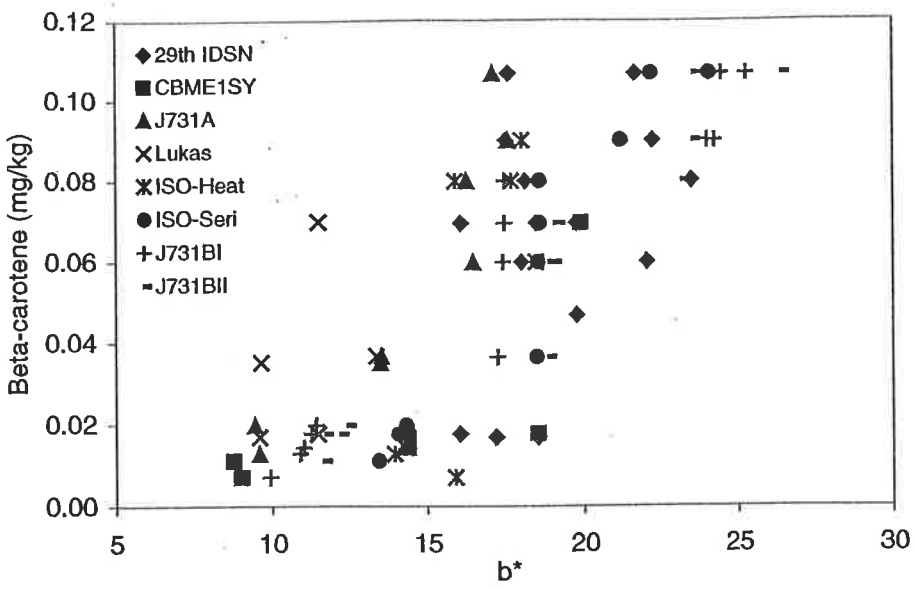


Figure 2.1.5 Beta-carotene concentration (mg/kg) for high, mid and low range b* values of the whole-meal fraction of individual wheat groups. Error bars were removed for clarity and were within 5% of the mean. For each point n=3.

Alpha-carotene concentrations were not significantly different between b* value ranges, with the exception of wheat groups J731BI and J731BII. In these wheat groups there was a difference between high and low b* value range ($p < 0.003$), also between high and mid b* value range ($p < 0.008$) for α-carotene concentration. The same two wheat groups were the only ones to have different ($p < 0.001$) β-carotene concentrations between the b* value ranges.

The bread wheat groups J731A, J731BI, J731BII, ISO-Seri and the durum wheat group 29th IDSN had similar maximal concentrations of β-carotene despite their high b* values differing.

The b* groupings (high, mid, low) identify lines differing in carotenoids whereas a* and L* do not. Therefore the correlation between b* and carotenoids is probably a meaningful relationship and not just due to chance.

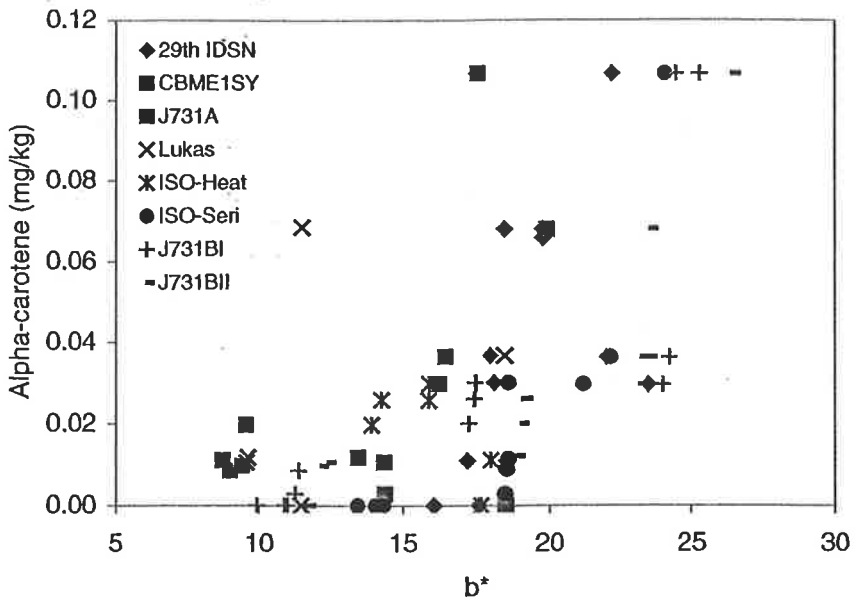


Figure 2.1.6 Alpha-carotene concentration (mg/kg) for high, mid and low range b* values of the whole-meal fraction of individual wheat groups. Error bars were removed for clarity and were within 5% of the mean. For each point n=3.

2.1.3 c Correlation between Colour and Carotenoids

CIE L*a*b* colour values were correlated with carotenoid concentration, and all colour-carotenoid relationships are shown in **Appendix B (Tables B1, B2 & B3)**. For the maximal model of separate regressions, the residual vs fitted plot of lutein vs b* showed that the variance changes as the two variables increased. Therefore, variance was normalised by a square root transformation (sqrt) **Appendix B (Table B4)**. As the wheat group 29th IDSN was a different wheat species (*Triticum turgidum*) that is bred for high pigmentation, it was not expected to comply to the same relationship with the variable as the remaining varieties. The analysis of sqrt (lutein) vs b* disregarding 29th IDSN suggested that at the 5% level there was no significant difference (p value = 0.19) between a distinct regression model with different slope and different intercept parameters and a sub-model with the same intercept and different slope parameters. There was very strong evidence (p = 0) that a common regression line for all varieties is not required and therefore the common intercept/different slope model was retained.

The analysis of sqrt (lutein) vs b* including 29th IDSN showed that at the 5% level there was very strong evidence (p value = 0) that different varieties required different

regressions, even after lutein had been transformed using its square root. Regression equations are shown in **Table 2.1.2.** Analysis of sqrt (β-carotene) vs b* showed that at the 10% level there was some evidence (p value = 0.055) that each regression described a different relationship between variables. At the 5% level this could be deemed as not strong enough and the model can be reduced to a model with the same intercept for each regression line but different slopes. Analysis of sqrt (α-carotene) vs b* suggested that at the 5% level there was no significant difference (p value = 0.074) between the slopes of the regressions. This suggests that the slopes of each regression are parallel and that each of the intercepts are different. There was significant evidence (p value = 0.018) that the intercepts are different and therefore the model may not be reduced further.

Table 2.1.2 Regression equations between b* and lutein, α-carotene and β-carotene for all wheat groups for square root transformed data.

Wheat Group	b* Lutein	b* α-carotene	b* β-carotene
29th IDSN	-1.42 + 0.13 b*	-0.12 + 0.005 b*	-0.04 + 0.015 b*
CBME1SY	-0.15 + 0.035 b*	-0.12 – 0.001 b*	-0.04 + 0.013 b*
ISO-Heat	-0.15 + 0.038 b*	-0.12 – 0.013 b*	-0.04 + 0.015 b*
ISO-Seri	-0.15 + 0.056 b*	-0.12 + 0.019 b*	-0.04 + 0.015 b*
J731A	-0.15 + 0.04 b*	-0.12 – 0.002 b*	-0.04 + 0.19 b*
J731BI	-0.15 + 0.05 b*	-0.12 – 0.004 b*	-0.04 + 0.015 b*
J731BII	-0.15 + 0.03 b*	-0.12 + 0.009 b*	-0.04 + 0.015 b*
Lukas	-0.15 + 0.04 b*	-0.12 + 0.016 b*	-0.04 + 0.018 b*

Of all the correlations between the colour values, L*, a* and b*, and carotenoid concentration, by far the strongest was that between the b* value and lutein concentration (strong correlation indicated by $r > 0.7$, $p<0.001$) **Appendix B, (Tables B1, B2, B3)** & **Figure 2.1.4**. The correlation between lutein concentration and b* value was less strong when all wheat groups were combined (r = 0.62), indicating that

individual regression equations most accurately explain the correlations. Correlations between L* value and lutein concentration, and a* value and lutein concentration were only strong in wheat group Lukas. In this wheat group lutein concentration was positively correlated with a* value and negatively correlated with L* value.

The b* value was also strongly correlated with β-carotene concentration in most wheat groups; wheat groups Lukas and 29th IDSN (durum) did not have this strong correlation (**Figure 2.1.5**). In several of the wheat groups there was a strong correlation between β-carotene concentration and a* value (CBME1SY, J731A, ISO-Seri, J731BI); and β-carotene concentration with L* value (CBME1SY, ISO-Seri). When the durum wheat group (29th IDSN) was removed from the data set there was a strong correlation between β-carotene and b*.

There were only two wheat groups, ISO-Seri and CBME1SY, that had a strong correlation between all three colour values, L*, a* and b*, and α-carotene concentration (**Figure 2.1.6**). When all wheat groups were combined there was not a strong correlation between b* value and α-carotene concentration.

2.1.3 d Retinol Activity Equivalent (RAE)

There was no significant difference between wheat groups for RAE at each b* range (**Table 2.1.3**). However, the low, mid and high b* range wheat varieties in each wheat group were different ($p < 0.001$) for RAE levels. There was no consistent correlation between RAE and any of the colour measures.

Table 2.1.3 Mean retinol activity equivalents per gram of wheat for high, mid and low b* range in all wheat groups, calculated using **Formula 2.1.1**.

	Low	Mid	High		Low	Mid	High
29th IDSN	0.006	0.007	0.008	**ISO-Seri**	0.001	0.006	0.011
CBME1SY	0.001	0.002	0.005	**ISO-Heat**	0.002	0.005	0.007
Lukas	0.003	0.005	0.005	**J731A**	0.002	0.003	0.009
J731BI	0.001	0.007	0.011	**J731BII**	0.002	0.006	0.01

2.1.4 Discussion

In order to determine the relationship between flour colour and specific carotenoids, both of these variables were measured in an arbitrary manner. The flour colour quantified by the L*, a* and b* values obtained from the Minolta Chroma Meter was correlated to concentrations of specific carotenoids measured by HPLC. Determination of a relationship between these variables will allow the use of a colour value to identify high carotenoid wheat varieties.

Colour relationship between wheat fractions

In all wheat groups the flour colour values (L*, a*, b*) (**Table 2.1.1**) were well defined by those of the whole-meal fraction (**Figures 2.1.1, 2.1.2, &2.1.3**), and this was attributed to the predominance of the flour fraction in the wheat grain. It is therefore possible to extrapolate colour values from the easily accessible, whole-meal samples, to those of the flour fraction, which is the main proportion consumed.

Correlation between colour and carotenoids

There was a strong positive correlation ($r > 0.75$) between b* values and lutein concentration in all wheat groups, with the strongest correlation seen in the wheat group with the highest b* values, 29th IDSN, the durum (**Appendix B, Table B1**). Despite this, the correlation was less strong when all wheat groups were combined ($r = 0.62$), which was attributed to the contribution of coloured constituents other than carotenoids to the b* value. The other colour parameters, L* and a*, showed little correlation with lutein (**Appendix B, Table B1**). Therefore the concentration of lutein in wheat flour (**Figure 2.1.4**) can be best estimated from the b* value, and wheat varieties can be ranked for lutein concentration through a comparison of this colour value.

Generally, b* values were strongly correlated to both α- and β-carotene concentration (**Appendix B, Tables B2 & B3**), indicating that b* values are useful for identifying high concentrations of these provitamin A carotenoids as well as lutein. The wheat groups without strong correlations between the b* value and β-carotene were those with the lowest b* values (Lukas) and the highest b* values (29th IDSN). Interestingly, the wheat group 29th IDSN, which had the highest b* value and lutein concentration, had a relatively low β-carotene concentration (**Figure 2.1.5**), and additionally was one of the wheat groups not to show a strong correlation between b* and β-carotene. It may well be that the accumulation of lutein and the relatively low levels of β-carotene indicate the dominance of the lutein side chain of the carotenoid biosynthetic pathway in durum wheat (see literature review for biosynthetic pathway). The dominance of the lutein pathway over the β-carotene pathway could possibly be due to the selection for colour contributed by lutein in durum wheat. Lutein absorbs at 446 nm (yellow) while β-carotene absorbs at 452 nm (orange), which results in different colour measures. Alternatively, the accumulation of lutein at the expense of β-carotene might be due to an interspecies differentiation between the durum (*Triticum turgidum*) and bread wheat (*Triticum aestivum*) for the enzymes regulating the biosynthetic pathway to β-carotene. Manipulation of these regulatory enzymes may be a possible approach for increasing or decreasing the concentration of certain carotenoids.

The strong correlation between α- and β-carotene and the b* value determined here does not agree with the findings of Takahata *et al.* (1993), who found that the a* value best correlated with β-carotene concentration in sweet-potato. Ramakrishnan and

Francis (1973) also found a strong relationship between a* and total carotenoid concentration in paprika, and it is possible that since the analyses which relate a* and carotenoid content were conducted on foods other than wheat, there is an effect of the plant species on the presence of a relationship. In addition, the low level of pigmentation in wheat in comparison with that of the other foods analysed may have an effect on the relationship between colour values and carotenoid concentrations.

The Retinol Activity Equivalents (RAE) (**Table 2.1.3**) were strongly correlated with b* in most wheat groups, identifying the b* value as a useful colour parameter for determining the RAE of wheat. This strong correlation was expected in wheat groups that had strong correlations with β-carotene, as this was the dominant carotene, and has a greater RAE value than α-carotene. The absence of a strong correlation in the durum wheat group (29th IDSN) could again be attributed to the dominance of lutein in this species over β-carotene. The determined correlation between the b* value and RAE is useful for the selection of wheat varieties high in provitamin A carotenoids, which can form the base of breeding programs to increase the nutritional value of this staple food. However, it is important to remember that the RAE does not take into account the complex interaction between carotenoids and the food matrix in which they are found or the nutritional status of the individual consumer, both of which affect the bioavailability and bioconversion of carotenoids. Therefore, the RAE does not accurately indicate the amount of vitamin A an individual will obtain from a food, but is at present the best measure of the potential of a food to provide vitamin A precursors.

The wheat samples analysed here for carotenoids were whole-meal, encompassing all parts of the wheat grain, while flour, which is principally comprised of endosperm is most commonly consumed, and it is acknowledged that there will be a difference in carotenoid concentrations between the flour and whole-meal. However, although the embryo (in the bran fraction here) is the most concentrated source of carotenoids, containing 14% of the total carotenoids in the grain, this only makes up ~10% of the whole-meal, while the endosperm with a lower concentration of carotenoids (6% of the total carotenoids) represents ~80% of the grain (Mac Masters *et al.*, 1971; Chen and Geddes, 1945 in Kruger and Reed, 1988; Orth and Shellenberger, 1988). Consequently endosperm carotenoid concentration has the greater influence on the total carotenoid concentration of wheat, and the carotenoid concentration determined here will not differ

vastly from that of flour. Hand-milling that replaces industrial milling in many villages leads to the inclusion of parts of the embryo in the consumed flour, and will increase the concentration of carotenoids that are obtained from wheat. However, retention of this oil containing plant-part reduces the storage of the flour due to rancidity.

In conclusion, the hypothesis that the CIE L*a*b* colorimetry method would be a useful tool for fast screening of both bread and durum wheat varieties for lutein content was supported, with a strong positive correlation shown between lutein and b*. The b* value was also indicative of the provitamin A carotenoid content in most bread wheat groups, though not in the durum wheat group (29th IDSN). Therefore, this method can be recommended for screening bread wheat varieties with higher concentrations of lutein and carotenes, and durum wheat varieties with higher concentrations of lutein.

CHAPTER 2.2

The Effect of Storage as Grain or Flour on Stability of Colour and Carotenoid Content in Wheat

Aim

To determine the dependence of colour / carotenoid content in wheat on time of storage has been stored as grain or flour at two temperatures.

2.2.1 Introduction

The year round demand by consumers for bakery products, and therefore all year demand for wheat by millers and bakers, necessitates storage of the majority of the annual harvest. The maximum time for which wheat can be stored is determined by a change in quality parameters specific to the end product, and such parameters include contamination and loss of baking quality. Wheat can be stored either as grain or as milled flour. Storage for the period between harvests as grain is common, and it is claimed that there is little decrease in quality with storage in this form (Zeleny, 1954). Wheat can also be stored as flour, and a minimum storage period of three weeks in this form is necessary to achieve quality parameters such as gluten stability for biscuits and cakes, while storage for up to eight weeks is necessary for continental breads. However, wheat is not stored as flour for periods longer than this in Australia due to contamination and degradation in other quality parameters (Lauke Mills, *pers. comm.*, 2001).

Numerous components make up flour quality, including protein concentration, which determines end use, mineral content and moisture content (Mailhot, 1988). Flour colour is another aspect of wheat quality to receive attention, and it can be either desirable or detrimental to quality again depending on the end product. Depending on end use, wheat quality can be either adversely or favourably affected by storage, and extensive studies have been conducted to elucidate many of the components that influence quality (Cuendet *et al.*, 1954; Bell *et al.*, 1979).

Of the reported studies into the level of colouration in various wheat varieties (Patton and Dishaw, 1968; Skarsaune and Shuey, 1975; Watson and Shuey, 1977) only one

measured colour deterioration during storage (Watson and Shuey, 1977). This study of wheat stored as flour for 2-3 months at room temperature failed to detect any colour change. However, this study used the Agtron method of colour evaluation, which uses a green scale and does not consider the role of carotenoid pigments (Patton and Dishaw, 1968). For this reason the Agtron method was not deemed suitable for the experiment undertaken here.

A study that investigated change in the concentration of lutein in wheat flour during storage (Farrington *et al.*, 1981) found that lutein exhibited a linear decline during the storage period, though changes in colour were not reported. Other studies that have reported changes in the carotenoid composition of wheat grain under various storage conditions found that total lutein was not reduced during storage as grain (Kaneko *et al.*, 1995; Pinzino, 1999). However, the focus of these studies was on the changes in lutein ester proportions, and did not relate free-lutein concentration to colour.

The following study investigates the correlation between colour measured using the CIE-Lab method, and carotenoids measured using HPLC, during storage as both grain and flour at 5 °C and 35 °C.

2.2.2 Material and Methods

2.2.2 a Grain and Flour Source

Two kilograms of grain from each of the 4 wheat varieties; Spear, Worrakatta, Krichauff and Tamaroi (a durum) were obtained from the 1999 harvest at Roseworthy Agricultural College, South Australia. For each variety there were 5 treatments: 1) unstored, 2) stored as grain at 5 °C for 6 months, 3) stored as grain at 35 °C for 6 months, 4) stored as flour at 5 °C for 6 months, 5) stored as flour at 35 °C for 6 months. Samples were stored in paper bags. The moisture content of the grain was approximately 11% before and after storage. Each treatment was replicated 4 times and each replicate involved 100 g of wheat.

Flour was obtained by milling the wheat on a Quadrumat Junior Mill (Brabender, Germany) located at the South Australian Research and Development Institute Wheat Quality Laboratory, at the Waite Campus of the University of Adelaide, Australia. This

process resembles the commercial milling process, albeit in a simplified form, and removes the outer layers of the grain leaving flour that is primarily composed of endosperm.

Krichauff is exceptional in that it has a b* value above the Australian standard acceptable range of 9-11 (Krichauff b* ~13), while the other bread wheats used in this study had b* values in the acceptable range.

2.2.2 b Storage Conditions

Grain and flour samples were stored in brown paper bags at constant temperatures of 5°C or 35 °C in the dark, at a relative humidity of ~5%.

2.2.2 c Colour Analysis

Regardless of whether the wheat was stored as grain or flour, colour analysis was performed on flour. Colour analysis was performed as detailed in **Chapter 2.1.**

2.2.2 d Carotenoid Analysis

Carotenoids were extracted from all samples using the method detailed in **Chapter 2.1**; further details are given in **Appendix A1.1.** HPLC analysis of the carotenoid profile was conducted as detailed in **Chapter 2.1**.

2.2.3 Results

2.2.3 a Colour

The greatest decrease in b* value for wheat stored as grain was brought about by an increase in storage temperature from 5 °C to 35 °C (**Figure 2.2.1**). The wheat variety Krichauff was an exception, with similar decreases in b* under all storage conditions. In wheat stored as flour the greatest decrease in b* value was as a result of storage for 6 months (**Figure 2.2.2**).

Despite the decreases in b* in both grain and flour, only Spear and Worrakatta, stored for 6 months at 35 °C, had a b* value acceptable to the Australian Wheat Board for bread wheat (b*< 11). The durum wheat variety Tamaroi recorded the highest b* values in both the grain and flour forms under all storage conditions.

In wheat stored as flour the overall decrease in b* values was not significantly different between varieties. Therefore, as there was a significant difference between varieties for initial b* values there was no relationship between the initial b* value and the overall decrease in b* value. The wheat varieties with the lowest and highest b* values, Spear and Tamaroi respectively, had equal decreases in overall b*. While the wheat varieties with mid b* values, Worrakatta and Krichauff, had comparatively less decrease in colour.

2.2.3 b Lutein

In all varieties, wheat stored both as grain and flour recorded a significant drop in lutein concentration when stored for 6 months as compared to samples that were analysed at the beginning of the study (**Figure 2.2.1, Figure 2.2.2**). A significant decrease in lutein concentration was also seen as storage temperature increased from 5 °C to 35 °C, again in both flour and grain forms and in all varieties ().

In the majority of varieties the greatest decrease in lutein concentration was due to an increase in storage time from 0 to 6 months with little further degradation attributable to an increase in temperature from 5 °C to 35 °C. The bread wheat variety Spear was the exception, and had a similar decrease in lutein concentration in both grain and flour forms despite a greater decrease in b* value when stored as flour.

In wheat stored as grain there were similar overall lutein concentrations in all varieties, with the exception of Spear, the variety with the absolute lowest initial concentration. The greatest decrease in lutein concentration in wheat stored as grain was due to an increase in temperature, and was similar in all wheat varieties except Spear, where the decrease was less.

In all varieties the overall decrease in lutein concentration was doubled by storage as flour rather than grain, with the exception of the durum. When stored as flour, the durum (Tamaroi) recorded a decrease in lutein concentration four times that when stored as grain.

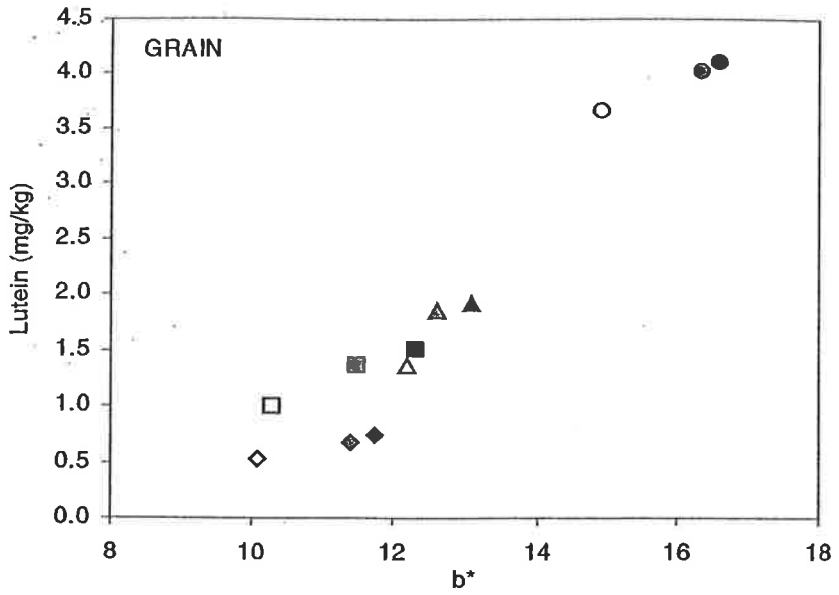


Figure 2.2.1 Lutein concentration (mg/kg) and b* values for wheat stored as grain under all conditions for all wheat varieties. Spear - ◇; Worrakatta - □; Krichauff - Δ; Tamaroi - O. Filled icon indicates unstored wheat, grey shaded icon indicates wheat stored at 5 °C for 6 months, and unfilled icon indicates wheat stored at 35 °C for 6 months. Values are the mean of 4 measurements, error bars were omitted for clarity but standard errors were within 5 % of the mean.

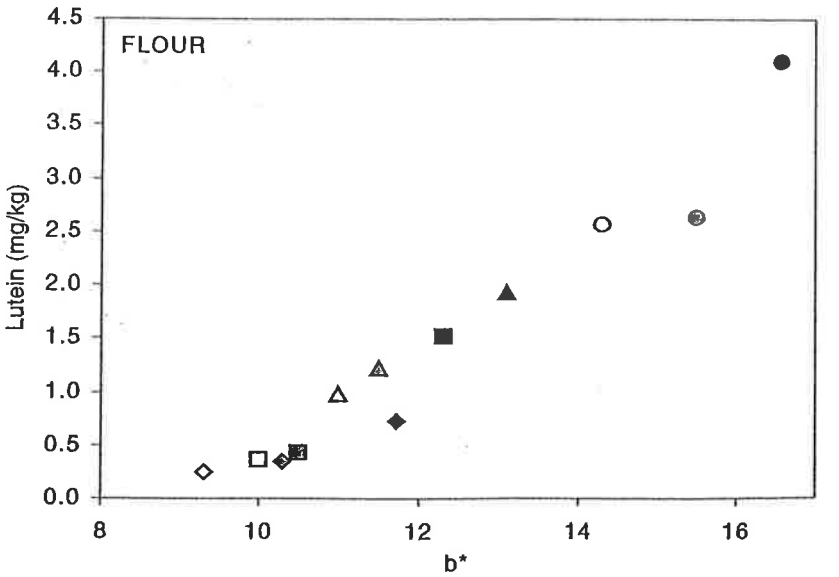


Figure 2.2.2 Lutein concentration (mg/kg) and b* values for wheat stored as flour under all conditions for all wheat varieties. Explanation of symbols is given above. Values are the mean of 4 measurements, error bars were omitted for clarity but standard errors were within 5 % of the mean.

In wheat stored as flour the greatest overall decrease in lutein concentration was seen in the variety with the highest initial lutein concentration, which was the durum variety Tamaroi.he least decrease was seen in the variety with the lowest lutein concentration initially, Spear. The greatest decrease in lutein concentration in wheat stored as flour was seen between wheat that was unstored and stored at 5 °C.

In wheat stored as grain, the varieties with the least and greatest b* values showed proportional decreases in b* value and lutein concentration, while in Krichauff and Worrakatta the decrease in b* value was greater than the decrease in the lutein concentration (**Table 2.2.1**).

Table 2.2.1 Summary of mean overall percent decrease in b* value and lutein concentration for all wheat varieties stored as grain. Reading both down columns and across lines different superscripts indicate significant difference. Standard errors were within 5 % of the mean, n= 4.

GRAIN	b*	Lutein	Overall
Spear	22% [a]	28% [a]	b* = lutein
Worrakatta	16% [b]	35% [c]	b* < lutein
Krichauff	7% [d]	29% [e]	b* < lutein
Tamaroi	11% [f]	11% [f]	b* = lutein

In wheat stored as flour the overall decrease in b* value was less than the overall decrease in lutein concentration in all wheat varieties (**Table 2.2.2**). There was a similar decrease in b* value, but the more pigmented varieties ie Krichauff and Tamaroi had less proportional decrease in lutein concentration than the other varieties.

Table 2.2.2 Summary of mean overall percent decrease in b* value and lutein concentration for all wheat varieties stored as flour. Different superscrips indicate significant difference. Standard errors were within 5 % of the mean, n=4.

FLOUR	b*	Lutein	Overall
Spear	18% [a]	76% [b]	b* < lutein
Worrakatta	18% [a]	77% [b]	b* < lutein
Krichauff	15% [a]	50% [c]	b* < lutein
Tamaroi	14% [a]	40% [c]	b* < lutein

2.2.3 c Carotenes

Alpha-carotene was undetectable in all samples stored as grain and flour, while low concentrations of β-carotene were detected in all varieties under all storage conditions. Krichauff had the highest concentration of β-carotene under all storage conditions. There was little decrease in β-carotene concentration with increased storage time and temperature in the grain form (**Figure 2.2.3**). In wheat stored as flour the greatest decrease in β-carotene concentration was brought about by increased storage time, most notable in Worrakatta (**Figure 2.2.4**), regardless of temperature. Krichauff showed similar decreases in β-carotene concentration in both the grain and flour forms while all other varieties had greater decreases in the flour form.

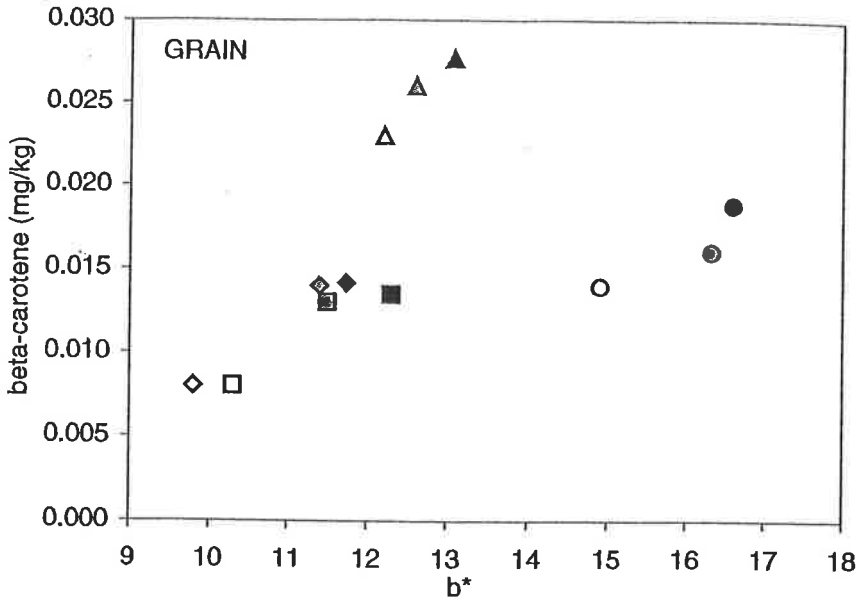


Figure 2.2.3 Beta-carotene concentration (mg/kg) and b* values of wheat stored as grain under all conditions for al wheat varieties. Spear - ◇; Worrakatta - □; Krichauff - Δ; Tamaroi - ○. Filled icon indicates unstored wheat, grey shaded icon indicates wheat stored at 5 °C for 6 months, and unfilled icon indicates wheat stored at 35° C for 6 months. Values are the mean of 4 measurements, error bars were omitted for clarity but errors were within 5 % of the mean.

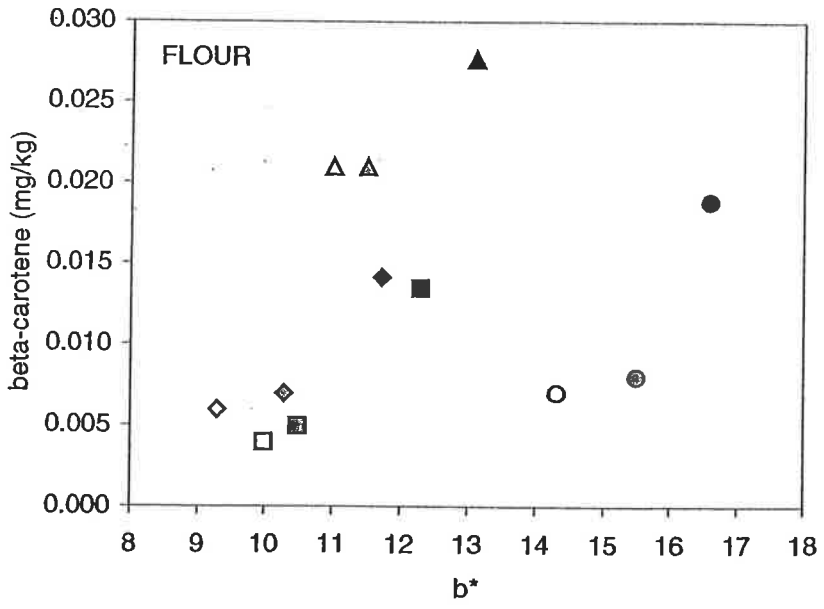


Figure 2.2.4 Beta-carotene concentration and b* values for flour stored under all conditions for all wheat varieties. Explanation of symbols is given above. Values are the mean of 4 measurements, error bars were omitted for clarity but errors were within 5 % of the mean.

2.2.3 d Correlations

CIE L*a*b* colour values were correlated with carotenoid concentrations, and all colour-carotenoid relationships are shown in **Appendix B (Tables B5, B6, B7 & B8)**. In wheat stored as both grain and flour there were strong correlations between b* value and lutein concentration. There were strong correlations also between b* value and β-carotene concentration in all wheat groups (**Appendix B Table B6 & B8**).

2.2.4 Discussion

The aim of this work was to determine if there was a strong relationship between colour measured by b* and specific carotenoids following periods of storage under different conditions. Determination of such a correlation would allow fast prediction of the carotenoid value of wheat following storage in either the grain or flour form. A change in grain/flour colour may represent an increase or decrease in quality depending on the end use of the flour. As flour colour is of such great importance in wheat quality, the pigments responsible for colour have been extensively investigated. Consistent with previous findings, the study presented here found that lutein was the major carotenoid present (**Figures 2.2.1, 2.2.2**) (Lepage and Sims, 1968), and that lutein concentration decreased in wheat during storage as flour (Farrington *et al.*, 1981), with less decrease seen in wheat stored as grain (Kaneko *et al.*, 1995). Unfortunately, neither of the previous studies into the effects of storage on carotenoids in wheat recorded colour changes, therefore direct comparisons with the colour to carotenoid correlation in current study are not possible.

Decreases in b* value were accompanied by a decline in lutein concentration (**Figure 2.2.1, 2.2.2**). However, the decrease in b* value was generally less than the decrease in lutein concentration (**Tables 2.2.1, 2.2.2**), indicating that not only all-*trans*-lutein concentration is responsible for the b* value. The greater proportional decrease in lutein concentration than b* value noted here, may be due to the production of lutein esters during storage (not quantified here) (Kaneko *et al.*, 1995). These compounds absorb at the same wavelength as all-*trans*-lutein (446 nm) and therefore contribute equally to the b* value. The difference between the varieties stored as grain for proportional decreases in b* value and lutein concentration may have been due to

varietal differences in the rate of esterification (Kaneko and Oyanagi, 1995). That the same varieties cannot be grouped together for their proportional differences in lutein concentration in wheat stored as flour as when stored as grain can be attributed to a combination of lutein esterification and oxidative degradation in this form.

In addition to lutein, β-carotene was also detected at very low concentrations in all samples analysed (**Figures 2.2.3, 2.2.4**), including those stored as flour at 35 °C where the greatest decrease in carotenoids was observed. The presence of lutein and β-carotene indicate that both side chains in the carotenoid biosynthetic pathway are present, while the absence of α-carotene may be attributed to hydroxylation of this hydrocarbon resulting in lutein.

The recently released high yielding bread wheat variety, Krichauff, is popular with farmers, but has been downgraded by the Australian Wheat Board because of its strong pigmentation (b* > 11). The effect of storage on the colour and carotenoid content of this variety is therefore of particular interest to growers and millers alike. Of all the wheat varieties analysed here Krichauff showed the least decrease in b* value with storage as grain (**Table 2.2.1**), indicating that the yellow colour of this highly pigmented bread wheat cannot be reduced significantly by storage in this form. However, in Krichauff stored as flour the overall decrease in lutein and β-carotene concentration was the least seen in all varieties (**Table 2.2.2**).

In contrast, when stored as flour Krichauff showed the least overall decrease in b* value associated with the least overall decrease in lutein and β-carotene concentration of the bread wheat varieties (**Table 2.2.2**). However, the overall decrease in b* value in Krichauff was sufficient to reach an acceptable b* value for millers (b* = 11). Krichauff can therefore attain an acceptable colour level while still retaining a proportionally higher concentration of lutein than the other bread wheat varieties even when stored as flour at 35 °C for 6 months. This may indicate that this variety has a reduced rate of esterification. Additionally, this wheat variety had a higher concentration of β-carotene than the other wheat varieties analysed here.

Generally, the greatest decrease in total lutein and β-carotene concentration was associated with storage for 6 months as flour. Little further decrease in the concentration of these carotenoids was found when storage temperature was increased

from 5 °C to 35 °C for the 6 month period. As the samples were stored in paper bags which allowed free flow of air during the storage period the majority of the decrease in carotenoids was attributed to oxidative degradation.

The presence of a relatively high concentration of lutein and associated high b* value of the durum, Tamaroi, can be attributed to the importance of yellow colour in pasta production based on durum wheat, and selection for this trait in breeding programs. Additionally, the durum showed little decrease in b* value in grain stored at 5 °C, with the majority of decrease occurring at 35 °C. Importantly for the pasta industry, storage of grain at 5 °C for up to 6 months will result in only a small loss of the desired yellow colour. It is proposed that the greater concentration of lutein proportional to β-carotene in the durum wheat resulted in a greater effect on b* value by this carotenoid than in the bread wheat varieties, resulting in the poor correlation between b* value and β-carotene concentration.

In conclusion, this study supported the hypothesis that that the CIE L*a*b* colorimetry method is a useful method for determining carotenoid concentrations in wheat under different storage conditions. Results indicate that in wheat stored as grain, an increase in temperature from 5 °C to 35 °C results in a considerable decrease in b* value, lutein and β-carotene concentration, which was minimal when stored at 5 °C. However, in wheat stored as flour, a large decrease in b* value and carotenoid concentration is caused by storage for 6 months, regardless of temperature, and this is attributed to oxidative degradation. There was a strong correlation in all wheat varieties between b* value and lutein concentration and b* value and β-carotene concentration.

CHAPTER 3

Distribution of Lutein, Zeaxanthin and Related Geometrical Isomers in Fruit, Vegetables, Wheat and Pasta Products

The work in this chapter was conducted at the University of Maryland (USA), in the Department of Chemistry and Biochemistry, under the supervision of Dr. F. Khachik.

Aim

To determine the concentration of lutein, zeaxanthin and their isomers in fruit, vegetables, wheat and pasta products commonly consumed in the USA.

3.1 Introduction

There have been numerous reports on the qualitative and quantitative distribution of carotenoids in fruit and vegetables (Khachik *et al.*, 1992a; Khachik *et al.*, 1999c). However, quantitative analysis of dietary lutein, zeaxanthin and their (*E/Z*)-geometrical isomers are scarce, and generally only the combined concentrations of these two carotenoids and their isomers are reported. Epidemiological and observational studies have shown that lutein and zeaxanthin accumulate in the human macula and may have implications in the prevention of age-related macula degeneration (AMD) (Bone *et al.*, 1993). Additionally, the consumption of dietary lutein and zeaxanthin has been shown to increase the carotenoid pigments found in the human macula.

The amount of lutein present in human blood is much greater than zeaxanthin and is a reflection of the proportions in food, however this is not reflected in the human macula where the ratio is 1:1. Additionally, the 1:1 ratio of *E*:*Z* isomers of lutein and zeaxanthin in food is again reflected in blood and tissue samples, though this is not found in the human eye, where the *Z* isomers are dominant. These ratios have important implications in the prevention of AMD, as sufferers have been found to have a reduced concentration of lutein and zeaxanthin *Z* isomers. In order to determine the dietary source of macular pigments, an accurate measure of the concentration of these carotenoid isomers in food is essential.

3.2 Materials and Methods

3.2.1 Source of Fruit and Vegetables

Fruit and vegetables analysed were obtained from a local supermarket (Maryland, USA) and included kale (*Brassica oleracea*), collard (. *oleracea*), broccoli (*B. oleracea*), spinach (*Spinacia oleracea*), green beans (*Phaseolus vulgaris*), fresh parsley (*Petroselinum crispum*), butternut pumpkin (*Cucurbita moschata*), orange (*Citrus sinensis*), papaya (*Carica papaya*), mango (*Mangifera indica*), nectarine (*Prunus persica*) and lettuce (*Lactuca sativa*). Canned sweet corn (*Zea mays*), peas (*Pisum sativum*) and Lima beans (*Phaseolus lunatus* L. (syn. *P. limensis*)) were analysed as the most commonly consumed form of these vegetables. Lasagne and egg noodles were purchased in a dehydrated form, and wheat was from the 1998 harvest in Maryland, USA. Freekeh (a green-harvested wheat) was imported from Australia as a commercial product.

Classification

The type of fruit or vegetable and the plant part consumed was used to classify the samples into the following groups: leafy green vegetables, non-leafy green vegetables, yellow/orange fruit and vegetables, and wheat and pasta products.

3.2.2 Explanation of terms used in text

The terms *E* and *Z* replace *trans* and *cis* respectively, in accordance with international convention on the nomenclature of chemical isomers, and a further explanation of these can be found in **Chapter 1**.

Total lutein is the combination of all *E*-lutein, 3'epilutein, and total *Z*-lutein concentration.

3.2.3 Extraction of Carotenoids

Carotenoids were extracted from samples using the method of (Khachik *et al.*, 1992a), with details in **Appendix A1.2**.

3.2.4 HPLC Analysis of carotenoids

The HPLC method was that of Kahcihck (*pers comm.*, 1999). Extracted samples were removed from –20° C and allowed to reach room temperature before analysis by HPLC.

Apparatus

Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Hewlett-Packard 1040A HPLC with a rapid scanning UV/visible photodiode array detector. The data were stored and processed by a Hewlett-Packard 85-B computing system that was operated with a Hewlett-Packard Model 9121 disk drive and 7470A plotter. The absorption spectra of the carotenoids were recorded between 200 and 600nm at the rate of 12 spectra/minute The HP-85B computer with a built in integration program was used to evaluate the peak area and peak height. Absorption spectra of isolated components in various solvents were recorded on a Beckman DU-7 UV/visible spectrophotometer.

3.2.4 a Reverse Phase Separations

Column

Separations were performed on a stainless steel (25 cm x 4.6 mm i.d.) Microsorb C_{18} (5 µm spherical particles) column (Rainin instrument Co.), which was protected with a Brownlee guard cartridge (3 cm length x 4.6 mm i.d.) packed with spheri-5-C_{18} (5 µm particle size).

Solvents and Method

Solvent A: acetonitrile (90%), methanol (10%)

Solvent B: 45% dichloromethane, 45% hexane, 10% methanol and 0.1% DIPEA

Method: At time 0 minutes, 95% solvent A, (5% solvent B); gradient resulting in 45%A at time 40 minutes; from time 45 minutes to 50 minutes gradient resulting in 95% A. The flow rate was a constant 0.7 ml/min

Injection solvent: 40% acetonitrile, 20% methanol, 20% dichloromethane, 20% hexane, 0.1% DIPEA.

3.2.4 b Nitrile bonded column separations for lutein and zeaxanthin isomer quantification

Column

Separations were performed on a Phenosphere (25 cm length x 4.6 mm i.d.) CN (5 µm particle size) column (Phenomenex, NZ, protected by a Phenosphere guard cartridge (3 cm length x 4.6 mm i.d.) packed with CN (5 µm particle size).

Solvents and Method

Solvent: 75% hexane, 25% dichloromethane, 0.25% methanol, 0.1% DIPEA.

Method: isocratic for 40 minutes, with a constant flow rate of 0.7 ml/min

Injection solvent: 75% hexane, 25% dichloromethane, 0.25% methanol and 0.1% DIPEA.

NB The amount of methanol is critical to this separation and extreme care should be taken to ensure that there is no contamination from outside the system.

3.3 Results

3.3.1 Lutein and Lutein Isomers

3.3.1 a Green Vegetables

Figures 3.1 & 3.2 show the most prolific of the lutein isomers in green vegetables to be all-*E*-lutein, greater even than the combination of all-*Z*-lutein, 9*Z*-lutein and 13*Z*-lutein, (shown as Total *Z*-lutein in **Figures 3.1 & 3.2**). Consequently, the green vegetables that had the highest concentration of this isomer also had the highest total lutein concentration. In leafy green vegetables (**Figure 3.2**) the concentration of total *Z*-lutein was proportional to the concentration of total lutein, whereas in non-leafy green vegetables (**Figure 3.1**) the total *Z*-lutein concentration was not related to the total lutein concentration.

Parsley (0.25 mg/kg) and peas (0.13 mg/kg) were the only green vegetables that had measurable concentrations of 3'epilutein. The presence of this lutein isomer was not associated with a particular concentration of total lutein and was detected both in green vegetables with the highest and lowest total lutein concentrations.

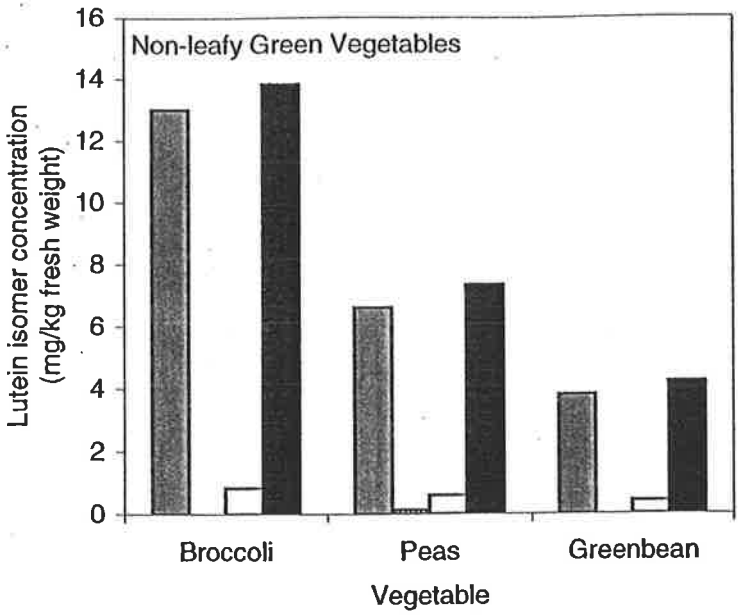


Figure 3.1 Concentration of: all *E*-lutein (■), 3'epilutein (▨) total *Z*-lutein (comprising all *Z*-lutein, 9*Z*-lutein and 13*Z*-lutein) (□), and total lutein (■) in non-leafy green vegetables, in order of descending total lutein concentration.

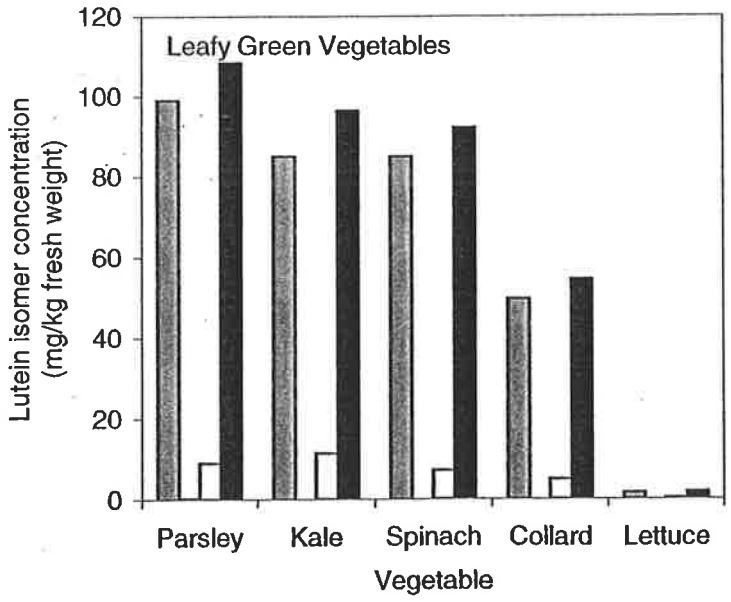


Figure 3.2 Concentration of: all *E*-lutein (■), 3'epilutein (▨), total *Z*-lutein (comprising all *Z*-lutein, 9*Z*-lutein and 13*Z*-lutein) (□), and total lutein (■) in leafy-green vegetables, in order of descending total lutein concentration.

3.3.1 b Yellow/Orange Fruit and Vegetables

The highest concentration of all *E*-lutein, total *Z*-lutein, and total lutein in the yellow/orange fruit and vegetables was detected in butternut pumpkin. As in the green vegetables, all *E*-lutein represented the major component of total lutein and the concentrations of lutein and lutein isomers are shown in **Figure 3.3.** Sweet corn, Lima bean (0.099 mg/kg) and nectarine were the only yellow/orange fruit and vegetables to have a measurable concentration of 3'epilutein. Sweet corn had the highest concentration of 3'epilutein of all the samples analysed.

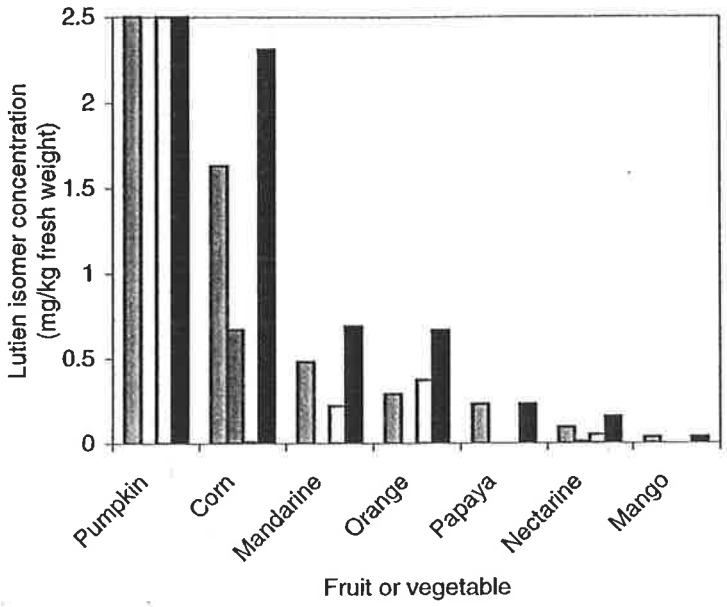


Figure 3.3 Concentration of: all *E*-lutein (■), 3'epilutein (■) total *Z*-lutein (comprising all *Z*-lutein, 9*Z*-lutein, 9'*Z*-lutein and 13*Z*-lutein), (□) and total lutein (■) in yellow/orange fruit and vegetables, in order of descending total lutein content. For butternut pumpkin: all *E*-lutein: 18mg/kg, total *Z*-lutein: 6 mg/kg, total lutein: 24 mg/kg.

None of the *Z*-lutein isomers were detected in the yellow/orange fruit papaya and mango. Additionally, mango had the lowest detectable concentration of all *E*-lutein of all the samples analysed. The 9*Z*-lutein isomer was undetectable in some of the yellow/orange fruit and vegetables (sweet corn, papaya and mango); the absence of this

isomer was restricted to the yellow/orange fruit and vegetables group, yet the concentration of this isomer in this group spanned the range obtained for all food analysed. All of the yellow/orange fruit and vegetables had undetectable concentrations of at least one of the lutein isomers. However, total lutein in the yellow/orange fruit and vegetables ranged from 24 mg/kg in butternut pumpkin to 3.45 x 10^{-2} mg/kg in mango.

3.3.1 c Wheat and Pasta Products

In wheat and pasta products (**Figure 3.4**) all *E*-lutein was the dominant isomer, and all of the samples contained both all *E*-lutein and at least one *Z*-lutein isomer. Egg noodles had the highest concentration of all *E*-lutein (1.1 mg/kg) and total *Z*-lutein (0.29 mg/kg), leading to the highest total lutein concentration (1.39 mg/kg). Although Freekeh had a similar concentration of total *Z*-lutein (0.23 mg/kg) to egg noodles it had a concentration of all *E*-lutein (0.58 mg/kg) equal to only half that of egg noodles. Catoctin a North American bread wheat, and lasagne had similar concentrations of total lutein (0.32 mg/kg & 0.29 mg/kg respectively), higher than that of Pioneer (0.22 mg/kg), another North American bread wheat. However, Catoctin and lasagne had different concentrations of all *E*-lutein (0.29, 0.23 mg/kg respectively) and total *Z*-lutein (0.036, 0.056 mg/kg respectively). 3'epilutein was undetectable in all samples in the wheat and pasta group.

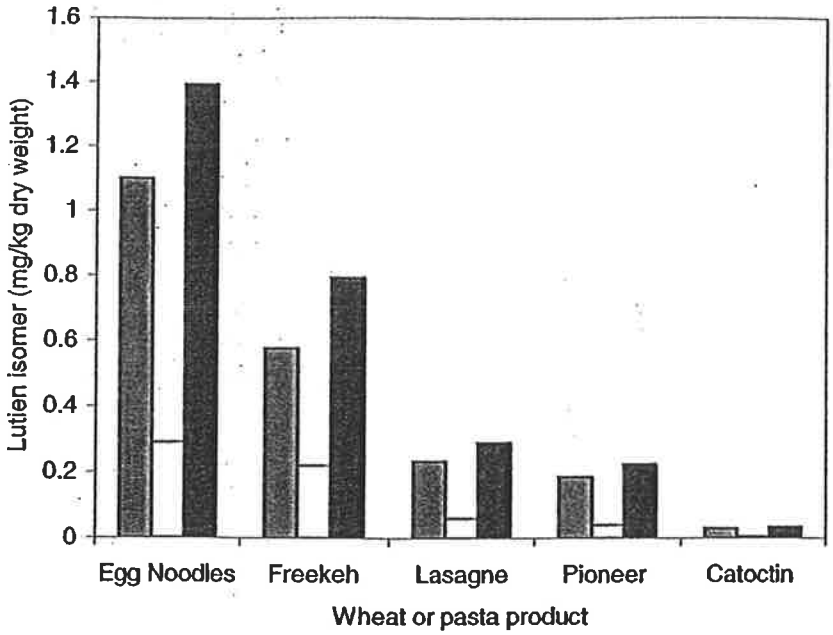


Figure 3.4 Concentration of: all *E*-lutein (■), total *Z*-lutein (□) (comprising all *Z*-lutein, 9*Z*-lutein, 9'*Z*-lutein and 13*Z*-lutein) and total lutein (■) in egg noodles and lasagne, two US wheat varieties, (Pioneer and Catoctin) and a green wheat product Freekeh, in order of descending total lutein content.

3.3.1 d Overall Comparison of Lutein Concentration

The green vegetables generally had the higher concentrations of total lutein. All *E*-lutein was the dominant isomer in all foods analysed and therefore is a good indicator of the total lutein concentration. Few of the samples analysed had detectable concentrations of 3'epilutein. These included parsley (0.25 mg/kg), peas (0.13 mg/kg), Lima beans (0.099 mg/kg), sweet corn (0.67 mg/kg) and nectarine (0.093 mg/kg). Several of the lutein isomers were undetectable in the yellow/orange fruit and vegetables, while 3'epilutein was the only isomer undetectable in the green vegetables and the wheat and pasta products.

3.3.2 Ratio of E:Z lutein

As noted in the introduction the consistent and natural ratio of 1:1 for the *E*:*Z* isomers of lutein is not reflected in the macular region of the eye where the *Z* isomers are dominant. People with a degenerative disease of the eye have been found to have reduced levels of the *Z* lutein isomers and so dietary sources with low *E*:*Z* ratios may be important in the prevention of this disease.

3.3.2 a Green Vegetables

Lima beans had the lowest ratio of all the green vegetables (**Figure 3.5**), though this was due to low levels of both the *E* and *Z* isomers. Kale had one of the lower ratios of the green vegetables combined with a high concentration of *Z* isomers. Parsley and peas had the same ratios but very different concentrations of the two isomers. Peas had less than 1/15 the concentration of all-E-lutein (6.6 mg/kg) of parsley (99 mg/kg), and total *Z*-lutein.

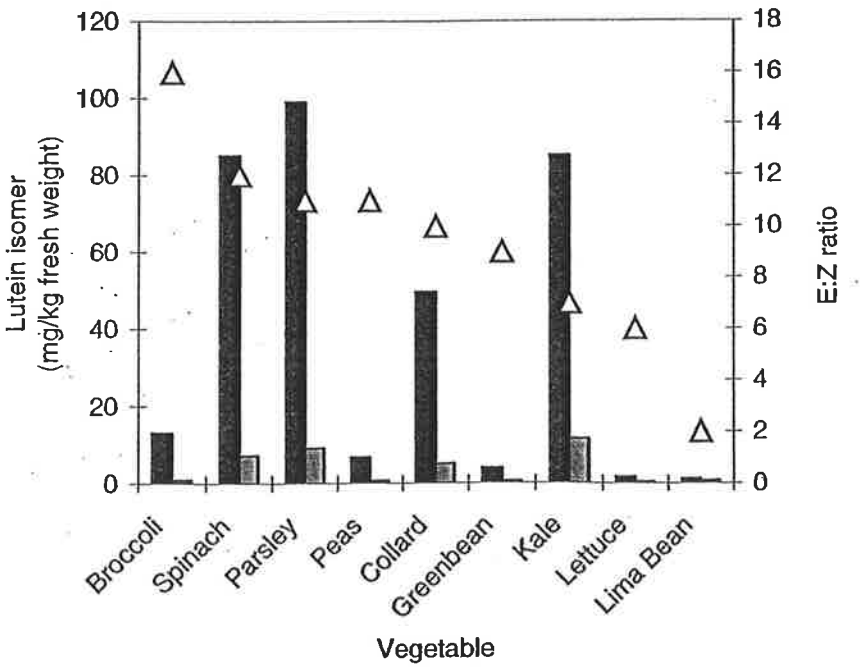


Figure 3.5 Concentrations of all-*E*-lutein (■), total *Z*-lutein (■) and *E*:*Z* ratio (△) in green vegetables, in order of descending ratio.

3.3.2 b Yellow / Orange Fruit and Vegetables

Of the yellow/orange fruit and vegetables (**Figure 3.6**) butternut pumpkin, nectarine and mandarine had low ratios of 3, 2 & 2 respectively. Additionally the comparatively high concentration of the *Z* isomers in butternut pumpkin makes this a valuable dietary source of these isomers. Sweet corn had the highest ratio (163) of the yellow/orange fruit and vegetables. Due to the absence of *Z*-lutein isomers a ratio could not be recorded for papaya and mango.

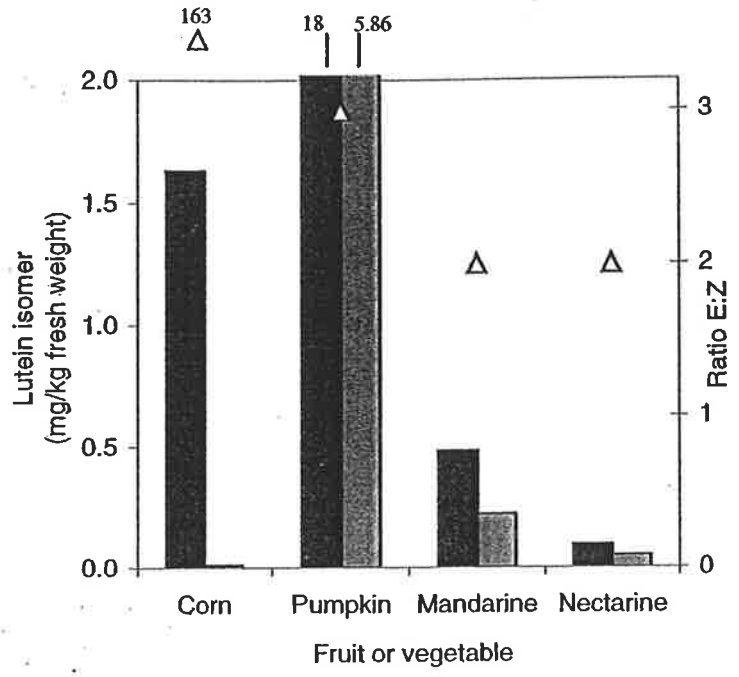


Figure 3.6 Concentrations of all-*E*-lutein (■), total *Z*-lutein (▩) and *E:Z* ratio (Δ) in yellow/orange fruit and vegetables, in order of descending ratio. Numbers above bars indicate concentration of carotenoid. Δ with number indicates ratio.

3.3.2 c Wheat and Pasta Products

Of the wheat and pasta products (**Figure 3.7**) the processed and unprocessed samples had similar ratios of *E* to *Z* lutein. The green harvested wheat, Freekeh, had the lowest ratio, and Catoctin had the highest ratio. There was a significant difference between the two wheat varieties for the ratio. Interestingly the green harvested wheat, Freekeh, had a similar concentration of the *Z* isomers as egg noodles.

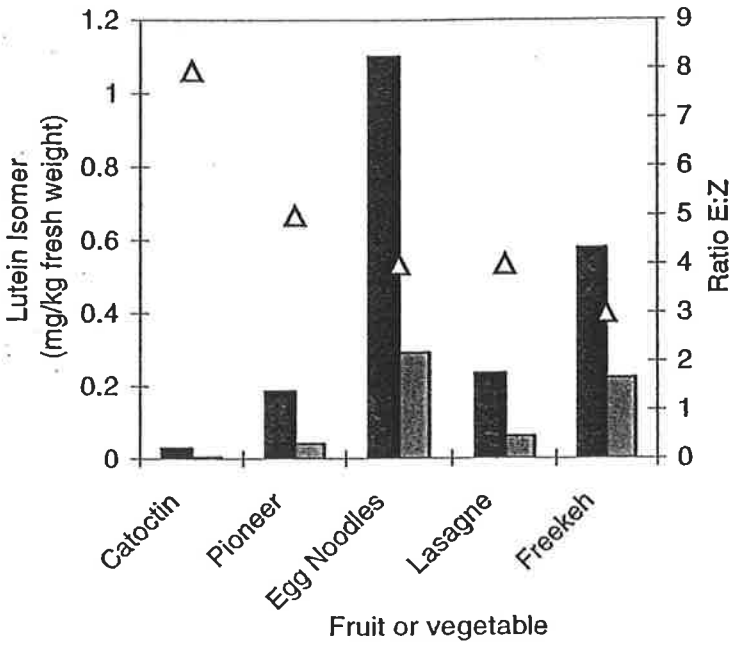


Figure 3.7 Concentration of all-*E*-lutein (■), total *Z*-lutein (■)and *E:Z* ratio (△) in wheat and pasta products, in order of descending ratio.

3.3.2 d Overall Comparison of *E*-:*Z*-Lutein Ratio

Generally the yellow/orange fruit and vegetables had the lowest ratios. The overall lowest ratio was found in Lima beans and nectarine though these had low levels of both isomers. Butternut pumpkin had a relatively low *E:Z* ratio combined with a relatively high concentration of the *Z* isomers. Sweet corn had by far the highest ratio of *E*-:*Z*-lutein although the *Z*-lutein isomer concentration in this vegetable was extremely low compared with other fruit and vegetables, contributing to the high ratio.

3.3.3 Zeaxanthin and Zeaxanthin Isomers

3.3.3 a Green Vegetables

Of the green vegetables (**Figure 3.8**) spinach and parsley had the highest concentration of 9Z-zeaxanthin (3.9 mg/kg and 3.7 mg/kg respectively), and equal amounts of all Z-zeaxanthin (1.3 mg/kg). Kale and collard had approximately half the amount of 9Z-zeaxanthin (1.71 mg/kg, 1.25 mg/kg) of spinach and parsley. However, these two vegetables were not similar in their concentration of all Z-zeaxanthin. Kale had the lowest concentration of all green vegetables, less than 0.5% of the highest (spinach & parsley), while collard had 9% of the highest concentration. None of the green vegetables had detectable concentration of 13Z-zeaxanthin.

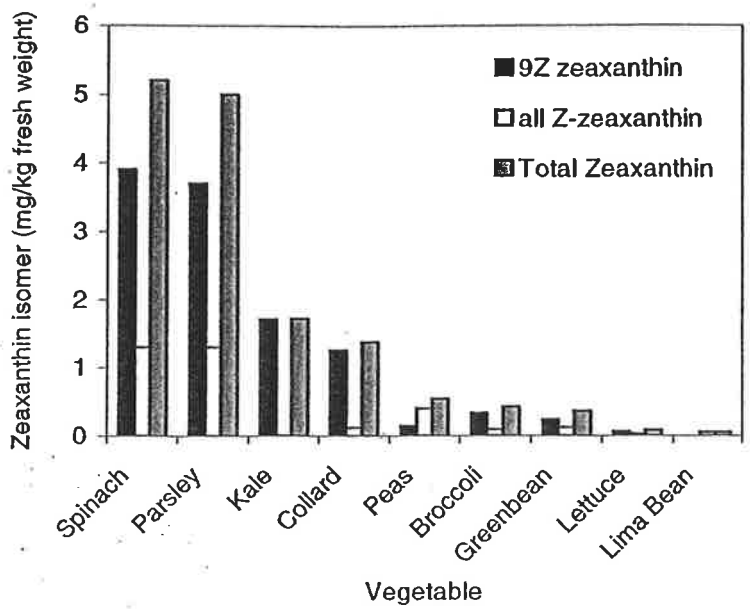


Figure 3.8 Concentration of 9Z-zeaxanthin (■), all Z-zeaxanthin (□) and total zeaxanthin (■) in green vegetables, in order of descending total zeaxanthin content.

Peas were the only green vegetable to have a higher concentration of all Z-zeaxanthin (0.4 mg/kg) than 9Z-zeaxanthin (0.14 mg/kg). However, this did not contribute greatly to the total zeaxanthin concentration which was almost 1/10 that of spinach (0.54 mg/kg). Lima beans were the only vegetable to have an undetectable concentration of 9Z-zeaxanthin. As this isomer generally represented the majority of the total zeaxanthin

concentration, with the exception of peas, Lima beans had the lowest total zeaxanthin concentration of the green vegetables.

3.3.3 b Yellow / Orange Fruit and Vegetables

Of the yellow/orange fruit and vegetables shown in **Figure 3.9**, sweet corn and butternut pumpkin had the highest two total zeaxanthin concentrations (3.3 mg/kg & 2.8 mg/kg), however the concentration of the individual isomers was quite different. All Z-zeaxanthin represented 90% of the total zeaxanthin concentration in sweet corn, while 9Z zeaxanthin was a minor component. This was also the case for mandarine. In contrast to corn, butternut pumpkin had an undetectable concentration of all Z-zeaxanthin, thus 9Z zeaxanthin represented 100% of the total lutein concentration.

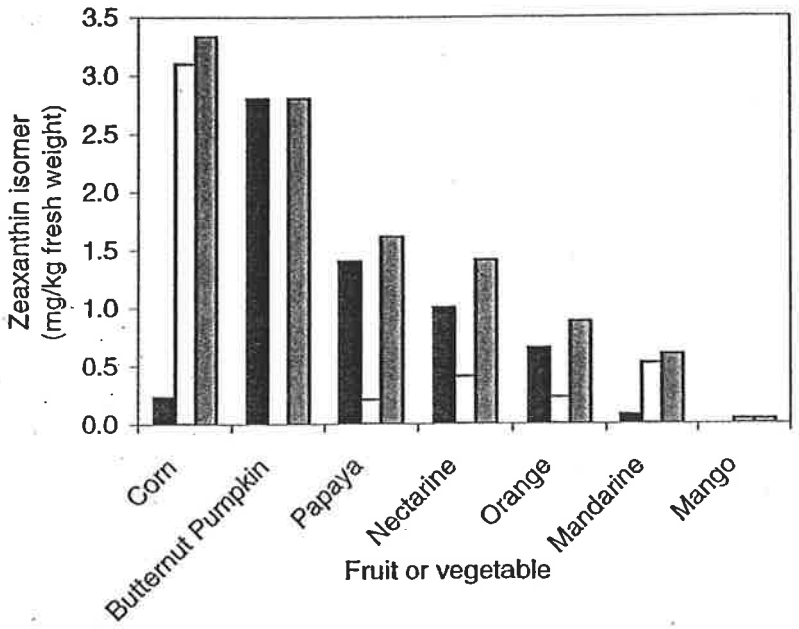


Figure 3.9 Concentration of 9Z-zeaxanthin (■), all Z-zeaxanthin (□) and total zeaxanthin (▣) in yellow/orange fruit and vegetables, in order of descending total zeaxanthin content.

Butternut pumpkin was the only sample analysed that had an undetectable concentration of all Z-zeaxanthin. Papaya, nectarine and orange followed the same trend in

zeaxanthin isomer concentrations as butternut pumpkin with 9Z-zeaxanthin the major contributer to total zeaxanthin

Mango, although brightly coloured, had an undetectable concentration of 9Z-zeaxanthin and a very low concentration of all Z-zeaxanthin ($3.8x10^{-2}$ mg/kg), resulting in the lowest total zeaxanthin concentration of the yellow/orange fruit and vegetables. None of the yellow/orange fruit and vegetables had detectable concentration of 13Z zeaxanthin.

3.3.3 c Wheat and Pasta Products

Not all samples in the wheat and pasta products group had a detectable concentration of 13Z-zeaxanthin, however, this was the only group where this isomer was detected. The 13Z-zeaxanthin isomer was detected in egg noodles, Freekeh and lasagne (shown in **Figure 3.10**); however the rest of the isomer profile of these three samples varied considerably. The 13Z-zeaxanthin isomer made up the greatest proportion of the total zeaxanthin concentration in egg noodles, and the least proportion in Freekeh. All Z-zeaxanthin made up the greatest proportion in Freekeh and 9Z-zeaxanthin and the least proportion in egg noodles.

Lasagne had a very low concentration of all three isomers, however the relative proportions of the isomers were similar to that of Freekeh. The North American bread wheat varieties, Catoctin and Pioneer were also very low in all zeaxanthin isomer concentrations with proportions of all-Z-zeaxanthin to 9Z-zeaxanthin similar to that of Freekeh.

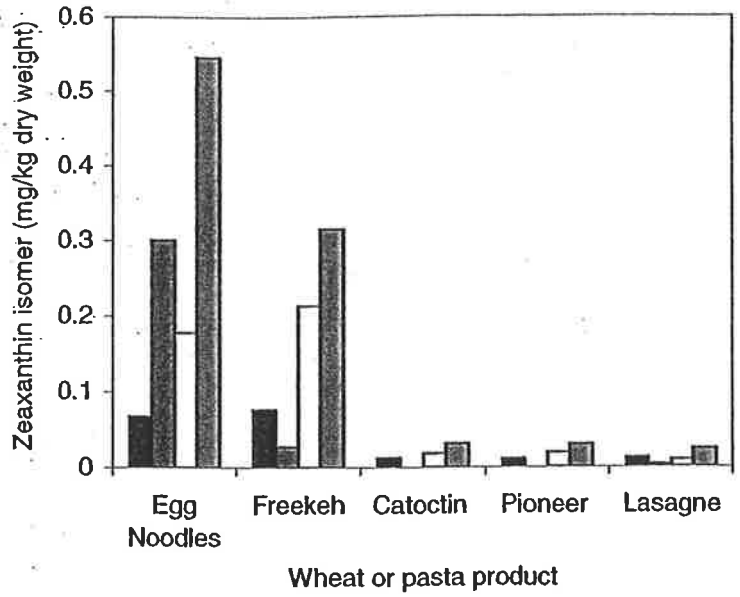


Figure 3.10 Concentration of 9Z-zeaxanthin (■), 13Z-zeaxanthin (■), all Z-zeaxanthin (□) and total zeaxanthin (■) in egg noodles, lasagne; two US bread wheat varieties, Catoctin and Pioneer; and Freekeh, in order of descending total zeaxanthin content.

3.3.3 d Overall Comparison of Zeaxanthin

The highest overall concentration of zeaxanthin was detected in spinach and parsley closely followed by corn and butternut pumpkin. Wheat and pasta products had the lowest overall zeaxanthin concentration with the exception of egg noodles.

3.3.4 Lutein: Zeaxanthin Ratio

The ratio of lutein to zeaxanthin represents the relative amounts of lutein when compared to zeaxanthin. The ratio of lutein to zeaxanthin in the macular region of the eye is 1:1. This ratio is important when trying to identify dietary sources that may contribute to achieve the ratio required to maintain an healthy eye.

3.3.4 a Green Vegetables

Of all the green vegetables parsley and spinach had the lowest ratios despite their higher concentration of lutein owing to a high concentration of zeaxanthin (**Figure 3.11**). Kale had the highest lutein to zeaxanthin ratio, attributable to a combination of a high concentration of lutein (96 mg/kg) and a low concentration (1.7 mg/kg) of zeaxanthin.

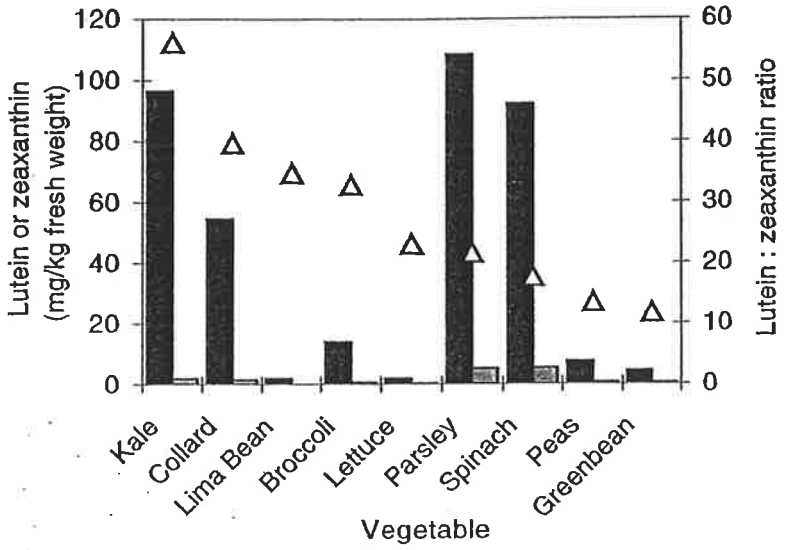


Figure 3.11 Concentration of lutein (■), zeaxanthin (■)and ratio of lutein to zeaxanthin (Δ) in green vegetables, in order of descending ratio.

3.3.4 b Yellow/Orange Fruit and Vegetables

Mandarine, mango and sweet corn had ratios of lutein to zeaxanthin closest to that found in the human eye. Although butternut pumpkin had the highest zeaxanthin concentration, with the exception of sweet corn, the lutein to zeaxanthin ratio was much higher than 1:1 indicating the larger concentration of lutein that zeaxanthin. Ratios in the yellow/orange fruit and vegetables were lower than in the green vegetables (**Figure 3.12**).

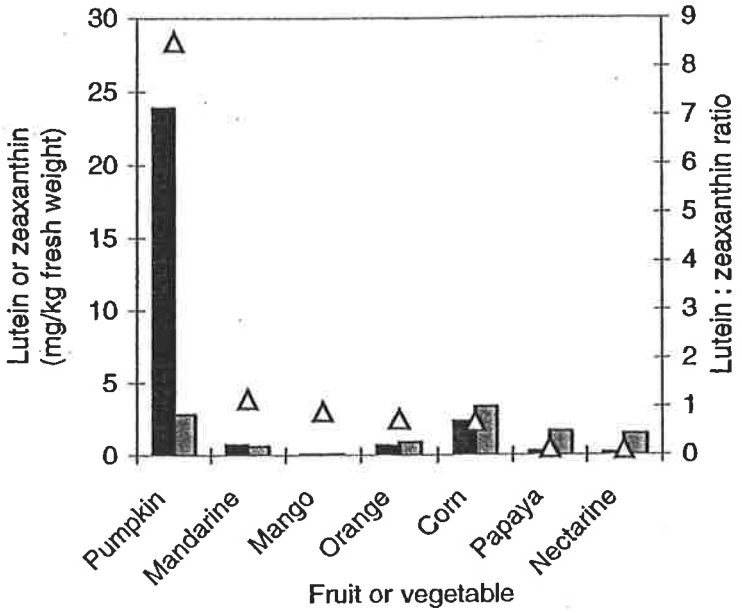


Figure 3.12 Concentration of lutein (■), zeaxanthin (■)and ratio of lutein to zeaxanthin (Δ) in yellow/orange fruit and vegetables in order of descending ratio.

3.3.4 c Wheat and Pasta Products

Ratios of lutein to zeaxanthin in wheat and pasta products were higher than for the yellow/orange fruit and vegetables due to the low lutein concentration of the latter (**Figure 3.13**). Zeaxanthin concentrations were lower than in the yellow/orange fruit and vegetables even in egg noodles which would have obtained this carotenoid from gg yolk. Only egg noodles and Freekeh had a lutein to zeaxanthin ratio similar to that found in the peripheral region of the macular region of the eye.

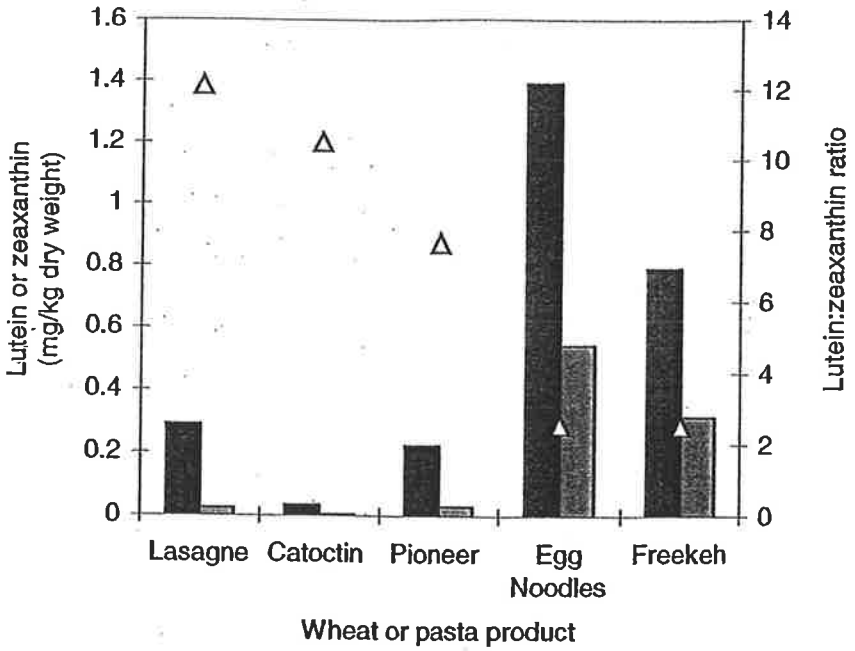


Figure 3.13 Concentration of lutein (■), zeaxanthin (■)and ratio of lutein to zeaxanthin (Δ) in wheat and pasta products in order of descending ratio.

3.3.4 d Overall Comparison of Lutein to Zeaxanthin Ratio

Generally the green vegetables had higher lutein to zeaxanthin ratios than the wheat and pasta products and the yellow/orange fruit and vegetables.

3.3.5 Other Carotenoids

3.3.5 a Green Vegetables

Lutein, zeaxanthin and their isomers were not the only carotenoids detected in green vegetables. As shown in **Figure 3.14,** α-, β- and γ-carotene, neurosporene and α- and β-cryptoxanthin were detected in all of the green vegetables analysed, with the exception of lettuce that had no detectable concentration of neurosporene. However, lactucaxanthin was detected in lettuce. With the exception of lettuce, the leafy green vegetables had the highest concentration of β- and γ-carotene. The concentration of β-

carotene (62.8 - 75.5 mg/kg) were over eight times that of the highest non-leafy green vegetable broccoli (8.8 mg/kg), while the concentration of γ-carotene (2.7 - 3.8 mg/kg) were nearly seven times that of the highest non-leafy green vegetable, again broccoli (0.4 mg/kg). However, concentration of α-carotene did not follow the same trend, and green beans had the highest concentration (0.4 mg/kg) followed by the leafy green vegetables and Lima beans, at half the concentration of green beans (0.2 mg/kg). The leafy green vegetables had the highest concentration of α-cryptoxanthin with concentration at least 1 1/2 times that of the non-leafy green vegetables.

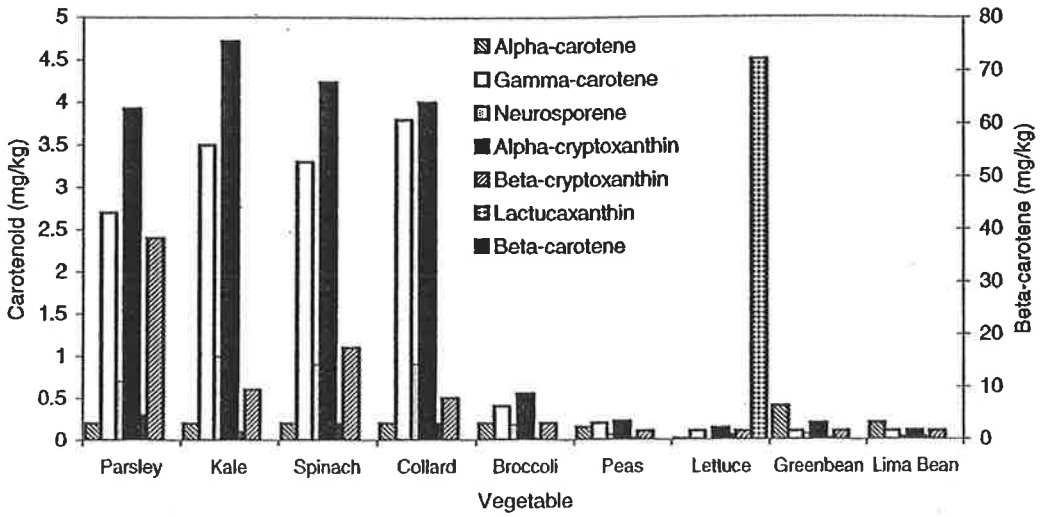


Figure 3.14 Carotenoids in green vegetables excluding lutein and zeaxanthin, showing β-carotene (on second y-axis), α-carotene, γ-carotene, neurosporene, α-cryptoxanthin, β-cryptoxanthin and lactucaxanthin.

3.3.5 b Yellow/Orange Fruit and Vegetables

Of the yellow/orange fruit and vegetables (**Figure 3.15**) butternut pumpkin had the highest concentration of α-carotene (18.5 mg/kg), γ-carotene (3.6 mg/kg) and α-cryptoxanthin (1.8 mg/kg), considerably higher than found in mandarine as yellow/orange vegetable with the second highest concentration of the carotenoids.

Butternut pumpkin also had the highest concentration of ζ-carotene (7.2 mg/kg), neurosporene (1.4 mg/kg) and β-carotene (52.1 mg/kg) again at a considerably greater concentration than that of papaya, that had the second highest concentrations of these carotenoids. Mandarine contained the greatest concentration of β-cryptoxanthin (20.2 mg/kg), nearly three times the concentration of that of the papaya. Papaya had the highest concentration of phytoene (6.8 mg/kg) and phytofluene (4.4 mg/kg).

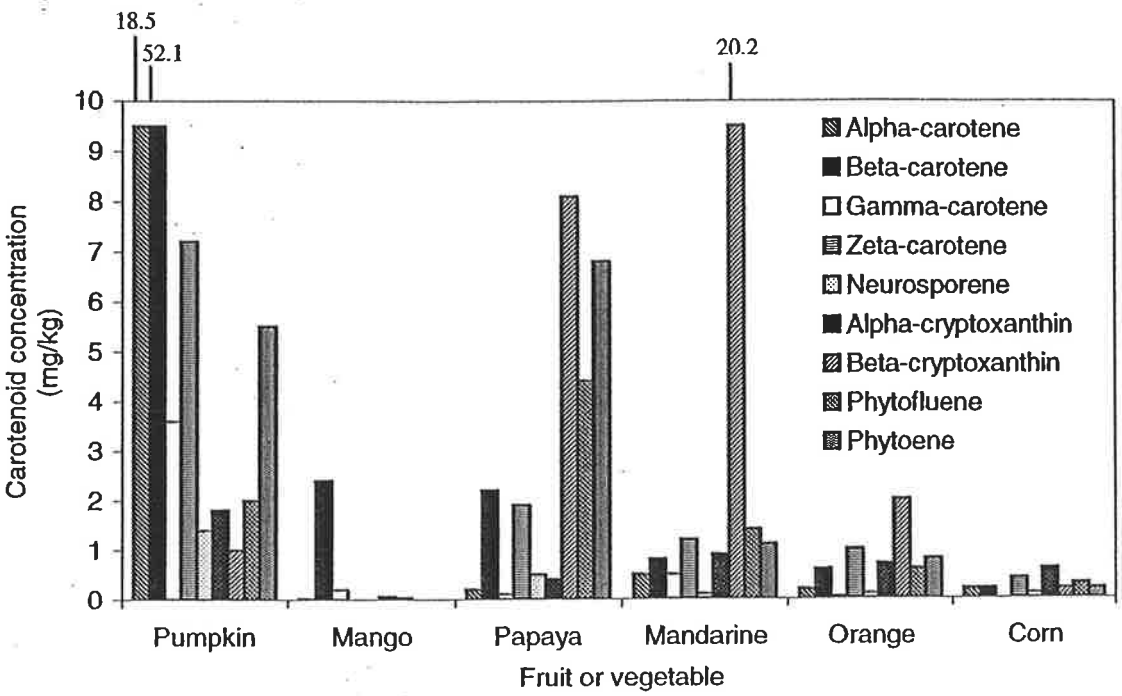


Figure 3.15 Concentration of α-carotene, β-carotene, γ-carotene, ζ-carotene, neurosporene, α-cryptoxanthin, β-cryptoxanthin, phytofluene and phytoene in yellow/orange fruit and vegetables. In order of descending β-carotene concentration. Numbers above bars indicate concentration of carotenoid in mg/kg.

Overall carotenoid concentration was highest in the leafy green vegetables and butternut pumpkin (**Figure 3.16**). Wheat and pasta products had the lowest overall carotenoid concentration.

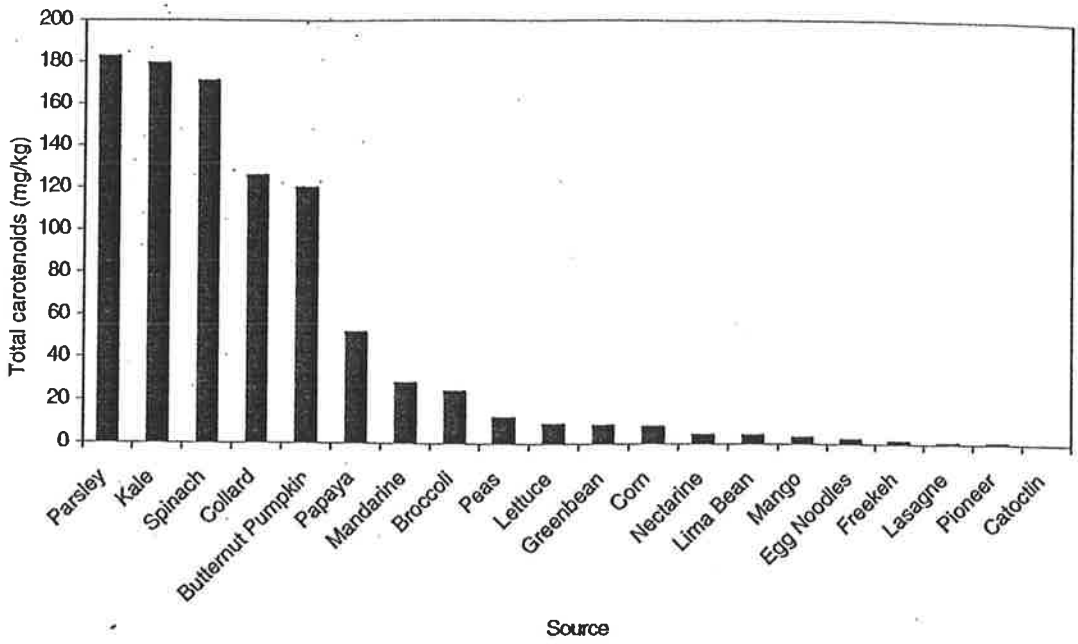


Figure 3.16 Total carotenoid concentration in fruit and vegetables in order of descending carotenoid content.

There was little difference between the total carotenoid content of butternut pumpkin and the non-leafy green vegetables, and this was attributed to the ability of some plants to retain chlorophyll via a defect in the chlorophyll degradation pathway as well as synthesise carotenoids as they mature (Hornero-Mendez and Minguez-Mosquera, 2000). As the retention of chlorophyll does not interfere with carotenoid biosynthesis, green and yellow/orange fruit and vegetables can therefore have similar concentrations of carotenoids.

3.4 Discussion

The association between the type of fruit or vegetable, ie leafy or non-leafy, green or yellow/orange; and the concentration of a wide range of carotenoids has been previously reported (Khachik *et al.*, 1986), though the individual lutein and zeaxanthin components were not separated. Due to the small proportion of zeaxanthin in relation to lutein in most plants (Khachik *et al.*, 1986) the current study in which lutein and zeaxanthin are separately quantified was not expected to change the reported relationships.

The abundance of lutein over zeaxanthin in nature is due to the dominant role of lutein in photosynthesis (Mathis and Schenck, 1982), and the observed greater concentration of lutein over zeaxanthin in green vegetables, butternut pumpkin and wheat and pasta products (**Figures 3.11, 3.12, 3.13**) was therefore expected. The abundance of lutein in fruit and vegetables commonly consumed is reflected in blood and tissue levels of these carotenoids (Khachik *et al.*, 1991b; Khachik *et al.*, 1992a; Khachik *et al.*, 1997b). However, in the macular region of the eye the ratio of lutein to zeaxanthin does not consistently reflect that seen in food and other body tissues; the concentration of lutein is greater than zeaxanthin in the peripheral region of the macula, while zeaxanthin is more abundant in the central region (Bone *et al.*, 1988; Bone and Landrum, 1992; Sommerburg *et al.*, 2000).

In contrast to the green vegetables, all of the yellow/orange fruit and vegetables analysed here, with the exception of butternut pumpkin, had a zeaxanthin concentration greater than or equal to the lutein concentration (**Figure 3.12**). The abundance of zeaxanthin over lutein against the trend in nature might be attributed to a propensity of these foods to accumulate β-carotene and therefore more of the oxidation product zeaxanthin. However, the concentration of β-carotene did not account for the difference (**Figure 3.15**).

The abundance of zeaxanthin is significant with regard to the concentration of this carotenoid in the central region of the macula, and it is feasible that increased consumption of the yellow/orange fruit and vegetables can reduce the incidence of AMD associated with decreased serum concentrations of lutein and zeaxanthin (The Eye Disease Case Control Study Group, 1992 & 1993).

The leafy green vegetables had a higher concentration of lutein than the non-leafy green vegetables (**Figures 3.1, 3.2**) and other samples analysed (**Figures 3.3, 3.4**), and this difference may be attributed to the plant part consumed (and analysed) and the role that it plays in light harvesting for photosynthesis (Goodwin, 1980). The possibility of a relationship between carotenoid concentration and the photosynthetic activity of edible portions of vegetables was briefly commented on by Khachik *et al.* (1986). As leaves are the major site for photosynthesis they have a high concentration of lutein, which along with other carotenoids is essential for the protection of plants from damage during this light harvesting process (Mathis and Schenck, 1982; Koyama, 1991). The analysed portions of the leafy green vegetables were consequently expected to have the higher concentrations of the photoprotecting carotenoids, lutein and zeaxanthin.

The enclosure of the edible portion in a pod, as seen in peas and Lima beans, may reduce the requirement for photoprotective compounds in the enclosed portion. This was reflected in the lutein concentration of these vegetables, which was low compared with other green vegetables.

Lettuce was an exception to the high lutein levels found in the leafy green vegetables, with a total lutein concentration lower than all other green vegetables, sweet corn and butternut pumpkin. The lighter green colour of lettuce, when compared to the other leafy green vegetables analysed may indicate a lower concentration of photosynthetic pigments (Phillip and Young, 1995), and this is likely to reduce the requirement for radical scavenging and therefore lutein. Additionally, the reduced concentration of total lutein in lettuce when compared to the other leafy green vegetables has been partially attributed to the presence of lactucaxanthin (dihydroxy -ε,ε-carotene), an isomer of lutein (dihydroxy-β,ε-carotene) and zeaxanthin (dihydroxy-β,β-carotene) (Demmig-Adams, 1998). Lactucaxanthin is only found in a taxonomically restricted group of plants (Siefermann-Harms *et al.*, 1981; Phillip and Young, 1995) and can replace lutein in the xanthophyll cycle of light harvesting complex II of the photosynthetic pathway (Phillip and Young, 1995). However, the concentration of lactucaxanthin detected in lettuce was not great enough to account for the difference in lutein concentration between this vegetable and collard, the leafy green vegetable with the lowest concentration of lutein (**Figure 3.14**). There has been no report on the human nutritional value of lactucaxanthin, though it has been identified in samples of human blood at low concentrations (Khachik pers comm, unpublished results).

Lutein : zeaxanthin ratio

As mentioned previously the ratio of lutein to zeaxanthin decreases towards the central region of the macula, and foods that have this lower ratio are therefore important in maintaining eye health. The yellow/orange fruit and vegetables, with the exception of butternut pumpkin, were the only samples analysed that had a ratio similar to that found in the central region of the macula by Bone *et al.* (1985) (**Figure 3.6**).

A low lutein concentration in some fruit and vegetables has been previously attributed to the accumulation of α-carotene resulting in a low rate of conversion to lutein (Khachik *et al.*, 1986). Green beans had a α-carotene concentration double that of all other green vegetables and the above mechanism provides a possible explanation for the low lutein concentration found in this vegetable (**Figure 3.14**). However, the concentration of α-carotene detected does not account for the difference in lutein between green beans and the other green vegetables and so other factors must also be contributing.

E:Z isomer ratio

Carotenoids are most commonly found in nature in the *E* form, and as expected all-*E*-lutein was the major component of the total lutein concentration in all samples analysed here. In the presence of light the *E* form is isomerised to the *Z* form, and as the major points for light harvesting, leaves are most likely to undergo this form of isomerisation. In this study a high concentration of the *Z*-lutein isomers was unique to leafy green vegetables, and was attributed to the relative exposure to light.

The natural dominance of *E* isomers is changed by the heat and oxygenation during processing, resulting in an increase in the concentration of *Z* isomers (Khachik *pers. comm.*, 1999) and therefore a decrease in the *E*:*Z* ratio. The expected change in the isomer ratio was not noted in any of the samples that had undergone processing, and the *E*:*Z* ratio in canned sweet corn was the highest of all samples analysed with a substantially greater concentration of *E* isomers than *Z* isomers.

Although a decrease in the *E*:*Z* ratio was not observed in the processed foods analysed here, several *Z* isomers were found only in these foods. 13-*Z*-zeaxanthin was only detected in lasagne, egg noodles and Freekeh which are subject to crushing / milling and heat processing. Exposure to oxygen appears to have an important role in the presence of this isomer as it was absent in corn and Lima beans, which were canned. The

presence of a detectable concentration of 3'epilutein, an oxidation product of lutein, was not related to food type or processing.

In conclusion, this study supports the hypothesis that commonly consumed fruit and vegetables have varying concentrations of lutein and zeaxanthin isomers. This study of carotenoids and their related geometrical isomers in commonly consumed fruit and vegetables has important implications for the health of Western populations in addition to the basic nutritional needs of developing countries. The significance of lutein, zeaxanthin and their isomers is becoming increasingly evident in eye health, specifically in relation to AMD and its possible prevention. These are the only carotenoids found in the macular region of the eye, and though the mechanisms of uptake and metabolism in relation to accumulation of these carotenoids in the eye are unknown, a relationship between reduced plasma concentration of these isomers and an increase in the incidence of AMD has been identified (The Eye-Disease Case Control Study Group, 1992; The Eye-Disease Case Control Study Group, 1993; Seddon *et al.*, 1994; Snodderly, 1995). It is acknowledged that further work is necessary to determine the optimum intake of lutein and zeaxanthin to reduce the incidence of AMD.

Leafy green vegetables were the best source of lutein, zeaxanthin and their isomers, while sweet corn and butternut pumpkin were additional good sources of zeaxanthin isomers. Zeaxanthin concentration was not related to food type, nor were the zeaxanthin to lutein isomer ratios. A combination of carotenoid synthesis replacing chlorophyll degradation, and parallel carotenoid synthesis and chlorophyll degradation was thought to be responsible for the carotenoid concentration seen in the fruit and vegetables analysed here (**Figure 3.16**). However, it is acknowledged that the photosynthetic mechanism involved in the formation of xanthophylls is affected by genetic and environmental factors such as cultivar, location and growing season (Khachik *et al.*, 1986). Identifying sources of these natural pigments is important as they can form the basis for improved dietary recommendations. They may also serve as a starting point for further research into the role of these carotenoids in eye health.

CHAPTER 4

Effect of Lutein on the Iron Status of Chickens

Aim

To determine the effect of lutein, supplied both as a supplement at two concentrations and in a food matrix, on iron status of chickens depleted in iron and retinol.

4.1 Introduction

Previous chapters have focused on the determination of the carotenoid profile of staples, especially wheat, and fruit and vegetables. The aim of these investigations was to identify foods with the ability to improve carotenoid nutrition in human populations. Complete nutrition however, is dependent on a complex combination of nutrients, and interactions have an important role to play in achieving optimal health. One such interaction that has created considerable interest is that between vitamin A and iron. Several studies have reported a positive interaction between vitamin A and iron in both animals (Hodges *et al.*, 1978; Mejia *et al.*, 1979; Mejia and Arroyave, 1982, Mejia and Chew, 1988, Bloem *et al.*, 1989; Roodenburg *et al.*, 1994; Roodenberg *et al.*, 1996) and humans (Mohanram *et al.*, 1977; Suharno *et al.*, 1993 Shatrugna *et al.*, 1997; Muhilal *et al.*, 1998; Mwanri *et al.*, 2000). Beta-carotene supplementation has also been observed to improve non-haem iron absorption from wheat, rice and corn to a greater degree than vitamin A supplementation in a human population (Garcia-Casal *et al.*, 1998).

The effect of vitamin A and β-carotene on iron absorption was attributed to their ability to bind iron liberated during digestion and form a complex that acts as a chelating agent preventing the inhibitory effects of other food compounds (Garcia-Casal *et al.*, 1998). Given the antioxidant potential of lutein, a xanthophyll, it is possible that this compound may also enhance iron absorption.

In order to determine if there was an interaction between lutein and iron, an animal model that closely resembled the human absorption and metabolism of both iron and xanthophylls was required. The chicken was selected as a model in the following study due to its ability to absorb xanthophylls intact as in humans, and its suitability for iron repletion tests (Lee *et al.*, 1999) (discussed in **Chapter 1**). Depletion of chickens of

vitamin A or iron as a basis for further studies has been previously reported (Williams *et al.*, 1962; Davis *et al.*, 1968; Pla and Fritz, 1970; Erdman *et al.*, 1986; Poor *et al.*, 1987). However, there are variations in the age at which dietary intervention commenced, diet formulation, and in the time required to achieve depleted / marginal concentrations of vitamin A and iron. Therefore, a suitable period of depletion was determined here, prior to the commencement of the treatment diets.

4.2 Materials and Methods

4.2.1 Animal Ethics and Care

The experimental protocol for the studies in this chapter was approved by the Animal Ethics Committees of The University of Adelaide and Primary Industries and Resources of South Australia (PIRSA). Chickens were maintained at the PIRSA Pig and Poultry Production Institute at the Roseworthy Campus of Adelaide University.

4.2.2 Experimental Method

Sixty male leghorn chickens (*Gallus domesticus*) were obtained from Steggles hatchery, housed as a group and fed normal starter diet from 1 day old (arrival day) to 14 days old (14 DO). Due to the known significant effect of sex on iron and vitamin A concentrations and mobilisation (Mohannah and Nys, 1998), results from any female birds were removed from the data sets.

At 14 DO, 50 chickens were weighed, identified by leg bands and fed the depletion diet outlined in **Table 4.1** for two weeks. The remaining 10 chickens were weighed, identified by leg bands, and fed a commercial finisher diet replete in all nutrients (**Table 4.1**, with adequate vitamins and minerals in vitamin / mineral premix shown in **Appendix C, Table C3**). These 10 birds remained on the commercial diet throughout the study.

Blood samples were collected on day 0 (14 DO), day 7 (21 DO) and day 14 (28 DO) from five birds on both the depletion and commercial diets and analysed for blood iron, haemoglobin, haematocrit, carotenoids and retinol. After two weeks on the depletion diet (28 DO), birds were determined to have achieved depleted blood and tissue concentrations of iron, carotenoids and retinol.

At 28 DO, ten birds were allocated to each of the five treatment diets, based on individual body weight at the end of the depletion period (14 – 28 DO). These diets were balanced for energy and protein and all other nutrients other than vitamin A and iron (**Table 4.2**). Birds were housed in treatment groups for the three week treatment period. Blood samples were taken weekly from five birds from each treatment (**Figure 4.1**). Birds that remained on the depletion diet for a further week were culled at 35 DO.

At 48 DO all birds were blood sampled and at 49 DO all birds were euthanased and tissue samples collected for analysis.

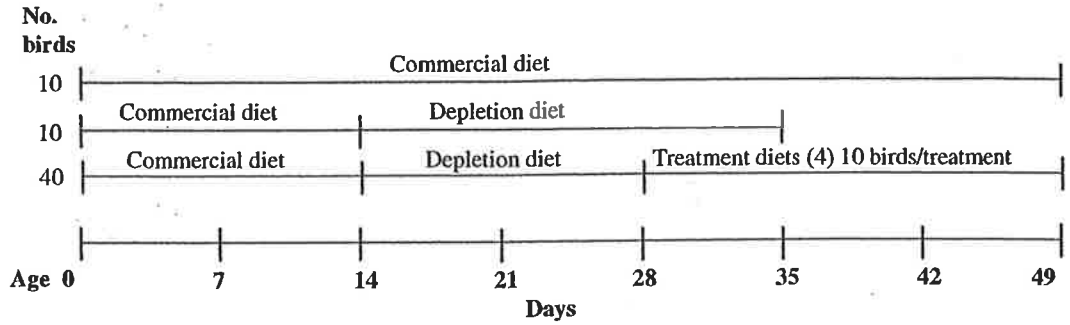


Figure 4.1 Number of birds allocated to each treatment diet and the age of birds during treatment periods.

4.2.3 Feed Components

The depletion diet (**Table 4.1**) contained all essential nutrients at adequate concentrations with the exception of vitamin A and iron. These nutrients were not added to the vitamin / mineral premix, though low concentrations of β-carotene (0.3 µg/T) and iron (5g/T) were identified in the wheat component of the diet.

Table 4.1 Components of the depletion diet and their proportions in g/kg.

Ingredient	Quantity (g/kg)	Quantity in 20kg (g)[2]
Wheat	800	16000
Casein	152	3040
Dicalcium phosphate	20	400
Calcium carbonate	11	220
DL-methionine	7	140
Vitamin / mineral premix [1]	5	100
Sodium chloride	3	60
Choline chloride (60%)	2	40

[1] see **Appendix C, Table C3** for individual diet vitamin /mineral premix composition.

[2] 20 kg amounts of feed were made up and pelletised, ensuring the ingredients were completely mixed to avoid selective feeding by the birds.

The treatment diets were developed to investigate the influence of lutein on iron bioavailability, and concentrations of these nutrients are shown in **Table 4.2**. All other nutrients were adequate and equal, and were made up of the components in **Table 4.1**. The form and formulae of the vitamins and minerals are given in **Appendix C, Table C2.**

The water used was analysed for mineral content (data not shown). Iron concentrations were negligible (<0.007 mg/l) and did not contribute substantial iron to the diet.

Table 4.2 Vitamin A, β-carotene, lutein and iron concentrations of all diets. All nutrients per kg feed.

Diet	Conditions	Vitamin A	β-carotene	Lutein	Iron
Commercial	Commercial diet	3 g [a]	-	-	50 g [d]
Depletion	Depletion diet	-	0.3 µg	-	5 mg
Beta-carotene	Vitamin A requirement present as β-carotene	-	18.2 mg [b]	-	19 mg [d]
High lutein	High supplemental lutein	225 µg [a]	-	40 mg [c]	19 mg [d]
Wheat	Wheat naturally high in lutein	225 µg [a]		18 mg	19 mg [d]
Equal lutein	Supplemental lutein equal to that in wheat treatment diet	225 µg [a]	-	18 mg [c]	19 mg [d]

[a] vitamin A present as retinol palmitate
[b] β-carotene present as *E*-β-carotene (**Sigma, Australia**)
[c] lutein present as Chromophyll 20 ™ (Bioquimex Reka, Australia) 82.5 % all-*E*-lutein 17.5 % other non-provitamin A carotenoids
[d] iron present as ferric chloride

4.2.4 Depletion Period

After two weeks on the depletion diet, plasma concentrations of carotenoids and retinol were significantly decreased and plasma retinol concentrations were at the lower end of the marginal retinol range of 0.1 - 0.2 mg/L (West *et al.*, 1992). Consequently, two weeks were determined as a suitable period of depletion before the introduction of the treatment diets.

4.2.5 Blood Collection and Analysis

Blood was collected from the wing vein directly into evacuated heparinised plastic tubes and immediately placed in iced water. One millilitre of whole blood was retained for haematocrit, haemoglobin and blood iron analysis. The remaining blood was centrifuged at 3000 rpm for 10 minutes, plasma collected and stored at -20 °C until required for retinol and carotenoid extraction and analysis (see **4.2.5 b** below).

4.2.5 a Iron Indices

Haematocrit and Haemoglobin

Haematocrit was measured manually using micro-haematocrit tubes, filled with heparinised blood via capillary action. Tubes were spun for 10 minutes at 3000 rpm in a haematocrit centrifuge. Percent packed cell volume was measured using a sliding scale haematocrit plate. Each sample was duplicated.

Haemoglobin determination was performed manually using a total haemoglobin test kit (SIGMA diagnostics Cat. No. 525-A).

Whole blood iron concentrations were analysed by inductively coupled plasma-atomic emission spectra (ICP-AES) by Waite Analytical Services (Waite Campus, Adelaide University). Details of the method are given in **Appendix A3.1**.

4.2.5 b Plasma Carotenoids

Plasma samples were thawed, and an equal volume of ethanol was added to the plasma and the sample vigorously shaken. A volume of ether equal to that of the original sample was then added and the sample again shaken vigorously. Samples were centrifuged at 3000 rpm at 4 °C for 5 minutes and the upper ether layer collected. The remaining sample was then re-extracted with ether. The combined fractions were

evaporated under a stream of high purity nitrogen and stored at -20 °C until required for HPLC analysis. Further details are presented in **Appendix A1.3.**

4.2.6 Tissue Collection and Analysis

Following euthanasia, whole liver, gastrointestinal tract, breast muscle, and abdominal fat samples were collected. The upper (ileal) and duodenal (jejunal) regions of the gastrointestinal tract were separated and washed with saline PBS pH 7.4 to remove remaining digesta. Liver and gastrointestinal tract samples were labelled, wrapped in aluminium foil and immediately placed in liquid nitrogen and stored at -80 °C. Abdominal fat and breast muscle samples were identified, placed in plastic bags on ice for storage at -20 °C.

4.2.6 a Iron Analysis

Muscle iron concentrations were analysed by ICP-AES, with method details given in **Appendix A3.2.**

Hepatic non-haem iron

Non-haem iron was analysed according to the method of Whittaker and Vanderveen, (1990). Liver samples were thawed and blotted, and 2 g was weighed into 50 ml Falcon tubes. Samples were homogenised using an ultra torax in 5 ml Milli-Q water. Following homogenisation the blender blades were rinsed with Milli-Q water and samples were made up to 15 ml with Milli-Q water. 1.5 ml was transferred to 10 ml Falcon tubes and 5 ml acid reagent was added. (Acid reagent was prepared by the combination of 250 ml each of 6 M hydrochloric acid, and 1.2 M trichloroacetic acid). Samples were vortexed and heated at 65 °C for 20 hours. Samples were removed from the oven, cooled at room temperature and centrifuged at 1500 x G for 20 minutes.

For iron analysis, 200 µl of the supernatant was transferred to a disposable test tube adding 1.8 ml of the colour reagent. Colour reagent was prepared on the day of use by combining 1 ml bathochromic reagent, 20 ml saturated sodium acetate (4.5 M) and 20 ml Milli-Q water. Bathochromic reagent was prepared by dissolving 62.5 mg bathophenanthroline sulphonate in 5 ml Milli-Q water and kept in the dark. An aliquot of 0.25 ml of thioglycolic acid was added, the solution was transferred to a 25 ml volumetric flask and made up to 25 ml with Milli-Q water.

Absorbance was read at 535 nm on a spectrophotometer against iron standards treated in the same manner, with a blank made up of 200 µl Milli-Q water and 1.8 ml colour reagent.

Whole-body haemoglobin-iron (whole-body Hb-Fe)

In order to take into account difference in body weights coinciding with different blood volume, whole-body haemoglobin-iron, as used by Shah and Belonje (1985) and Gordon and Godber (1989), was calculated using the following formula (Anderson *et al.*, 1940):

Formula 4.1 Calculation of Whole-body Haemoglobin-iron

Whole-body Hb-Fe =

Hb (mg/l) x body wt (kg) x Fe content of Hb (%) x blood volume (% of body wt)

where chicken Hb contains 0.335% (w/w) iron, and blood volume was taken as 7.5% of body weight (Scott *et al.*, 1982)

Haemoglobin regeneration efficiency (HRE)

Haemoglobin regeneration efficiency (HRE) takes into account the difference in bioavailability of iron from different diets. Even when dietary iron concentrations are equal differences in feed consumption will result in differences in uptake. HRE makes a correction for the different iron intakes (Mahoney *et al.*, 1974) and is achieved by dividing the whole-body Hb-iron gain by the amount of iron ingested according to the following equation (Mahoney and Hendricks, 1982):

Formula 4.2 Calculation of Haemoglobin Regeneration Efficiency (HRE)

$$HRE_{day\ x} = \frac{\text{whole-body Hb-Fe}_{day\ x}\ (mg) - \text{whole-body Hb-Fe}_{day\ 0}\ (mg)}{\text{Fe intake}_{day\ 0\ to\ x}\ (mg)}$$

4.2.6 b Carotenoid Analysis

Carotenoid extraction and sample thawing were performed under decreased light conditions and exposure to oxygen was minimised.

Muscle, abdominal fat and gastrointestinal tract carotenoids

Breast muscle, abdominal fat and gastrointestinal tract sections were removed from the freezer, allowed to come to room temperature and weighed. Approximately 2 g of muscle, fat and gastrointestinal samples were sonicated in 200 ml tetrahydrofuran (THF) on ice (< room temperature) for 2 hours with 10% the weight of sample of Na_2CO_3.

Hepatic carotenoids

Liver samples were removed from the freezer, allowed at thaw to room temperature, blotted and weighed. Approximately 2 g of the livers were then sonicated for 30 minutes in THF with 10% weight of sample Na_2CO_3. The liquid was collected and the remaining liver was ground with a mortar and pestle to aid extraction, before a further 30 minutes sonication. Grinding and sonication were repeated.

For all samples the collected liquid fractions were combined and filtered through a Buchner filter funnel with a No.1 Whatman filter paper. The filtrate was collected and transferred to a ball flask for evaporation. Any water in the samples was removed by partitioning the sample with equal amounts of water and CH_2Cl_2. The organic phase was collected and evaporated to dryness. Samples were transferred to a darkened glass vial using minimal amounts of injection solvents and evaporated to complete dryness under nitrogen for storage at -20 °C until required for HPLC analysis.

4.3 Results

4.3.1 Haem Iron Indices

Haemoglobin

Haemoglobin concentrations (**Figure 4.1**) decreased significantly during the period on the depletion diet, and approached depleted concentrations (<5 mg/dl) (Pla and Fritz, 1970) at 28 DO. The introduction of the treatment diets (β-carotene, high supplemental lutein, wheat high in lutein, and supplemental lutein at a concentration equal to that found in the wheat) resulted in an immediate increase in haemoglobin concentrations in all birds. At the conclusion of the experiment (48 DO) birds on the high lutein and β-carotene diets had significantly higher haemoglobin concentrations that birds on other diets, including those on the commercial diet.

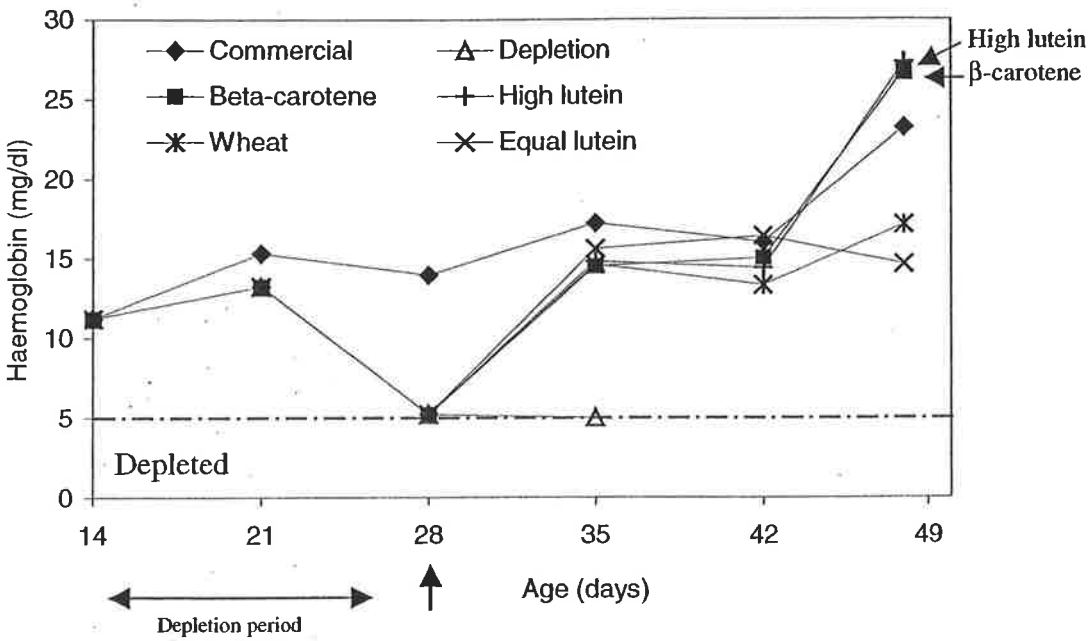


Figure 4.1 Time course of haemoglobin concentrations (mg/dl) for birds on all diets. Values are the mean for 10 birds. Error bars were omitted for clarity but standard errors were within 5 % of the mean. Birds were on a depletion diet from 14-28 DO except those on the commercial diet. The arrow on the x-axis indicates the commencement of treatment diets at 28 DO. An explanation of treatment diet conditions is given in **Table 4.2**. The area below the dashed line indicates depleted concentration (<5 mg/dl) as determined by Pla and Fritz (1970).

Whole-body haemoglobin-iron and haemoglobin regeneration efficiency

The whole-body haemoglobin-iron showed the same changes during the experimental period as haemoglobin (**Appendix C, Figure C3**). Birds on the high lutein and β-carotene diets had a higher ($p<0.001$) haemoglobin regeneration efficiency in the final week of the experiment (42-48 DO) than birds on all other diets (**Figure 4.2**).

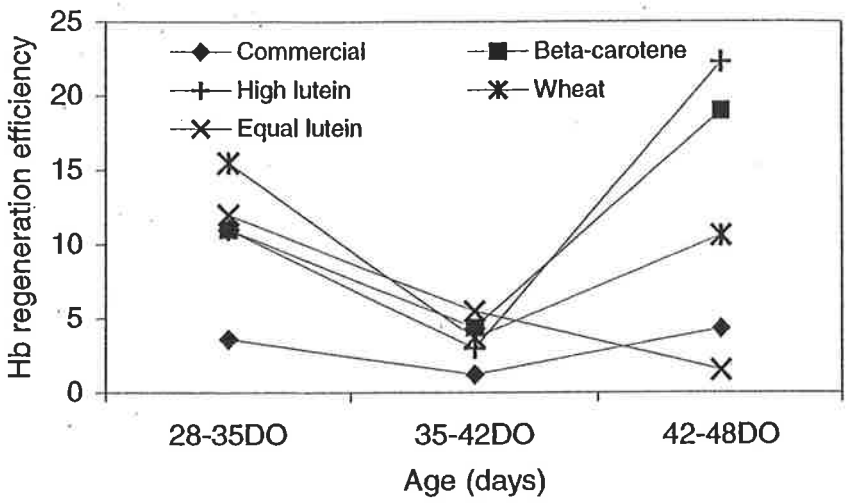


Figure 4.2 Haemoglobin regeneration efficiency in birds on all diets, calculated using **Formula 4.2**. Values are the mean for 10 birds, error bars were omitted for clarity but standard errors were within 5 % of the mean.

Haematocrit

Haematocrit (**Figure 4.3**) significantly increased after one week on the depletion diet, and decreased again (significantly) to below that of the first sampling after a second week on the depletion diet. Haematocrit at 28 DO (commencement of treatment diets) was significantly lower in birds on the depletion diet than in those on the commercial diet, and depletion concentrations (<24%) (Pla and Fritz, 1970) were approached.

At the conclusion of the experiment (48 DO) birds on the equal lutein diet had a significantly higher haematocrit percentage that birds on all other diets. Haematocrit percentages from birds on the wheat, high lutein and β-carotene diets were not significantly different; however in birds on the wheat and β-carotene diets, percentages were significantly higher than those of birds on the commercial diet.

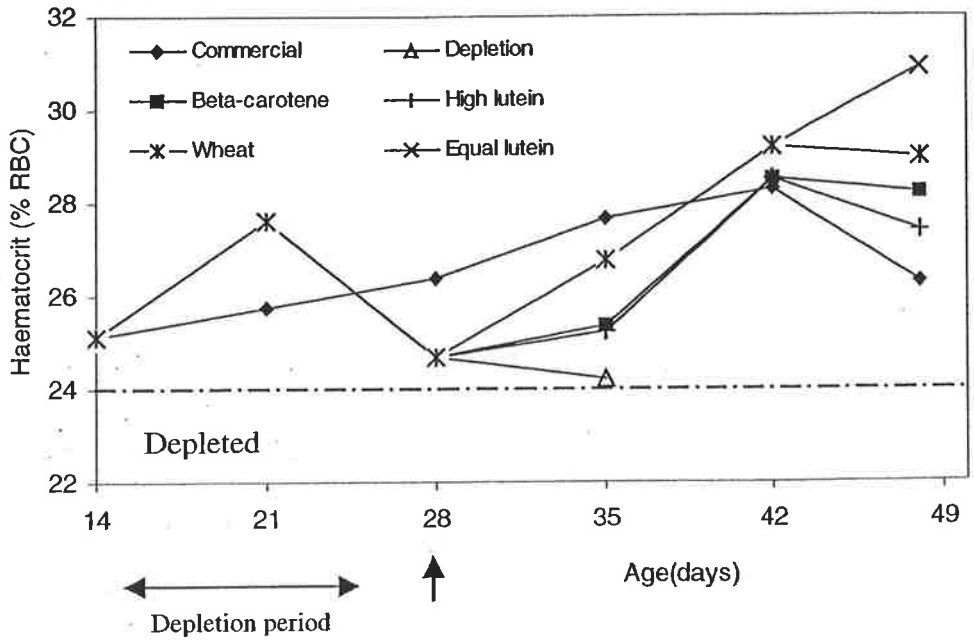


Figure 4.3 Time course of haematocrit percentage for birds on all diets. Values are the mean for 10 birds, error bars were omitted for clarity but standard errors were within 5 % of the mean. Birds were on a depletion diet from 14-28 DO except those on the commercial diet. The arrow on the x-axis indicates commencement of treatment diets at 28 DO. An explanation of diet conditions is given in **Table 4.2**. The area below the dashed line indicates depleted concentration (<24%) determined by Pla and Fritz (1970).

Blood Iron

Blood iron concentration measured by ICP-AES (**Figure 4.4**) decreased significantly during the depletion diet period (14-28 DO). Birds that remained on the depletion diet for a further week showed no further significant decrease in blood iron.

After one week on the treatment diets (35 DO), a significant increase in blood iron concentration was observed in all birds, with the exception of those on the depletion diet. At 48 DO, birds on the commercial, wheat and equal lutein diets had significantly higher blood iron concentrations than birds on the β-carotene and high lutein diets.

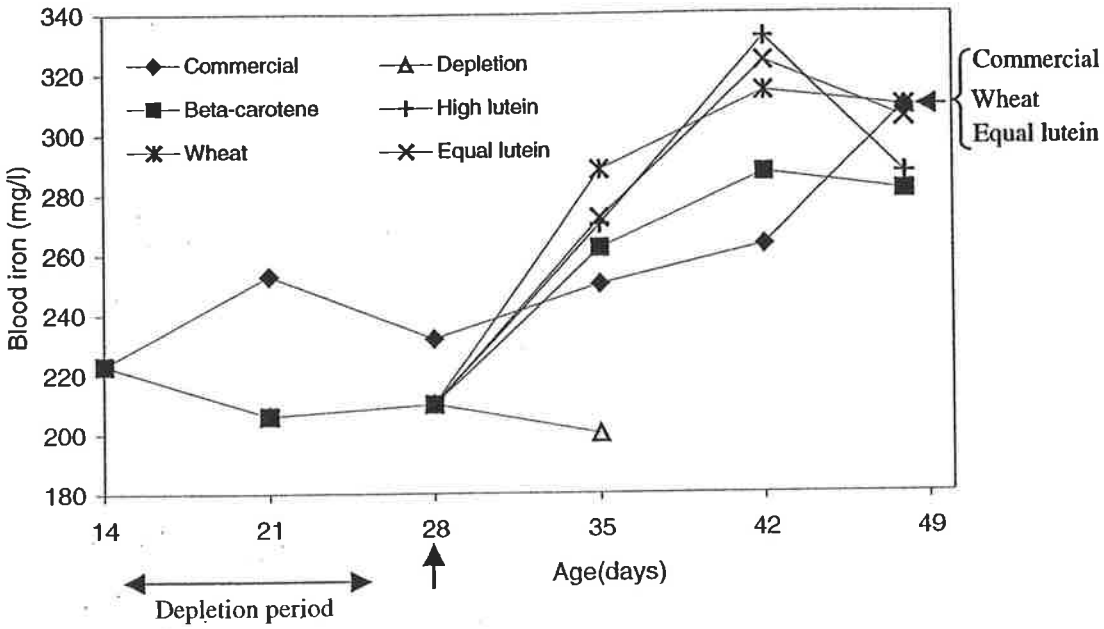


Figure 4.4 Time course of blood iron (mg/dl) concentrations measured by ICP-AES for birds on all diets. Values are the mean for 10 birds, error bars were omitted for clarity but standard errors were within 5 % of the mean. All birds were on a depletion diet from 14-28 DO except those on the commercial diet. The arrow on the x-axis indicates commencement of treatment diets at 28 DO. An explanation of treatment diet conditions is given in **Table 4.2.**

4.3.2 Tissue Iron

There was no significant difference between birds on the treatment diets for hepatic non-haem iron, muscle iron and gastrointestinal iron concentrations (**Appendix C, Table C4**).

4.3.3 Plasma Retinol

There was no significant difference between birds on the treatment diets for plasma retinol (**Figure 4.5**) at the conclusion of the study, with the exception of those on the commercial diet which were significantly higher. Birds on the treatment diets had marginal concentrations of retinol. Plasma retinol concentrations were significantly decreased by two weeks on the depletion diet. All birds, except those remaining on the depletion diet, had a significant increase in plasma retinol after one week on the treatment diets (35 DO). Birds on the high-lutein diet had a higher plasma retinol concentration ($p<0.001$) that all other birds at 35 and 42 DO, but not at the conclusion of the experiment (49 DO)

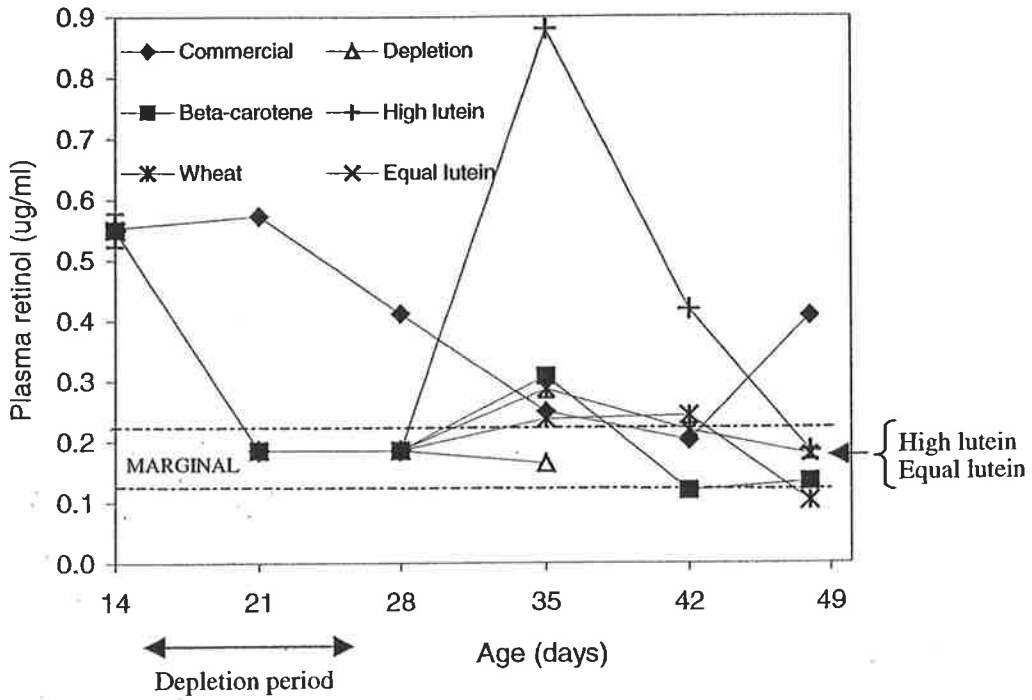


Figure 4.5 Time course of plasma retinol concentrations (ug/ml) for birds on all diets. Values are the mean for 10 birds, error bars were omitted for clarity but standard errors were within 5 % of the mean. All birds were on a depletion diet from 14-28 DO except those on the commercial diet. The arrow on the x-axis indicates commencement of treatment diets at 28 DO. Area within the dashed lines indicates the marginal range of retinol (0.2-0.1 µg/ml plasma) in chickens (West *et al.*, 1992). An explanation of the treatment conditions is given in **Table 4.2**.

4.3.4 Tissue retinol

Liver retinol concentrations (**Table 4.3**) were significantly ($p < 0.002$) higher in birds on the commercial diet than in birds on the other diets. Birds on the depletion and high-lutein diet had significantly lower hepatic retinol concentrations than birds on the other diets. Hepatic retinol concentrations of birds on the wheat and equal-lutein diets were not significantly different. Birds on all diets had significantly higher hepatic retinol concentration than those birds on the depletion diet. Retinol was undetectable in the fat and muscle samples.

Table 4.3 Retinol concentrations (μg/g) in jejunal and ileal sections of intestine, and liver, in birds on all diets and significance of difference between treatments. Values are means ± standard error.

	Jejunum	Ileum	Liver
Commercial n=8	0.041 ± 0.006 [a]	0.04 ± 0.003 [c]	4.95 ± 1 [d]
Depletion n=8	nd	nd	0.03 ± 0.001 [g]
β-carotene n=9	nd	0.016 ± 0.004 [c]	0.19 ± 0.01 [e]
High-lutein n=8	0.005 ± 0.006 [a]	0.48 ± 0.03 [c]	0.09 ± 0.01 [f]
Wheat n=10	0.02 ± 0.006 [a]	0.019 ± 0.002 [c]	0.15 ± 0.04 [e]
Equal-lutein n=8	0.36 ± 0.006 [b]	0.34 ± 0.005 [c]	0.15 ± 0.03 [e]
Significance	p<0.004	ns	p<0.002

Explanation of treatment conditions given in **Table 4.2.**

Different superscripts indicate significant differences down columns.

4.3.5 Plasma Lutein

Plasma lutein (**Figure 4.6**) was significantly reduced in birds on the depletion diet when compared to birds on the commercial diet. After one week on the treatment diets birds on diets containing supplemental lutein had significantly higher plasma lutein concentrations than birds on the other diets. The following weeks resulted in a drop in the plasma lutein concentrations in these birds. However, at the conclusion of the experiment birds on the high- and equal-lutein diets still had significantly higher plasma lutein than birds on the other diets. Birds on the diet containing β-carotene had significantly lower plasma lutein concentrations than other birds.

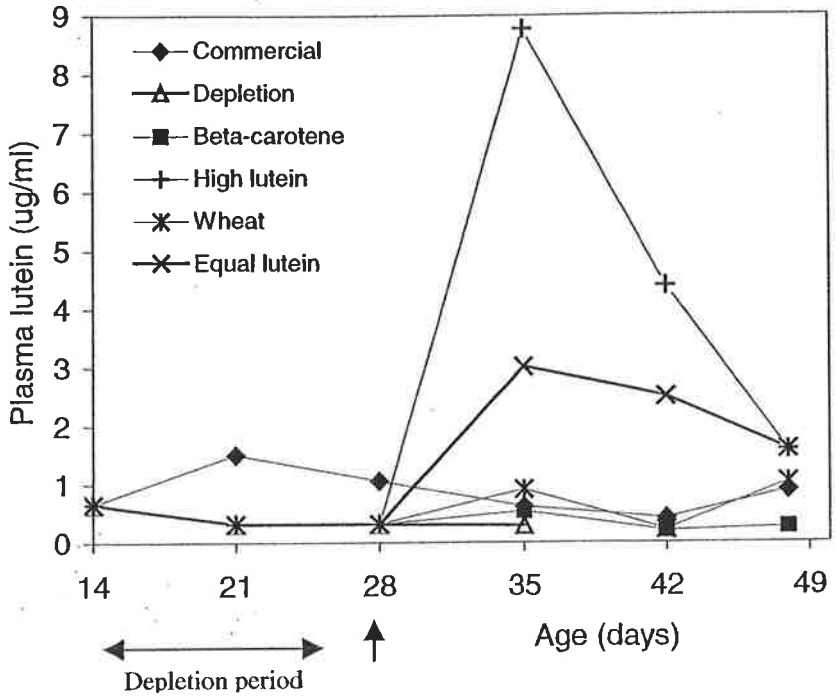


Figure 4.6 Plasma lutein concentration (ug/ml plasma) for birds on all treatments. Values are the mean for 10 birds, error bars were omitted for clarity but standard errors were within 5 % of the mean. All birds were on a depletion diet from 14-28 DO except those on the commercial diet. The arrow on the x-axis indicates commencement of treatment diets at 28 DO. Lutein concentration in birds on the high lutein treatment diet at 35 DO was 8.5 µg/ml plasma. An explanation of treatment diet conditions is given in **Table 4.2.**

4.3.6 Tissue Lutein

Birds on the high-lutein diet had significantly higher jejunal concentrations of lutein than birds on the equal-lutein and wheat diets. All other birds had undetectable concentrations. In all birds but those on the high-lutein diet, the jejunal region of the gastrointestinal tract had higher concentrations of lutein than the ileal region (**Table 4.4**). Birds on the high-lutein diet had equal concentrations of lutein in both regions of the gastrointestinal tract, and there was no significant difference between birds on the different diets for ileal lutein concentration. Birds on diets containing supplemental lutein had significantly higher hepatic lutein concentrations than birds on the other diets, including those on the commercial diet.

Only birds with lutein in their diets had detectable concentrations of this carotenoid in their adipose tissue (**Table 4.4**), which was proportional to the concentrations in the feed. Lutein was undetectable in muscle samples.

Table 4.4 Lutein concentrations (μg/g) in fat, jejunal and ileal sections of intestine, and liver, in birds on all treatment diets and significance of difference between treatments. Values are means ± standard error. An explanation of treatment diet conditions is given in **Table 4.2**. Different superscripts indicate significant difference down coloumns.

	Fat	Jejunum	Ileum	Liver
Commercial n=8	nd	0.079 ± 0.006 ^{c}	0.018 ± 0.001	0.12 ± 0.01 ^{i}
Depletion n=8	nd	0.0132 ^{g}	nd	0.001 ± 0.0001 ^{l}
β-carotene n=9	nd	0.016 ± 0.002 ^{f}	0.002 ± 0.0008	0.007 ± 0.003 ^{k}
High lutein n=8	0.009 ± 0.004 ^{b}	0.048 ± 0.003 ^{d}	0.043 ± 0.001	0.189 ± 0.02 ^{h}
Wheat n=10	0.003 ± 0.001 ^{a}	0.02 ± 0.001 ^{f}	0.007 ± 0.001	0.042 ± 0.01 ^{j}
Equal lutein n=8	0.006 ± 0.003 ^{ab}	0.036 ± 0.004 ^{e}	0.019 ± 0.001	0.2 ± 0.01 ^{h}
Significance	**p<0.001**	**p<0.008**	**ns**	**p<0.03**

4.3.7 General Observations

Only sub-clinical signs of iron and vitamin A deficiency were noted in birds at the completion of the depletion period (28 DO). After three weeks on the treatment diets birds on the high-lutein diet showed noticeable yellowing of skin on the legs and around the eyes, beak and feather follicles **(Plate No 4.1 a & b)**. Yellowing of the shank was also noted in birds on the equal lutein diet, but not in any other birds.

On dissection some birds were observed to have larger testes than other birds, however this was not restricted to any one of the diets and included the commercial diet that was replete in vitamin A and iron. Enlarged testes have been previously observed as a response to vitamin A deficiency by Nockles and Kienholz (1967).

There was no significant effect of the depletion diet (14-28 DO) on actual growth rate (weight gain / day) (**Figure 4.7 A**). However, birds remaining on the depletion diet from 28-35 DO recorded a significant decrease in actual growth rate during this period. At the conclusion of the experiment there was no significant difference in actual growth rate between the treatment diets. There was no significant difference in feed consumption between birds on any of the treatment diets and those on the commercial diet (**Figure 4.7 B**).

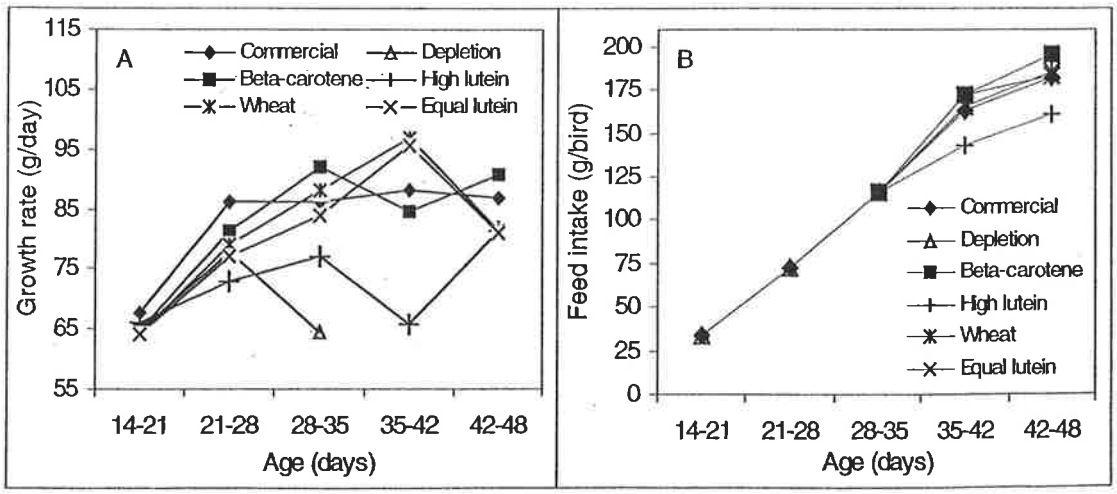


Figure 4.7 Time course of growth rate (g/bird) (**Graph A**) and feed intake (g/bird) (**Graph B**) for birds on all diets. All birds were on the depletion diet until 28DO except those on the commercial diet. Values are the means for 10 birds, error bars were omitted for clarity but standard errors were within 5 % of the mean.

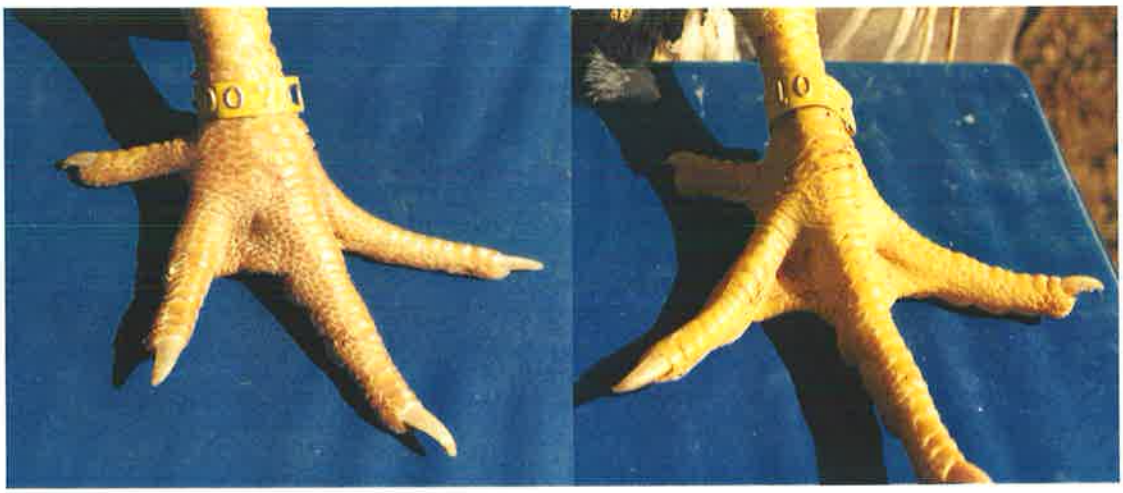


Plate 4 a Pigmentation of chicken feet from birds on commercial diet (left) and high-lutein diet (right).

Plate 4 b Pigmentation of beak, skin and feather follicles from birds on high-lutein diet.

4.4 Discussion

To understand the responses in iron indices to the diets, the established interaction between vitamin A and iron and the effects of this interaction must be taken into consideration. Therefore both iron and retinol responses will be discussed together where appropriate.

Main Study (28-48 DO)

At the conclusion of the experiment, haemoglobin concentrations in birds on the β-carotene and high-lutein diets were significantly higher than in birds on the commercial and other diets (**Figure 4.1**). The increase in haemoglobin concentration can be attributed to one of two mechanisms; either 1) an increase in iron bioavailability, or 2) remobilisation of hepatic iron stores as a response to depletion. There was no significant difference between birds on the different diets for hepatic non-haem iron (**Appendix C, Table C4**), indicating that the significantly higher concentrations were not due to remobilisation of hepatic stores as a response to iron deficiency (Mac Phail, 1998), but increased availability of dietary iron. The increase in haemoglobin concentrations with supplemental β-carotene has been previously reported, however, this is the first report to my knowledge of an increase in haemoglobin concentration with supplemental lutein. Haemoglobin regeneration efficiency (**Figure 4.2**) was also significantly higher in birds on the high lutein and β-carotene diets than in birds on all other diets at the completion of the experiment (48 DO). The significantly higher haemoglobin concentration was not reflected in the other blood iron parameters, haematocrit (**Figure 4.3**) and blood iron (**Figure 4.4**).

Birds on all treatment diets showed and increase in iron indices after on week on the treatment diets. Although this might be attributed to the minimal concentration of iron added to these diets, it could also be a homeostatic response to the previous weeks' iron depleted diet in the form of increased remobilisation of hepatic stores of iron as a response. If remobilisation were responsible for the increase in these iron indices then the increase would also be expected to appear in birds that remained on the depletion diet for a further week. The increase in iron indices was continued in the following week in heamatocrit and blood iron but not in haemoglobin that showed a plateau. The increase may be attributed to an up regulation of iron absorption as a response to the

depletion period. In the final week the haematocrit and blood iron concentration of most birds decreased or remained constant, the reason for this is unknown.

Blood iron and haematocrit determine the overall effect of iron deficiency, and indicate a general response to iron availability. However, haemoglobin more accurately indicates a response to iron availability from a diet (Henry and Miller, 1995). Haemoglobin, and in particular haemoglobin regeneration after iron depletion, has been used as the response criterion in determining iron bioavailability (Henry and Miller, 1995). Consequently, haemoglobin repletion is the method recommended by the AOAC for analysis of iron bioavailability (AOAC, 1995).

There was no significant difference between birds on the different diets for plasma retinol (**Figure 4.5**) at the conclusion of the experiment, excluding birds on the commercial diet. This lack of difference in plasma retinol after supplementation has also been observed with another xanthophyll, canthaxanthin, and β-carotene (Blakely *et al.* 1991) using the rat as the animal model. However, there are significant differences between the two studies; including diets with adequate / excess retinol (120,000 RE/kg diet) (Blakely *et al.* 1991) and the different animal model. Blakely *et al.* (1991) observed that canthaxanthin lowered liver non-haem iron concentrations, but not significantly when compared to β-carotene combined with an excess concentration of retinol. The current study did not find a significant difference in hepatic non-haem iron concentrations (**Appendic C, Table C4**). Other indicators of iron status were not reported in the study by Blakely *et al.* (1991), leaving some doubt as to the response in iron concentrations to canthaxanthin.

After one week on the experimental diets (35 DO) birds on the high-lutein diet had significantly higher plasma retinol concentrations than birds on the other diets (**Figure 4.5**). Lutein is a non-provitamin A carotenoid and therefore the noted increase in plasma retinol cannot be related to an increase in the dietary supply. The increase in plasma retinol may have resulted from a remobilisation of hepatic stores as a response to vitamin A deficiency. This is reinforced by the hepatic retinol concentrations (**Table 4.3**), which were significantly lower in birds on the high-lutein diets.

The potential for xanthophylls to be reduced to form precursors of retinol, as reported in fish most recently by Schiedt *et al.* (1985) and rats (Matsuno, 1991), may also present a

possible reason for the increase in plasma retinol concentrations without an apparent dietary relationship. This mechanism has not been confirmed in birds and to determine this was beyond the scope of this project.

Another conceivable explanation for the increase in plasma retinol concentrations is that lutein may have a similar site of storage to retinol (Tyczkowski and Hamilton, 1986), and the high concentration of lutein may have in some way displaced retinol from the liver, leading to the increased retinol concentrations in the plasma. The liver as a storage site for lutein has been confirmed (Tyczkowski and Hamilton, 1986), and the concentration of lutein in the livers of birds on the high-lutein diet was significantly higher than in birds on the commercial diet (**Table 4.4**); however not significantly higher than in birds on the equal-lutein diet, further confusing the observed response in hepatic retinol concentration. To determine if the increase in plasma retinol in birds on the high-lutein diet was due to displacement of retinol by lutein, a similar experiment could be conducted including weekly culls, allowing concurrent analysis of the liver and plasma.

To determine if the increase in haemoglobin was due to an interaction between carotenoids and iron at the site of absorption, two regions of the gastrointestinal tract were analysed. The jejunum, as the site for the absorption of both iron and xanthophylls (ie lutein) (Tyczkowski and Hamilton, 1986; Ganguly *et al.*, 1959), and the ileum as the major site for absorption of provitamin A carotenoids and retinol (Tyczkowski and Hamilton, 1986), and a minor site for iron absorption. However, analysis revealed that there was no significant difference between birds on the different diets for intestinal iron concentrations (**Appendix C, Table C4**). In fact intra-diet variation was high.

Chickens absorb xanthophylls intact (Lee *et al.*, 1999; Tyczkowski and Hamilton, 1986), while carotenes are converted to retinol in the intestinal wall and transported to the liver in this form (Ganguly *et al.*, 1959; Tyczkowski and Hamilton, 1986). Consequently, β-carotene was not detected in any samples of plasma or tissue, while birds on the high-lutein diet had a marked and significant increase in plasma lutein concentration after one week on the treatment diets (**Figure 4.6**). The increase in plasma lutein was also seen in birds on the equal-lutein diet, though not to the same extent, which can be attributed to the lower concentration of lutein in the diet. A linear

relationship between total dietary carotenoids and serum carotenoids has been previously reported (Dua *et al.*, 1967; Combs and Nicholson, 1963).

As the xanthophylls are absorbed preferentially in the jejunal region of the gastrointestinal tract (Ganguly *et al.*, 1959) the high concentration detected in this region in birds on the high-lutein diet (**Table 4.4**) was expected. Interestingly, these birds had equal concentrations of lutein in both the jejunal and ileal region, and it is reasonable to suggest that this is a result of the high dietary concentration of lutein, conceivably leading to absorption in the lower region.

After two weeks on the treatment diets birds on the β-carotene diet had plasma retinol concentrations that were marginal and significantly lower than in birds on the other diets (**Figure 4.5**). Beta-carotene was the only source of vitamin A for birds on this diet, and the concentration of β-carotene took into account the conversion factor to retinol, and was supplied at a concentration used by others to achieve adequate vitamin A concentrations (Poor *et al.*, 1987; Mayne and Parker, 1986). Degradation of β-carotene in the diet due to oxidation was discounted by analysis that revealed no appreciable loss of this vitamin A precursor. As β-carotene is converted to retinol in the intestinal wall and transported as retinol in the plasma, this carotenoid cannot have been competing with retinol for transport in the plasma.

It must be noted that birds on the β-carotene diet received adequate vitamin A from this precursor source, while birds on the high-lutein diet sourced marginal vitamin A from a different form, retinol palmitate. Additionally, the commercial diet (**Appendix C, Table C1**) was very different in composition to the treatment diets, and served only as an indicator of normal responses to an adequate concentration of nutrients and age related changes in body weight. Therefore, the higher haemoglobin concentration (**Figure 4.1**) in birds on the high lutein and β-carotene diets in comparison to birds on the commercial diet has limited significance. However, haemoglobin concentration was significantly higher than in birds on the other diets also, which more closely resembled the composition of the high lutein and β-carotene diets.

Iron deficiency anaemia caused by bleeding or dietary deficiency has been reported to lead to an increase in absorptive activity at the main sites for iron absorption, the duodenum and jejunum (Chirasiri and Izak, 1966). In the current study the amount of

blood removed during blood sampling was not at a volume sufficient to induce anaemia or affect blood iron indices. However, dietary deficiency of iron during the depletion period may have led to an increase in absorptive activity, which combined with marginal concentrations of dietary iron possibly resulted in the significant increase in blood iron indices after one week on the treatment diets. The increased absorption mechanism would be turned off when adequate iron concentrations were reached.

There was no significant difference in actual growth rate between birds on the different treatment diets at the conclusion of the experiment (**4.7A**), indicating that differences in blood and tissue nutrient concentration between birds on the different diets represent a response to the diets, rather than an effect of decreased feed consumption (**Figure 4.7B**) or a difference in body weight.

Depletion Period (14 – 28 DO)

The aimed for sub-clinical level of depletion in retinol and iron concentrations was achieved after two weeks on the depletion diet. The degree of retinol depletion and the age at which this was achieved has been previously reported, despite the prior depletion period commencing at hatching (Friedman and Sklan 1989a); the initial marked decrease in plasma retinol and the ensuing plateau has also been observed (Friedman and Sklan, 1989b). Therefore, no advantage can be gained from placing birds on the depletion diet at hatching at which they are subject to severe non-diet related effects of isolation (B. Hughes, *pers. com.*, 2000).

A significant increase in haematocrit concentration was observed after one week on the depletion diet. This was attributed to an increase in haematopoiesis (polycythaemia) as a physiological response to iron deficiency. Another potential explanation for the initial increase in haematocrit is haemoconcentration as a result of depressed water intake (Sure *et al.*, 1929; Nockles and Kienholz, 1967; Amine *et al.* 1970; Mahant and Eaton 1976; Mejia *et al.* 1979). Since water consumption was not measured in the current study this cannot be ruled out as a cause. However, as the previously reported decrease in water intake was a response to vitamin A and iron deficiency it would not be expected that birds remaining on a diet deficient in these nutrients would reacquire their thirst. Therefore, the drop in iron indices after a further week on the depletion diet in

the current study indicates that haemoconcentration due to depressed water intake was not the cause of the initial increase. Other known causes of haemoconcentration include extreme physiological stress [Fazio, 2001 #1303] not experienced by these birds; as a response to burns [Santos, 2001 #1302] which was not a factor in this study; and as a response to extreme cold temperature [Donaldson, 2001 #1305] which also was not a factor in this study, as the room was kept at a constant temperature of 25-27 °C.

While both the liver and bone marrow are stores for iron and red blood cells that can be remobilised to prevent iron deficiency, hepatic stores are more easily remobilised (Beard *et al.*, 1993). It is therefore proposed that the absence of a decrease in haemoglobin after one week on the depletion diet was due to remobilisation of hepatic iron stores as a response to a diet deficient in iron. However, analysis of the liver revealed erratic iron concentrations, with both higher and lower concentrations than normal (Saiz *et al.*, 1990) detected.

An increase in hepatic iron concentration as a response to vitamin A deficiency has been previously reported in chickens (Mejia *et al.*, 1979; Sklan *et al.*, 1986) and in rats (Mejia *et al.*, 1979; Roodenberg *et al.*, 1996). These studies found that vitamin A deficiency inhibited iron remobilisation from the liver resulting in high hepatic and low circulating iron concentrations, which concurs to some extent with the results found in birds on the depletion diet here. Further evidence for the role of vitamin A deficiency in affecting concentrations of circulating iron is provided by an increase in plasma iron with vitamin A supplementation (Mohanram *et al.*, 1977; Mejia and Arroyave, 1982; Mejia and Chew, 1988; Bloem *et al.*, 1989; Bloem *et al.*, 1990). It is therefore reasonable to suggest that both iron and vitamin A deficiency affected iron remobilisation in the current study; vitamin A deficiency inhibiting remobilisation in some birds, and iron deficiency enhancing remobilisation in others.

The significant decrease in iron indices after a second week on a diet deficient in retinol and iron observed here has been previously attributed to a depletion of hepatic iron stores (Pla and Fritz, 1970). However, the significant effect of vitamin A on iron remobilisation needs to be taken into account and it is feasible that inhibition of remobilisation also contributed to the decreased iron concentration in this study.

It is suggested that after one week on the depletion diet hepatic iron was remobilised as a response to iron deficiency, resulting in little change in the mean-cell-haemoglobin. The decreased retinol concentrations did not inhibit iron remobilisation at this point as they had not yet reached marginal concentrations. After a second week on the depletion diet, marginal retinol concentrations inhibited further iron remobilisation and, combined with remobilisation in the previous week, resulted in the erratic hepatic iron concentrations.

Despite the absence of provitamin A carotenoids and retinol in the depletion diet vitamin/mineral premix, birds remaining on this diet for a third week did not have further significantly decreased plasma concentrations of these nutrients; depleted concentrations of retinol <0.01 mg/L, (West *et al.*, 1992) were not reached. The reason for the disparity between the degree of depletion achieved here and previously, may be that the study by West *et al.* (1992) obtained depleted concentrations in chicks that were from depleted hens, and therefore had a marginal initial retinol concentration. In comparison, the current study used birds fed on nutritionally adequate diets for 14 days as a starting point, perhaps explaining the inability to achieve depleted plasma retinol concentrations. Marginal concentrations of vitamin A precursors were confirmed to be present in the wheat used as a base for the diet, which would have contributed to the plasma concentration observed. The unacceptable variability in plasma retinol concentrations found in chicks obtained from a commercial hatchery by West *et al.* (1992) was not observed in the current study, nor were the severe physical signs of vitamin A deficiency after two weeks on the depletion diet.

Haematocrit and haemoglobin were also not significantly decreased beyond the second week on the depletion diet. This may be due to a marginal buffered level of iron that is difficult to further decrease. It is also probable that the low concentration of iron in the wheat used as a base for the depletion diet contributed to the prevention of a further drop in iron indices.

The physical signs of deterioration due to vitamin A and iron deficiency, including decreased feed intake (Rechcigl *et al.*, 1962; Amine *et al.*, 1970; Davis and Sell, 1983; West *et al.*, 1992) and weight loss (Guilbert and Hart, 1935; Paul and Paul, 1946), were not seen in the current study. This indicates that the decreased concentrations of nutrients observed in the blood or plasma, depending on the parameter, were due to

marginal concentrations of nutrients in the diet rather than decreased feed consumption. Birds remaining on the depletion diet for a third week had a significant decrease in actual growth rate, agreeing with the results of Amine *et al.* (1970) who found a significant effect of a diet deficient in both vitamin A and iron on weight and feed intake in the rat.

Overall, after two weeks on the depletion diet circulating concentrations of iron and retinol were significantly decreased due to marginal concentrations of these nutrients in the diet, and apparent exhaustion of tissue stores. Hepatic iron stores were affected by depleted concentrations of both retinol and iron resulting in both remobilisation and retention and hepatic retinol concentrations were significantly decreased by the depletion diet.

In conclusion, the hypothesis that lutein could act in the same was as the established interaction between β-carotene and iron in humans and animals deficient in iron and vitamin A was confirmed in chickens in this study. Additionally, this study found that a diet containing a high concentration of supplemental lutein resulted in an increase in haemoglobin concentration, equal to that achieved with β-carotene. An increase in haemoglobin concentrations due to an interaction between dietary iron and this carotenoid found in several staple foods has the potential to decrease the incidence of iron deficiency anaemia world-wide. Further studies are necessary however to endorse the results of this preliminary study, and to confirm that the interaction is present in humans.

Chapter 5

General Discussion

The studies presented in this thesis investigated the carotenoid profile of some staple foods and the interaction of one carotenoid, lutein, with another essential nutrient, iron. Some of the main conclusions of these studies and their relevance to the health of populations in both developed and developing countries are discussed in this chapter.

5.1 Carotenoids in Staple Foods and their Role in Human Nutrition in Developing Countries

Staple foods such as wheat, rice and maize form the basis of diets in many populations, and may occasionally be the sole source of nutrition for resource-poor populations. Reliance on a single food has meant that the nutritional value of a staple food can have a dramatic influence on the health and nutrition of a population. Unfortunately, the primary staple foods consumed by impoverished people contain inadequate concentrations of many essential micronutrients (Mason and Garcia, 1993; McGuire, 1993). One such group of compounds is the carotenoids. As precursors to vitamin A, carotenes are important in the prevention of blindness and in normal immune function, and deficiency of vitamin A is evidenced by the over 100 million children affected and an equivalent number of adults affected annually (UNICEF, 2000). Xanthophylls are also important in the maintenance of visual acuity and as antioxidants.

Wheat is a staple to billions of people world-wide (FAO/WHO, 1991) and an increase in the carotenoid content of this grain therefore has the potential to improve the carotenoid intake of these people. As carotenoids contribute many of the red, orange and yellow colours in nature it was likely that a relationship between colour and carotenoids would be present in wheat. The relationship between b* and total yellow pigment (extracted by the American Association of Cereal Chemists method) has been previously reported (Mares *et al.*, 1997), however, the relationship between specific carotenoids and colour measured objectively was reported here for the first time. A strong correlation between yellow colour reported as the b* value of the CIE - L*a*b*

measurement and lutein concentration was determined in wheat obtained from an international wheat breeding facility in Mexico (CIMMYT) (**Chapter 2.1**), and was strongest in the highly pigmented durum wheat group. Other colour measures, L* and a* had little relationship with lutein concentration, in contrast to several studies in different foods where a* was determined to be the colour parameter most closely correlated with total carotenoid concentration (Shewfelt *et al.*, 1988; Ramakrishnan and Francis, 1973). Lutein is a non-provitamin A carotenoid and increased consumption of foods with high concentrations of this compound may have nutritional benefits from its role as an antioxidant, in maintenance of the macula region of the eye, and potentially in improving iron nutrition as investigated in **Chapter 4**.

The identified strong correlation between lutein and b* can be used as a fast and reliable method to select wheat varieties with higher concentrations of lutein. The concentration of lutein may then be further increased by crossing of these varieties in conventional breeding programs (Graham *et al.*, 1999), although environmental conditions are known to affect carotenoid concentration of the grain (Shuey, 1976). Although the lutein esters were not quantified here, investigations into the role of these commonly formed compounds in human health and their possible interaction with other nutrients is needed to determine their efficacy when compared to free lutein.

The overall correlation between b* value and β-carotene concentration was strong in wheat cultivars surveyed only after the durum wheat group was removed from the data set. This indicates that b* is a good indicator of both lutein and β-carotene concentrations in bread wheat varieties only. The β-carotene concentrations detected here and previously (Rosser, 1997) are not high enough to suggest that current wheat varieties can impact on nutritional blindness (xerophthalmia) caused by vitamin A deficiency. However, the number of wheat varieties surveyed here represents less than 0.1% of the available gene-bank. Additionally, varieties that have not been cultivated outside isolated geographic regions are yet to be explored for their carotenoid concentration. Investigation into this untapped resource may reveal natural variation for high carotenoid wheat varieties that can be introduced into breeding programs.

5.2 Sources of Carotenoids and their Role in Nutrition in Developed Countries

5.2.1 Managing the paradox between health and consumer appeal in bread wheat

The demand by millers for wheat varieties that mill to give white flour has resulted in plant breeders selecting against strong yellow pigmentation in bread wheats. However, an increased focus on the nutritional value of foods consumed in developed countries may lead to a better balance between nutrition and consumer appeal. This focus has resulted in the recent availability of nutritionally enhanced foods; for example, eggs with enhanced levels of omega-3-fatty acids, and a margarine containing cholesterol lowering plant sterols. These products command a premium in the market and demand often outweighs supply, indicating the popularity of products with perceived nutritional benefits.

The current consumer climate where health is a prominent motivation for product selection would be excellent to release yellow bread produced from the highly pigmented wheat, Krichauff. This variety has high concentrations of lutein in comparison with more commonly consumed bread wheat varieties, and the colour and carotenoid content can be conserved by storage at 5 °C (**Chapter 2.2**). The well reported nutritional advantages of consuming a diet rich in the carotenoids that cause this unique colour could be the focus of a marketing campaign, informing consumers of the potential benefits of such a product.

The levels of lutein in Krichauff are an indirect result of attempts to improve agronomic qualities of bread wheat varieties, and so are not subject to the current concern in relation to genetically modified foods. Additionally, because of the popularity of this variety with farmers, the possibility of consumer demand outweighing product supply is unlikely, ensuring a consistency in quality and supply to millers and bakers and therefore consumers. If the consumer appeal for yellow coloured bakery products cannot be raised, then pigmented wheat varieties will continue to be a negative factor for the milling/baking industry. However, results from **Chapter 2.2** show that the strength of colour can be reduced to acceptable levels.

A significantly greater reduction in both colour and lutein was found in wheat stored as flour rather than grain, and this can be attributed to the effects of oxidation on carotenoids when isolated from the protein complex by milling. The reduction in colour with storage provides a possible method for improving the usefulness of bread wheat with high b* values at harvest (such as Krichauff), and therefore has important implications for the Australian grain industry. Unfortunately, a reduction in carotenoid concentration is associated with a decrease in yellow colour in wheat, diminishing the potential health benefits of highly pigmented varieties.

5.2.2 Food sources of lutein and zeaxanthin and their role in age related macular degeneration

The role of carotenoids in sight extends beyond that of a precursor to retinol, essential for vision. Investigations into the role of non-provitamin A carotenoids, including lutein and zeaxanthin, have shown these compounds to also have important physiological functions in eye health, specifically prevention of age related macular degeneration (AMD) (Seddon *et al.*, 1994), which currently has no cure.

The yellow pigment in the macular region of the eye was preliminarily identified as a xanthophyll by Wald (1945), and subsequent studies (Bone *et al.*, 1985; Bone *et al.*, 1988; Handleman *et al.*, 1988) confirmed that the pigments were indeed lutein and zeaxanthin. These carotenoids are involved in the protection of this region of the eye from blue light damage. Further, the importance of these hydroxy carotenoids in prevention of AMD was illustrated by two studies that showed that low plasma concentrations of carotenoids indicate an increased risk of cataract (Yeum *et al.*, 1995) and AMD (The Eye-Diseases Case Control Study Group, 1992 & 1993; Seddon *et al.*, 1994; Snodderly, 1995).

Carotenoids cannot be synthesised *in vivo* and so these essential pigments must be obtained from the diet. Supplementation with lutein and zeaxanthin has been shown to increase the macular concentrations of these carotenoids (Hammond *et al.*, 1997b), as has consumption of a diet rich in fruit and vegetables containing high concentrations of lutein and zeaxanthin (Seddon *et al.*, 1994). However, the study by Seddon *et al.* (1994) did not differentiate between the types of fruit and vegetables consumed and the value of individual foods was not reported.

Several previous studies have identified the concentrations of carotenoids in fruit and vegetables without separately quantifying the levels of lutein and zeaxanthin. This has resulted from their structural similarities making separation for quantification difficult; this is to my knowledge the first study to report separately the levels of these xanthophylls and their isomers in several fruit and vegetables. Lutein is more abundant than zeaxanthin in food and this is reflected in the blood, though not in the macula region of the eye where there is an equal concentration of both lutein and zeaxanthin. Degeneration of this region of the eye has been related to low plasma concentrations of lutein and zeaxanthin. Spinach, parsley, corn and butternut pumpkin were all identified as having relatively high concentrations of zeaxanthin (**Chapter 3**). The yellow/orange fruit and vegetables had lutein to zeaxanthin ratios closest to that found in the macula. The wheat and pasta products contained low concentrations of lutein and zeaxanthin when compared to the fruit and vegetables. However, the role of wheat in improving carotenoid intake in populations where this grain represents a large proportion of the daily food intake is still important.

5.3 The Role of Carotenoids in Reducing Iron Deficiency Anaemia

The established interaction between vitamin A and iron (Mejia and Chew, 1988; Roodenburg *et al.*, 2000) and subsequently β-carotene and iron (Garcia-Casal *et al.*, 1998) in both animal and human populations was the base for the study into the role of lutein on iron indices (**Chapter 4**). That both retinol and β-carotene have similar effects on iron uptake indicates that the chemical form does not restrict this mechanism. However, the conversion of β-carotene to retinol would inevitably occur.

Supplementary lutein fed for three weeks was found to increase haemoglobin concentration in chickens, equivalent to that achieved with β-carotene as a source of retinol. However, quantitative studies are essential to determine the effect in human systems. The form and concentration of lutein used to achieve the response in haemoglobin here would not be readily available from an average diet in either developing or developed countries, however it is possible that a lower concentration could also induce this effect over a longer period of time. The minimal concentration and the time required to achieve such a response needs to be further refined.

Lutein in a food complex, as the form most readily available to populations, was represented by wheat in this study, and resulted in no enhancement of haemoglobin concentration. It is possible that the food complex reduces the availability of lutein and therefore the ability to enhance iron levels, perhaps by reducing the direct contact between these nutrients. The concentration of lutein in the wheat was substantially lower than in the supplemental lutein diet that produced the increase in haemoglobin concentration.

It is therefore most likely that the effect of lutein on iron is concentration dependent. The diet containing supplemental lutein at concentrations equal to that found in wheat still failed to achieve an increase in haemoglobin concentration. The differences haemoglobin concentrations in response to lutein may also be related to the period of supplementation; the lower concentrations of lutein may require a longer feeding period to achieve the increase in haemoglobin. If verified, the lutein-iron interaction will be important in reducing the incidence of iron deficiency anaemia and associated illnesses in over 3 billion people worldwide.

5.4 Considerations of Nutritionally Enhanced Staple Foods

The development of nutritionally enhanced staple foods can help to reduce reliance on supplementation programs as a solution to nutritional deficiencies (Combs *et* al., 1996; Graham, 1984; Graham and Welch, 1996; Welch and Graham, 1999). These programs are restricted in their ability to reach remote populations and by their reliance on funding to continue the intervention, resulting in inconsistent supplementation (WHO, 1992). Another restriction of supplementation programs is that they generally address deficiencies of single nutrients only, while nutritionally enhanced staple foods have the capacity to deliver several nutrients as well as caloric requirements (FAO/WHO, 1991).

The use of enhanced staple foods to prevent nutritional deficiencies must take into account agronomic and social factors that affect food acceptance and therefore consumption. These factors are important in maximising the adoption of high nutrient staples and are best addressed in the country where the crop will be produced and consumed. The established relationship between colour and carotenoids (**Chapter 2**) can contribute significantly to the identification of varieties with high concentrations

of carotenoids. This can be used in self-help breeding programs in areas where wheat represents a major proportion of daily caloric intake. However, education is essential to remove the association between strong pigmentation and poor quality, and to encourage populations to improve their nutrition using food based solutions. By encouraging the combination of food diversity with nutritionally enhanced staple foods the reliance on supplementation programs can be reduced leading to a sustainable improvement in health.

Fruit and vegetables are the base for many diets worldwide. The possible role of lutein and zeaxanthin in the prevention of AMD highlights the importance of determining the concentration of these carotenoids in commonly consumed foods (**Chapter 3**). Since lutein is prolific in green plants due to its association with photoprotection, foods high in this carotenoid should be readily available to people in both developing and developed countries. However, in developing countries the majority of green plant material available is present as grass and inedible leaves. Further studies are necessary to identify sources of lutein in arid areas where fruit and vegetables are unavailable. In developing countries fruit and vegetables with high concentrations of lutein and zeaxanthin are generally abundant though AMD continues to be reported. This may be attributed to a combination of low consumption of the fruit and vegetables related to poverty and poor education, and an increase in average age of the worlds population.

Enhanced concentrations of nutrients in staple foods are of no value to nutritionally deficient populations unless they are available for absorption and metabolism. Plant based nutrients are subject to endogenous and exogenous interactions that can increase or decrease their bioavailability. For example, carotenoids are fat-soluble compounds and it is therefore essential that some form of fat is present in the diet to enable absorption, either from the same food or from another source. Therefore, increasing the carotenoid content of any staple food without addressing the need for fat in the diet will result in little benefit from the enhanced concentrations. The majority of fat in grains is located in the embryo, which is removed during milling. Education of people about the nutritional benefits of whole-grains could result in increased consumption of essential fats, at the same time an increasing the intake of other nutrients removed during milling such as zinc and iron. The lack of fat to

facilitate the absorption of fat-soluble vitamins is not generally a problem in developed countries where over consumption of fat is more common. However, the assumption that all fat is detrimental to health is common, and weight reduction diets need to ensure a proportion of fat is consumed in order to ensure uptake of these essential nutrients.

Plant compounds such as phytates and polyphenols negatively interact with nutrients such as zinc and iron inhibiting their uptake from staple foods (Brune *et al.*, 1989); . Grains are particularly high in phytates, however, the aleurone contains the majority of this nutrient with little present in the endosperm which remains after milling (O'Dell *et al.*, 1972). Vitamin A and β-carotene (Layrisse *et al.*, 1997; Layrisse *et al.*, 1998) have been shown to ameliorate to some extent the effect of both phytates and polyphenols, further indicating the important role that these plant compounds have in human nutrition in increasing the availability of not only iron but other nutrients also.

Although the Retinol Activity Equivalent is a measure of the provitamin A value of a food, 100 % conversion of precursors to the active vitamin A precursors does not occur. Additionally, the bioavailability of precursors from the food matrix has important implications in the amount actually available to the consumer. Numerous studies have been undertaken to identify the bioavailability of provitamin A carotenoids from foods (de Pee *et al.*, 1998; Khan *et al.*, 1998; Tang *et al.*, 1999; van Lieshout *et al.*, 2001; van het Hof *et al.*, 1999;van het Hof *et al.*, 2000). These studies have shown that the bioavailability varies considerably between foods, depending on numerous factors.

As noted previously, exogenous interactions are also important in the bioavailability of nutrients, and the capacity of a food, including nutritionally enhanced staples, to deliver nutrients can be further enhanced by the consumption of foods that have synergistic effects on nutrient availability. Education as to food combinations that can increase the availability of nutrients is essential. However, the local and seasonal availability of foods need to be taken into account when making such recommendations.

Although nutritionally enhanced staple foods have the potential to replace the need for supplementation they can never replace a balanced diet with wide food variety, as the

unidentified nutritional benefit of food compounds may have essential roles to play in optimum health and nutrition. However, improved staple foods can be used to help combat the debilitating effects of nutritional deficiencies in populations where a wide range of food types are not readily available, and to reduce the reliance on supplementation programs.

5.5 Future Directions

- The Minolta Chroma Meter value b* was identified as a useful tool for selecting high carotenoid wheat varieties and this can be used fast screening of germplasms. Selected varieties can then be used in breeding programs to increase the concentration of carotenoids in this staple food.

- It would be interesting to compare the carotenoid concentration of the individual fruit and vegetables throughout the maturation process to determine if the carotenoid concentration at maturity was related in any way to the concentration throughout development. Colour could also be related to carotenoid concentration and compared with the observations of Hornero-Mendez and Minguez-Mosquera (2000) and Rosser (1997). Rosser (1997) compared wheat varieties for carotenoid concentration throughout grain development, determining that there were two peaks in carotenoid accumulation prior to maturity.

- The identified interaction between lutein and iron needs further qualification to determine the importance in human nutrition. The role of the time period required to record an effect of lutein on iron concentrations and the interaction with the concentration effect of this carotenoid need to be determined, and may reveal that current concentrations in some staple foods are adequate to initiate this mechanism. The possible reduction of lutein to β-carotene as noted in rats by (Matsuno, 1991) may have an important effect on the interaction between lutein and iron. Further studies into the interaction between iron, carotenoids, zinc and inulin, a non-digestible polysaccharide, are proposed.

APPENDIX A: Methods

A1 Extraction of Carotenoids from Biological Samples

Biological samples were extracted in the following manner (Kahchik *pers. comm*, 1999) with amendments included in the relevant chapters.

A1.1 Grain

Five grams of each wheat sample was ground in an IKA analytical mill for 60 seconds, weighed and extracted for 1 hour in 20 ml tetrahydrofuran (THF) and 10% of the sample weight sodium carbonate (Na_2CO_3). Samples were partitioned into equal parts of water and CH_2Cl_2 ~ 20 ml of each, to remove any water. Approximately 5 ml of an aqueous solution of 10% sodium chloride ($NaCl_{aq}$) was added to prevent the formation of an emulsion. Any remaining water in the collected lower organic phase was removed by the addition of ~ 2 g Na_2SO_4 (anhydrous) followed by vacuum filtration through a No.1 Whatman filter paper. The filtrate was evaporated *in vacuo* and the residue was transferred to a darkened glass vial through a 0.45 µm solvent resistant filter. This involved the use of enough injection solvent (CH_3ON 40%: MeOH 20%: CH_2Cl_2 20%: Hexane 20% + 0.1% amine (DIPEA)(N,N-Diisopropylethylamine ($[(CH_3)_2CH]_2NC_2H_5$) to ensure all of the sample was transferred. The sample was evaporated to dryness under a stream of high purity nitrogen, and stored at -20^{0}C until required for HPLC analysis.

A1.2 Fruit and Vegetables

Fruit and vegetables were diced, weighed and homogenised for 1 hour (Omni Mixer Homogeniser, Omni International) with 5% Na_2CO_3 and 20% celite to aid filtration and enough tetrahydrofuran (THF) to cover the sample by at least 1cm. Pasta products and wheat samples were ground to a fine powder and homogenised as for the fruit and vegetables.

The homogenate was filtered under vacuum via a Buchner filter funnel with a Whatman 15cm filter paper (Number 1), taking care that none of the sample remained on the homogeniser blades or in the homogeniser jar. The homogenised sample was washed with THF to ensure all of the carotenoids were removed from the remaining celite and fruit complexes, and vacuum filtration was allowed to continue until no further filtrate

could be extracted. The filtrate was then transferred to a ball flask with minimal THF and evaporated to dryness on a rotary evaporator. The evaporated filtrate was transferred from the ball flask to a partitioning flask with dichloromethane (CH_2Cl_2) + amine (N,N-Diisopropylethylamine ($[(CH_3)_2CH]_2NC_2H_5$)) and minimal THF where necessary. Any water–soluble substances were transferred from the ball flask to the partitioning flask with minimal H_2O.

The partitioning flask contained 5% NaCl solution (300ml H2O + 15g NaCl) and ~200ml CH_2Cl_2 + amine (N,N-Diisopropylethylamine ($[(CH_3)_2CH]_2NC_2H_5$)). NaCl was used to break the emulsion and enable 2 clear layers to form (water-soluble layer and organic solvent soluble layer) so that the carotenoid-containing layer (organic solvent soluble) could be collected.

The partitioning flask was stoppered and removed from the holder, inverted and gas build up released immediately. The flask was then shaken gently and gas released, the shaking was increased in vigor with gas releases between until no further gas was detected. The flask was then returned to the holder and the stopper immediately removed and washed with CH_2Cl_2. If two layers did not form then more NaCl (aqueous) was added and a little alcohol if necessary.

While waiting for partitioning to complete a beaker was prepared with ~65g Na_2SO_4 (anhydrous) mashing any lumps to maximise surface area for absorption of water in the collected organic soluble layer.

Following collection of the organic layer, H_2O equal to the amount of organic solvent remaining and minimal CH_2Cl_2 were added and the flask was gently swirled, not inverted, being careful not to disturb the lower layers too much. More of the carotenoids partitioned into this layer and were drained off into the beaker containing the previous partitioned layer. The remaining filtrate in the partitioning flask, which was colourless and contained water-soluble plant extracts, was discarded.

The partitioned fraction was stirred vigorously with a spatula to ensure maximal Na_2SO_4 (anhydrous) contact with water. H_2O will remain on the surface of the solution and therefore will not come in contact with the Na_2SO_4 if not stirred. If more water is present than the Na_2SO_4 is able to absorb, the sample may be filtered, re-separated and Na_2SO_4 (anhydrous) again added. To minimise time taken for this process, delay

addition of Na_2SO_4 (anhydrous) until the whole of the organic layer has been collected when the decision can be made as to re-separation.

The solution was then filtered through a Buchner enamel filter funnel, through a filter paper, using CH_2Cl_2 to wash the beaker. The funnel was removed from the vacuum when no further filtrate was flowing through, and transferred to a 500ml ball flask with CH_2Cl_2 for rotary evaporation. On completion of rotary evaporation the sample was transferred to a darkened glass vial using minimal CH_2Cl_2 dried under a stream of high purity nitrogen, and stored at -20^0C until required for HPLC analysis.

A1.3 Blood and Tissue Samples

(a) Blood

Blood was collected into 5 ml heparinised vacu-tubes, inverted to ensure complete mixing of the heparin with the blood sample and stored on ice in the dark. The production of oxides by heparin/EDTA does not affect carotenoids. Samples were centrifuged at 3000 rpm for 10 minutes at 0 °C. The upper pale yellow plasma fraction was pippetted into labeled 2 ml tubes and placed on ice for transportation, while the lower red layer containing the RBC was discarded.

Plasma volume was measured and an equal amount of ethanol was added. An amount of ether equal to the amount of plasma was added and the mixture shaken vigorously. The sample was centrifuged at 3000 rpm for 5 minutes and the supernatant pippetted into a darkened glass vial. The protein pellet that formed at the bottom of the centrifuge tube was extracted twice more with ether, centrifuged and the supernatant collected. At each extraction the pellet was broken up to ensure complete extraction of the carotenoids. Re-extraction of the pellet with ethanol is not recommended as the pellet will become hard making further extraction of the carotenoids difficult. THF and hexane are not recommended as an alternative to ether as they crystalise some carotenoids. The supernatant was dried under a stream of high purity nitrogen and the samples stored at -20 °C until required for HPLC analysis.

(b) Tissue Samples

Tissues were placed in either liquid nitrogen or on ice immediately following collection and stored at -20 °C. For extraction of carotenoids the samples were brought to room temperature. Tissue samples were sonicated with THF.

A2 Analysis of Carotenoids in Biological Samples

A2.1 High Performance Liquid Chromatography (HPLC)

Extracted samples were removed from -20 °C and allowed to come to room temperature before injection onto the HPLC. A 10ml leuger lock syringe was prepared by attaching a 25mm 0.45 μm GHD Acrodisc filter and adding 2-3ml of HPLC injection solvent. The sample was transferred to the syringe using a Pasteur pippette with 200 μl injection solvent, and the sample filtered. An aliquot of this solution was transferred with a Pasteur pipette to an HPLC auto sampler injection vial and capped. The sample was then analysed by reverse phase HPLC.

A3 Elemental Analysis

A3.1 Blood

Blood volume collected was measured and recorded. Four millilitres of nitric acid was added and the sample left overnight. The following day the samples were heated for 25 minutes at 80 °C, 30 minutes at 100 °C, 90 minutes at 120 °C, 60 minutes at 125 °C, 60 minutes at 135 °C and at 141 °C until 0.5 ml of the sample remained. Samples were cooled and diluted to 25 ml with 1% nitric acid. Analysis of iron, zinc, calcium, magnesium, sodium, potassium and phosphorus, was by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (ARL 3580 B) with plasma in radial mode using liquid nebulisation.

A3.2 Liver and Muscle

Liver and muscle samples were weighed and recorded. 6 ml of nitric acid was added and the sample left overnight. Th samples were then heated at 80 °C for 25 minutes, 100 °C for 20 minutes, 125 °C for 90 minutes and then at 140 °C until 1ml of the samples remained. The remaining solution was further digested in 10 ml of nitric acid and 1 ml of perchloric acid at 80 °C for 20 minutes, 100 °C for 20 minutes, 120 °C for

20 minutes, 130 °C for 60 minutes, 140 °C for 30 minutes, 150 °C for 50 minutes, 170 °C for 40 minutes, 180 °C for 15 minutes and 225 °C for 7 minutes.

Samples were cooled and diluted to 25 ml with 1% nitric acid. Analysis of iron, zinc, calcium, magnesium, sodium, potassium and phosphorus, was by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (ARL 3580 B) with plasma in radial mode using liquid nebulisation.

APPENDIX B: Carotenoid Colour Correlations

Table B1 Summary of the Linear Regression and Pearson Correlations between lutein and L*, a*, b*, for the whole-wheat fraction comparing samples selected for HPLC analysis.

Parameter	29th IDSN	CBME1SY	ISO-Heat	ISO-Seri	Lukas	J731BI	J731BII	J731A	Overall
L*									
Linear Regression	0.36	0.35	0.25	0.41	0.78	0.32	0.007	0.07	0.008
Correlation Coefficient	- 0.59	- 0.59	0.5	- 0.64	- 0.88	- 0.56	0.08	- 0.27	0.09
a*									
Linear Regression	0.35	0.28	0.008	0.29	0.75	0.56	0.5	0.24	0.002
Correlation Coefficient	0.59	0.53	0.09	0.54	0.87	0.75	0.7	0.5	0.04
b*									
Linear Regression	0.89	0.81	0.62	0.59	0.7	0.89	0.75	0.74	0.39
Correlation Coefficient	0.95	0.89	0.79	0.77	0.84	0.94	0.87	0.86	0.62

Table B2 Summary of the Linear Regression and Pearson Correlations between α-carotene and L*, a*, b*, for the whole-wheat fraction comparing samples selected for HPLC analysis.

Parameter	29th IDSN	CBME1SY	ISO-Heat	ISO-Seri	Lukas	J731BI	J731BII	J731A	Overall
L*									
Linear Regression	0.07	0.82	0.12	0.83	0.05	0.25	0.009	0.05	0.22
Correlation Coefficient	0.26	0.91	0.35	0.83	0.22	0.5	0.03	0.22	0.47
a*									
Linear Regression	0.01	0.79	0.5	0.75	0.05	0.43	0.54	0.26	0.29
Correlation Coefficient	0.1	0.89	0.7	0.87	0.22	0.65	0.74	0.5	0.53
b*									
Linear Regression	0.17	0.58	0.45	0.66	0.1	0.67	0.64	0.47	0.002
Correlation Coefficient	0.14	0.76	0.67	0.81	0.31	0.82	0.8	0.69	0.046

Table B3 Summary of the Linear Regression and Pearson Correlations β-carotene and L*, a*, b*, for the whole-wheat fraction comparing samples selected for HPLC analysis.

Parameter	29th IDSN	CBME1SY	ISO-Heat	ISO-Seri	Lukas	J731BI	J731BII	J731A	Overall
L*									
Linear Regression	0.04	0.91	0.08	0.81	0.03	0.06	0.31	0.27	0.22
Correlation Coefficient	0.2	0.95	0.28	0.9	0.17	0.24	0.56	0.51	0.47
a*									
Linear Regression	0.03	0.88	0.009	0.77	0.06	0.41	0.75	0.49	0.29
Correlation Coefficient	0.17	0.94	0.09	0.88	0.25	0.64	0.87	0.7	0.54
b*									
Linear Regression	0.14	0.52	0.66	0.9	0.28	0.86	0.9	0.84	0.002
Correlation Coefficient	0.37	0.72	0.8	0.95	0.53	0.93	0.95	0.92	0.045

Table B4 Regression equations between b* and lutein, alpha-carotene and beta-carotene for all wheat groups.

Wheat Group	Equation Sqrt (Lutein)	Equation Sqrt (alpha-carotene)	Equation Sqrt (beta-carotene)
29th IDSN	-1.42 + 0.13 b*	-0.12 + 0.005 b*	-0.04 + 0.015 b*
CBME1SY	-0.15 + 0.035 b*	-0.12 – 0.001 b*	-0.04 + 0.013 b*
ISO-Heat	-0.15 + 0.038 b*	-0.12 – 0.013 b*	-0.04 + 0.015 b*
ISO-Seri	-0.15 + 0.056 b*	-0.12 + 0.019 b*	-0.04 + 0.015 b*
J731A	-0.15 + 0.04 b*	-0.12 – 0.002 b*	-0.04 + 0.19 b*
J731BI	-0.15 + 0.05 b*	-0.12 – 0.004 b*	-0.04 + 0.015 b*
J731BII	-0.15 + 0.03 b*	-0.12 + 0.009 b*	-0.04 + 0.015 b*
Lukas	-0.15 + 0.04 b*	-0.12 + 0.016 b*	-0.04 + 0.018 b*

Table B5 Correlations between b* and lutein in all wheat varieties for grain stored at 0 °C, 5 °C and 35 °C, for 0 or 6 months.

Variety	Linerar regression	Correlation coefficient
Spear	0.9869	0.993
Worrakatta	0.983	0.992
Krichauff	0.812	0.901
Tamaroi	0.999	0.999

Table B6 Correlation between b* and β-carotene in all wheat varieties for grain stored at 0 °C, 5 °C and 35 °C, for 0 or 6 months.

Variety	Linerar regression	Correlation coefficient
Spear	0.971	0.986
Worrakatta	0.645	0.803
Krichauff	0.953	0.976
Tamaroi	0.785	0.886

Table B7 Correlations between b* and lutein in all wheat varieties for flour stored at 0 °C, 5 °C and 35 °C, for 0 or 6 months.

Variety	Linerar regression	Correlation coefficient
Spear	0.951	0.975
Worrakatta	0.974	0.987
Krichauff	0.999	0.999
Tamaroi	0.759	0.871

Table B8 Correlation between b* and β-carotene in all wheat varieties for flour stored at 0 °C, 5 °C and 35 °C, for 0 or 6 months.

Variety	Linerar regression	Correlation coefficient
Spear	0.908	0.953
Worrakatta	0.988	0.994
Krichauff	0.947	0.973
Tamaroi	0.789	0.888

APPENDIX C: Animal Study

C1 Commercial Diet

Table C1 Vitamin and mineral levels per tonne of feed in commercial diet.

Vitamin	Concentration	Mineral	Concentration
A	10 MIU [a]	Mn	80 g
D_3	2 MIU [b]	Zn	60 g
E	20 g	Fe	50 g
K	2 g	Cu	6 g
B_1	1 g	I	0.5 g
B_2	4 g	Se	0.1 g
B_6	2 g	Mo	0.25 g
B_{12}	10 mg	Ca Pantothenate	10 g
Niacin	30 g		
Biotin	100 mg		
Folic Acid	0.5 g		

[a] 1 MIU = 0.3 g retinol. MIU indicates Million International Units
[b] 1 MIU = 0.025 g.

C2 Form and formulae of vitamins and minerals

Table C2 Form and formulae of vitamins used in depletion diet vitamin / mineral premix.

Vitamin	Form	Formula
E	Alpha-tocopherol acetate	$C_{31}H_{52}O_3$
B_1	Thiamine hydrochloride	$C_{12}H_{17}ClN_4O_5 + HCl$
B_2	Riboflavin	$C_{17}H_{20}N_4O_6$
B_6	Pyridoxine hydrochloride	$C_8H_{11}NO_3 + HCl$
B_{12}	cyanocobalamin	
Niacin	D-pantothenic acid (D[+]-N-[2,4-dihydroxy-3,3-dimethylbutytyl]-β-alanine)	Hemicalcium salt $C_9H_{16}NO_5 + 1/2\ Ca$
Biotin	d-biotin (vitamin H)	$C_{10}H_{16}N_2O_3S$
Folic acid	Pteroylglutamic acid	$C_{19}H_{19}N_7O_6$

Table C3 Form and formulae of minerals used in depletion diet vitamin / mineral premix.

Mineral	Form	Formula
Copper	Copper sulphate	$CuSO_4$
Iodine	Potassium iodate	KIO_3
Manganese	Manganese sulphate	Mn_2SO_4
Molybdenum	Ammonium molybdate	MoO_3
Selenium	Sodium selenate	Na_2SeSO_4
Zinc	Zinc sulphate	$ZnSO_4$
Iron	Ferric chloride	Fe2O6Cl6

C3 Whole-body Hb-Fe

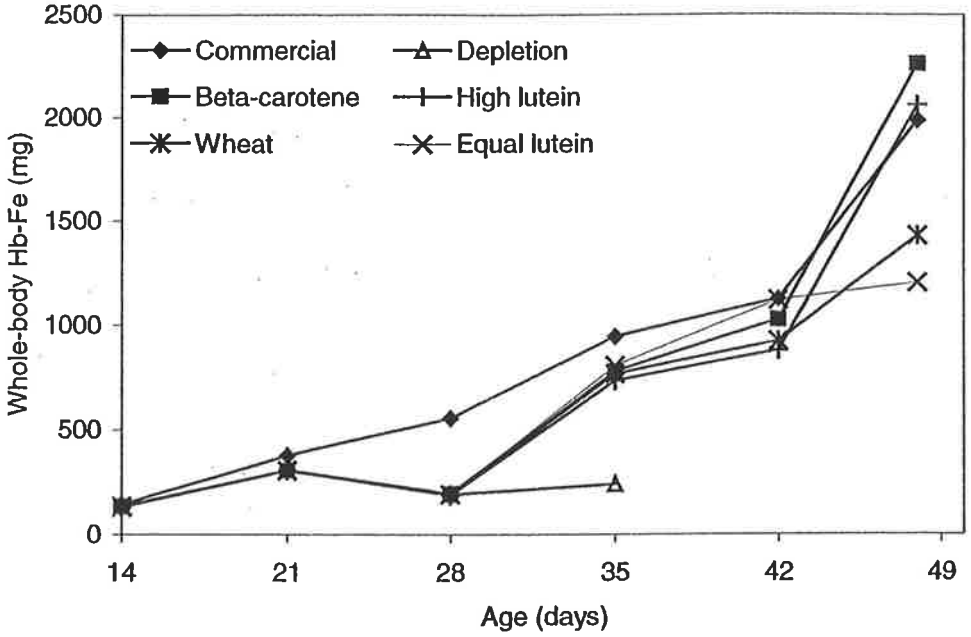


Figure C1 Whole-body Hb-Fe of birds on all diets. Values are the means for 10 birds, standard error bars were omitted for clarity but errors were within 5 % of the mean.

C4 Tissue Iron

Table C4 Iron concentrations (mg/kg) detected in liver, (haem and non-haem), muscle, and jejunal and ileal gastrointestinal tract sections. Values are means ± standard error.

	Hepatic Total Iron	Hepatic non-haem iron	Hepatic Haem iron	Muscle Iron	GI Jejunum	GI Ileaum
Commercial	113 ± 22	84 ± 9	29 ± 18	4.8 ± 0.88	13 ± 2	20 ± 4
Depletion	110 ± 32	98 ± 27	12 ± 15	5.8 ± 0.58	10 ± 3	15 ± 3
β-carotene	143 ± 24	100 ± 23	43 ± 13	5 ± 0.87	16 ± 15	30 ± 15
High lutein	136 ± 24	82 ± 25	54 ± 8	5 ± 0.83	19 ± 1	18 ± 1
Wheat	171 ± 54	112 ± 53	59 ± 14	4.9 ± 1	15 ± 10	28 ± 10
Equal lutein	122 ± 12	84 ± 20	38 ± 9	5.2 ± 0.65	14 ± 5	20 ± 5
Significance	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**

Bibliography

Ali, M., Samson, T. and Janson, H.P. (1994). Micro-nutrients and vegetables: a neglected food frontier. In *Workshop in food policy and agricultural technologies to improve diet quality and nutrition*, Bouis, H. (ed). International Food Policy Research Institute, Washington, DC: Annapolis, Maryland, USA. Jan 10-12.

Amine, E.K., Corey, J., Hegsted, D.M. and Hayes, K.C. (1970). Comparative hematology during deficiencies of iron and vitamin A in the rat. *J.Nutr.* **100**:1033-1040.

Anderson, H.D., McDonough, K.B. and Elvehjem, C.A. (1940). Relation of the dietary calcium-phosphorus ratio to iron assimilation. *J.Lab.Clin.Med.* **25**:464-471.

AOAC (1995). AOAC Official Method 974.31 Bioavailability of iron: Rat hemoglobin repletion assay. *AOAC* **45**:62-63.

Armstrong, G.A. and Hearst, J.E. (1996). Genetics and molecular biology of carotenoid pigment biosynthesis. *FASEB J.* **10**:228-237.

Austern, B.M. and Gawienowski, A.M. (1969). *In vitro* biosynthesis of β-carotene by bovine corpus luteum. *Lipids* 4:227-229

Beard, J.L., Connor, J.R. and Jones, B.C. (1993). Iron on the brain. *Nutr.Rev.* **10**:133-148.

Beisel, W.R. (1982). Single nutrients and immunity. *Am.J.Clin.Nutr.* **Feb (suppl)**:417-468.

Belitz, H.-D. and Grosch, W. (1987). Saponification. In *Food chemsitry*. Springer-Verlag: New York.

Bell, B.M., Chamberlain, N., Collins, T.H., Daniels, D.G.H. and Fisher, N. (1979). The composition, rheological properties and bread making behaviour of stored flours. *J.Sci.Food Agric.* **30**:111-1122.

Bhatt, G.M. and Mc Master, G.J. (1976). Variation in pigment content of flour colour in bread wheat (*Triticum aestivum* L.). *Euphytica* **25**:685-688.

Bierer, T.L., Merchen, N.R. and Erdman, J.W. (1995). Comparative absorption and transport of five common carotenoids in preruminant calves. *J.Nutr.* **125**:1569-1577.

Blakely, S.R., Mitchell, G.V., Jankins, M.J., Grundel, E. and Whittaker, P. (1991). Canthaxanthin and excess vitamin A alter α-tocopherol, carotenoid and iron status in adult rats. *J.Nutr.* **121**:1649-1655.

Bliss, A.F. (1951). The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase. *Arch.Biochem.* **31**:197-204.

Bloch, C.E. (1928). Decline in immunity as a symptom due to deficiency in A-vitamine and C-vitamine. *Acta Paediatr.* **7 (suppl 12)**:61-70.

Block, G. and L. Langseth (1994). Antioxidant vitamins and disease prevention. Food Technol. **48**: 80-84.

Bloem, M.L. (1995). Interdependence of vitamin A and iron: an important association for programmes of anaemia control. *Proc.Nutr.Soc.* **54**:501-508.

Bloem, M.W., Wedel, M., Egger, R.J., Speek, A.J., Schrijver, J., Saowakontha, S. and Schreurs, W.H.P. (1989). Iron-metabolism and vitamin-A-deficiency in children in Northeast Thailand. *Am.J.Clin.Nutr.* **50**:332-338.

Bloem, M.W., Wedel, M., van Agtmaal, E.J., Speek, A.J., Saowakontha, S. and Schreurs, W.H.P. (1990). Vitamin A intervention: short-term effects of a single, oral, massive dose on iron metabolism. *Am.J.Clin.Nutr.* **51**:76-79.

Boileau, A.C., Merchen, N.R., Wasson, K., Atkinson, C.A. and Erdman, J.W.J. (1999). *cis*-Lycopene is more bioavailable that *trans*-lycopene *in vitro* and also *in vivo* in the lymph canulated ferret. *J.Nutr.* **129**:1176-1181.

Bone, R.A. and Landrum, J.T. (1992). Distribution of macular pigment components, zeaxanthin and lutein, in human retina. *Methods in Enzymology* **213A**:360-366.

Bone, R.A., Landrum, J.T., Fernandez, L. and Tarsis, S.L. (1988). Analysis of the macular pigment by HPLC: Retinal distribution and age study. *Invest.Opthamol.Vis.Sci.* **29**:843-849.

Bone, R.A., Landrum, J.T. and Tarsis, S.L. (1985). Preliminary identification of the human macular pigment. *Vision Res.* **25**:1531-1535.

Bone, R.S., Landrum, J.T., Hime, G.W. and Cains, A. (1993). Stereochemistry of the human macular carotenoids. *Invest.Opthamol.Vis.Sci.* **34**:2033-2049.

Boynton, L.C. and Bradford, W.L. (1931). Effect of vitamin A and D on resistance to infection. *J.Nutr.* **4**:323-324.

Bramley, P. M. (1997). The Regulation and Genetic Manipulation of Carotenoid Biosynthesis in Tomato Fruit. *Pure Appl.Chem.* **69**(10): 2159-2162.

Britton, G. (1983). *The Biochemistry of Natural Pigments*. Cambridge University Press: Cambridge, UK.

Britton, G. (1991). Carotenoids. In *Methods in Plant Biochemistry .Vol. 7, Terpenoids*. Academic Press: New York, USA.

Britton, G. (1995). Structure and properties of carotenoids in relation to function. *FASEB J* **9**:1551-1559.

Britton, G., Singh, R.K., T.W., G. and Ben-Aziz, A. (1975). The carotenoids of *Rhodomicrobium vannielii* (Rhodospirllaceae) and the effect of diphenylamine on the carotenoid composition. *Phytochem.* **14**:2427-2433.

Broich, C.R., Gerber, L.E. and Erdman, J.W. (1983). Determination of lycopene, α- and β-carotene and retinyl esters in human serum. *Lipids* **18(3)**:235-258.

Brown, N.A.P., Bron, A.J., Harding, J.J. and Dewar, H.M. (1998). Nutrition supplements and the eye. *Eye* **12**:127-133.

Brune, M., Rossander, L. and Hallberg, L. (1989). Iron absorption and phenolic compounds: importance of different phenolic structures. *Eur.J.Clin.Nutr.* **43**:547-558.

Bruno, R.S. and Medeiros, D.M. (2000). Lutein, zeaxanthin and age-related macular degeneration. *J.Nutraceuticals Functional Medical Foods* **3**:79-85.

Burton, G.W. (1989). Antioxidant action of carotenoids. *J.Nutr.* **119**:109-111.

Burton, G.W. and Ingold, K.U. (1984). Beta-carotene - an unusual type of lipid antioxidant. *Science* **224**:569-571.

Byers, T. and Peery, G. (1992). Dietary carotenes, vitamin C and vitamin E as protective antioxidants in human cancers. *Ann.Rev.Nutr.* **12**:139-141.

Cao, J., Luo, X.G., Henry, P.R., Ammerman, C.B., Littell, R.C. and Miles, R.D. (1996). Effect of dietary iron concentration, age and length of iron feeding on feed intake and tissue iron concentration of broiler chickens for use as a bioassay of supplemental iron sources. *Poult.Sci.* **75**:495-504.

Castenmiller, J.J.M. and West, C.E. (1997). Bioavailability of Carotenoids. *Pure Appl.Chem.* **69**:2145-2150.

Chen, B.H. and Bailey, C.A. (1987). Separation of carotenoids in Turf Bermuda grasses by high performance liquid chromatography. *J. Chromatography* **393**:398-304.

Chen, K.-T. and Geddes, W.F. (1945). Studies on the wheat pigments. M.Sc. University of Minnesota, St Paul, Minnesota, USA.

Chew, B.P., Park, J.S., Wong, T.S., Weng, B.C., Kim, H.W., Byrne, K.M., Hayek, M.G. and Reinhardt, G.T. (1998). Role of dietary beta-carotene in modulating cell-mediated and humoral immune response in dogs. *FASEB J* **12**:A967 abs.

Chew, B.P., Wong, T.S., Michal, J.J., Standaert, F.E. and Heirman, L.R. (1991). Kinetic characteristics of beta-carotene uptake after an injection of beta-carotene in pigs. *J.Anim.Sci.* **69**:4883-4891.

Chirasiri, I. and Izak, G. (1966). The effect of acute haemorrage and acute haemolysis on intestinal iron absorption in the rat. *Brit. J. Haematol.* **12**:611-622.

Christian and West, K.P. (1998). Interactions between zinc and vitamin A: an update. *Am.J.Clin.Nutr.* **68(suppl)**:435 S-441 S.

Clark, R.M., Yao, L.L., She, L. and Furr, H.C. (1998). A comparison of lycopene and canthaxanthin absorption - using the rat to study the absorption of non-provitamin A carotenoids. *Lipids* **33**:159-163.

Clinton, S.K. (1998). Lycopene - chemistry, biology, and implications for human health and disease. *Nutr.Rev.* **56**:35-51.

Colditz, G.A., Branch, L.G., Lipnick, R.J., Willett, W.C., Rosner, B., Posner, B.M. and Henekens, C.H. (1985). Increased green and yellow vegetable intake and lowered cancer deaths in an elderly population. *Am.J.Clin.Nutr.* **41**:32-36.

Combs, G.F. and Nicholson, J.L. (1963). Report of Maryland broiler trials S-38. *Feedstuffs* **351**:36-38.

Combs, G.F., Welch, R.M., Duxbury, J.M., Uphoff, N.T. and nesheim, M.C. (1996). Food-based approaches to preventing micronutrient malnutrition: An international research agenda. Cornell International Institute for Food, Agriculture and development, Cornell University, Ithaca, NY, USA.

Connett, J.E., Kuller, L.H., Kjelsberg, M.O., Polk, B.F., Collins, G., Rider, A. and Hulley, S.B. (1989). Relationship between carotenoids and cancer: the multiple risk factor intervention trial (MRFIT) study. *Cancer* **64**:126-134.

Craft, N.E. (1992). Carotenoid RPHPLC methods. *Methods in Enzymology* **213**:185-205.

Craft, N.E., Sander, L.C. and Pierson, H.F. (1989). Determination of purity and isomer content of selected preparations of β-carotene. National Institute of Standards and Technology: Gaithersburg, Maryland, USA.

Cuendet, L.S., Larson, G., Morris, C.G. and Geddes, W.P. (1954). The influence of moisture content and other factors on the stability of wheat flours at 37.8 C. *Cereal Chem.* **31**:362-389.

Davies, B.H. (1976). *Chemistry and Biochemistry of Plant Pigments.* Vol. 2. Academic Press, New York, USA.

Davis, C.Y. and Sell, J.L. (1983). Effect of all-*trans* retinol and retinoic acid nutriture on the immune system of chicks. *J.Nutr.* **113**:1914-1919.

Davis, P.N., Norris, L.C. and Kratzer, F.H. (1968). Iron utilisation and metabolism in the chick. *J.Nutr.* **94**:407-417.

De Leenheer, A.P. and Neils, H.J. (1992). Profiling and quantiation of carotenoids by HPLC and photodiode array detection. *Methods in Enzymology* **213**:251-262.

de Pee, S., West, C.E., Muhilal, Karyadi, D. and Hautvast, J.G.A. (1995). Lack of improvement in vitamin A status with increased consumptionof dark-green leafy vegetables. *Lancet* **346**:75-81.

de Pee, S., West, C.E., Permaesih, D., Martuti, S., Muhilal and Hautvast, J.G.A.J. (1998a). Orange fruits are more effective in increasing serum concentratons of retinol and β-carotene than dark-green leafy vegetables in school children in Indonesia. *Am.J.Clin.Nutr.* **68**:1058-1067.

de Pee, S., West, C.E., Yuniar, Y. and Muhilal. (1998b). Evaluation of vitamin A status - reply. *Am.J.Clin.Nutr.* **67**:149-150.

Demmig-Adams, B. (1998). Survey of thermal energy dissipation and pigment composition in sun and shade leaves. *Plant Cell Physiol.* **39**:474-482.

Di Mascio, O., Kaiser, S. and Sies, S. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch.Biochem.Biophys.* **274**:532-538.

Di Mascio, P., Murphy, M.E. and Sies, H. (1991). Antioxidant defence systems: the role of carotenoids, tocopherols and thiols. *Am. J. Clin. Nutr.* **53**:194 S-200 S.

Dorey, C.K., Thomson, L., Kinert, K., Finger, M., Nichols, C., Cheng, K. and Craft, N. (1998). Effect of dietary zeaxanthin on age related changes in quail retinas. *Opthamol.Vis.Sci.* **39**:164 abs.

Dua, P.N., Day, E.J., Hill, J.E. and Grogan, C.O. (1967). Utilisation of xanthophylls from natural sources by the chick. *J.Agric.Food Chem.* **15**:324-328.

Duffus, D. and Slaughter, C. (1980). *Seeds and their uses*. John Wiley and Sons: New York, USA.

Dugas, T. R., D. W. Morel, et al. (1998). Impact of LDL Carotenoid and Alpha-Tocopherol Content On LDL Oxidation By Endothelial Cells in Culture. Journal of Lipid Research **39**(5): 999-1007.

Elschenbroch, C. and Salzer, A. (1992). Donor/acceptor ligands. In *Organometalics. A consise introduction*. VCH Publishers, Weinheim, Federal Republic of Germany.

Emodi, A.S. and Scialpi, L. (1980). Quality of bread fortified with 10 micronutrients. *Cereal Chem.* **57(1)**:1-3.

Erdman, J.W., Fahey, G.C. and White, C.B. (1986). Effects of purified dietarty fibre sources on β-carotene utilisation by the chick. *J.Nutr.* **116**:2415-2423.

Erdman, J.W.J., Bierer, T.L. and Gugger, E.T. (1993). Absorption and transport of carotenoids. *Ann.N.Y. Acad. Sci.* **691**:76-85.

FAO/WHO (1967). FAO Food and Nutrition Series / WHO Technical Report Series: Requirements of vitamin A, thiamine, riboflavin and niacin. FAO/WHO: Rome, Geneva.

FAO/WHO (1988). Requirements of vitamin A, iron, folate and vitamin B_{12}. In *Expert consultation: Food and Nutrition Series*, Vol. 23. FAO: Rome.

FAO/WHO (1991). Theme paper 6: Preventing specific micronutrient deficiencies. In *International conference on nutrition*: Rome, Italy.

Farrington, W.H.H., Warwick, M.J. and Shearer, G. (1981). Changes in the carotenoid and sterol fractions during prolonged storage of wheat flour. *J.Sci.Food Agric.* **32**:948-950.

Fortmann, K.L. and Joiner, R.R. (1988). Wheat pigments and flour colour. In *Wheat: Chemistry and Technology*, Pomeranz, Y. (ed), Vol. 1. pp. 493-522. American Association of Cereal Chemists Inc.: Minnesota, USA.

Fraser, P.D., Hedden, P., Cooke, D.T., Bird, C.R., Schuch, W. and Bramley, P.M. (1995). The effect of reduced activity of phytoene synthase on isoprenoid levels in tomato pericarp during fruit development and ripening. *Planta* **196**:321-326.

Fraser, P.D., Truesdale, M.R., Bird, C.R., Such, W. and Bramley, P.M. (1994). Carotenoid biosynthesis during tomato fruit development. *J.Plant Physiol.* **105**:405-413.

Friedman, A. and Sklan, D. (1989a). Impaired T-lymphocyte immune response in vitamin A depleted rats and chicks. *Brit.J.Nutr.* **62**:439-449.

Friedman, A. and Sklan, D. (1989b). Antigen-specific immune response impairment in the chicks as influenced by dietary vitamin A. *J.Nutr.* **119**:790-795.

Furr, H.C. and Clark, R.M. (1997). Intestinal absorption and tissue distribution of carotenoids. *J.Nutr.Biochem.* **8**:364-377.

Ganguly, J., Krishnamurthy, S. and Mahadevan, S. (1959). The transport of carotenoids, vitamin A and cholesterol across the intestines of rats and chickens. *Biochem J* **71**:756-762.

Garcia-Casal, M.N., Layrisse, M., Solano, L., Baron, M.A., Arguello, F., Llovera, D., Ramirez, J., Leets, I. and Tropper, E. (1998). Vitamin A and β–carotene can improve non-heme iron absorption from rice, wheat and corn by humans. *J.Nutr.* **128**:646-650.

Gibson, R.S. (1994). Zinc nutrition and public health in developing countries. *Nutr.Res.Rev.* **7**:151-173.

Goodwin, T.W. (1965). *Chemistry and Biochemistry of Plant Pigments*. Academic Press: New York, USA.

Goodwin, T.W. (1980). *The Biochemistry of the Carotenoids. Vol. 1 Plants.* Chapman and Hall: New York, USA.

Goodwin, T.W. (1984). *The Biochemistry of the Carotenoids.* Vol. 2, Animals. Chapman and Hall: London, UK.

Goodwin, T.W. (1986). Metabolism, nutrition and function of carotenoids. *Ann.Rev.Nutr.* **6**:273-297.

Goodwin, T.W. (1993). Biosynthesis of carotenoids: an overview. *Methods in Enzymology* **214**:339-340.

Gordon, D.T. and Godber, J.S. (1989). The enhancement of nonheme iron bioavailability by beef protein in the rat. *J.Nutr.* **119**:446-452.

Graham, R.D. (1984). Breeding for nutritional characteristics in cereals. *Adv.Plant Nutr.* **1**:57-102

Graham, R.D., Senadhira, D., Beebe, S., Iglasias, C. and Monasterio, I. (1999). Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. *Field Crops Res.* **60**:57-80

Graham, R.D. and Welch, R.M. (1999). Breeding for staple-food crops with high micronutrient density. Agricultural strategies for micronutrients. Working paper 3. International Food Policy Research Institute, Washington, DC, USA

Grangoud, R., Massonet, R., Conquy, T and Ridolfo, J. (1962). *Comp.Rend.Acad.Sci.Paris* **254**:579-581 (In Matsuno, 1991)

Grant, J.P. (1991). *The State of the World's Children.* Oxford University Press on behalf of UNICEF: New York, USA.

Griffiths, M., Sistrom, W.R., Cohen-Bazire, G. and Stanier, R.Y. (1955). Function of carotenoids in photosynthesis. *Nature* **176**:1211-1215.

Gross, J. and Budowski, P. (1966). Conversion of carotenoids into vitamin A_1 and A_2 in two species of freshwater fish. *Biochem.J.* **101**:747-754.

Guilbert, H.R. and Hart, G.H. (1935). Minimum vitamin A requirements with particular reference to cattle. *J.Nutr.* **10**:409-414.

Hammond, B.R., Johnson, E.J., Russell, R.M., Krinsky, N.I., Yeum, K.J., Edwards, R.B. and Snodderly, D.M. (1997b). Dietary modification of human macular pigment density. *Invest.Ophthalmol.Vis.Sci.* **38**:1795-1801.

Hammond, B.R., Wooten, B.R. and Snodderly, D.M. (1997a). Density of the human crystalline lens is related to the macular pigment carotenoids, lutein and zeaxanthin. *Opt.Vis.Sci.* **74**:499-504.

Handleman, G.J., Dratz, E.A., Reay, C.C. and van Kuijk, F.J.G.M. (1988). Carotenoids in the human macula and whole retina. *Invest. Opthamol.Vis.Sci.* **29**:850-855.

Heinonen, M., Ollilainen, V., Linkola, E., Varo, P. and Koivistoinen, P. (1989). Carotenoids and retinoids in Finnish foods: Cereal and bakery products. *Cereal Chem.* **66**:270-273.

Henry, P.R. and Miller, E.R. (1995). Iron Bioavailability. In *Bioavailabiltiy of nutrients for animals: amino acids, minerals and vitamins*, Ammerman, C.B., Baker, D.H. & Lewis, A.J. (eds) pp. 169-199. Academic Press: California, USA.

Hodges, R.E., Sauberlich, H.E., Canham, J.E., Wallace, D.L., Rucker, R.B., Mejia, L.A. and Mohanram, M. (1978). Hematopoietic studies in vitamin A deficiency. *Am.J.Clin.Nutr.* **31**:876-885.

Hornero-Mendez, D. and Minguez-Mosquera, M.I. (2000). Xanthophyll esterification accompanying carotenoid overaccumulation in chromoplast of *Capsicum annum* ripening fruit is a constitutive process and useful for ripeness index. *J.Agric.Food Chem.* **48**:1617-1622.

Hsieh, Y.-P. and Karel, M. (1983). Rapid extraction and determination of α- and β-carotene in foods. *J.Chrom.* **259**:515-518.

Iribarren, C., A. R. Folsom, *et al.* (1997). Association of serum vitamin levels, LDL susceptibility to oxidation, and autoantibodies against MDA-LDL with carotid atherosclerosis - a case-control study. *Arteriosclerosis Thrombosis Vasc.Biol.* **17**(6): 1171-1177.

Isler, O. (1971). Carotenoids. Birkhauser Verlag: Bazel.

Jacques, P.F. and Chylack, L.T. (1991). Epidemiological evidence of a role for the antioxidant vitamins and carotenoids in cataract prevention. *Am.J.Clin.Nutr.* **53**:532S-535S.

Jensen, C.D., Howes, T.W., Spiller, G.A. and Scala, J. (1986). Observations on the effects in humans of *cis* and *trans* β-carotene isomers. *Am.J.Clin.Nutr.* **43**:689-691.

Kaneko, S., Nagamine, T. and Yamada, T. (1995). Esterification of endosperm lutein with fatty acids during the storage of wheat seeds. *Biosci.Biotech.Biochem.* **59**:1-4.

Kaneko, S. and Oyanagi, A. (1995). Varietal differences in the rate of esterification of endosperm lutein during the storage of wheat seeds. *Biosci.Biotech.Biochem.* **59**:2312-2313.

Khachik, F., Beecher, G.R., Goli, M.B. and Lusby, W.R. (1991b). Separation, identification, and quantification of carotenoids in fruits, vegetables and human plasma by high-performance liquid-chromatography. *Pure Appl.Chem.* **63**:71-80.

Khachik, F., Beecher, G.R., Goli, M.B. and Lusby, W.R. (1992a). Separation and quantitation of carotenoids in foods. *Methods in Enzymology* **213**:347-359.

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

Khachik, F., Bernstein, P.S., Squires, A. and Rosser, J.M. (1999d). Identification of carotenoids and related metabolites in frog retina and liver: a useful non-primate model for studying the physiological role of macular carotenoids. In *12th International Carotenoid Symposium*: Cairns, Australia.

Khachik, F., Cohen, L.A. and Zhao, Z. (1999c). Metabolism of dietary carotenoids and their potential role in prevention of cancer and age-related macular degeneration. Oxford University Press, Oxford, UK.

Khachik, F., Spangler, C.J., Smith, C.J., Canfield, L.M., Steck, A. and Pfander, H. (1997b). Identification, quantification and relative concentrations of carotenoids and their metabolites in human milk and serum. *Analytical Chem.* **69**:1873-1881.

Khan, N.C., West, C.E., de Pee, S. and Khoi, H.H. (1998). Comparison of the effectiveness of carotenoids from dark-green leafy vegetables and yellow and orange fruits in improving vitamin A status of breast-feeding women in Vietnam. In *Report of the XVIII International Vitamin A Consultative Group Meeting*: Washington DC, USA.

King, T.J., Khachik, F., Bortkiewicz, H., Fukushima, L.H., Morioka, S. and Bertram, J.S. (1997). Metabolites of dietary carotenoids as potential cancer preventive agents. *Pure Appl.Chem.* **69**:2135-2140.

Koyama, Y. (1991). Structures and functions of carotenoids in photosynthetic systems. *J.Photochem. Photobiol. B:Biol.* **9**:265-280.

Krinski, N.I., Cornwell, D.G. and Oncley, J.L. (1958). The transport of vitamin A and carotenoids in human plasma. *Arch.Biochem.Biophys* **73**:233-246.

Krinsky, N.I. (1991). Effect of carotenoids in cellular animal systems. *Am.J.Clin.Nutr.* **53**:238S-246S.

Krishnan, S., Bhuyan, U.N., Talwar, G.P. and Ramaling, V. (1974). Effect of vitamin-A and protein-calorie undernutrition on immune-responses. *Immunol.* **27**:383.

Kruger, J.E. and Reed, G. (1988). Chapter 8: Enzymes and Colour. In *Wheat: Chemistry and Technology*, Pomeranz, Y. (ed), Vol. 1. American Association of Cereal Chemists: St Paul, Minnesota, USA.

Kwan, M., Niinikoski, J. and Hunt, T.K. (1972). *In vivo* measurement of oxygen tension in the cornea, aqueous humor, and anterior lens of the open eye. *Invest.Opthalmol.* **11**:108-111.

Lacroix, L.J. and Lier, J.B. (1975). Carotenoids in durum wheat: developmental patterns during two growing seasons. *Can.J.Plant Sci.* **55**:679-684.

Laval-Martin, D., Quennemet, J. and Moneger, R. (1975). Pigment evolution in *Lycopersicon esculentum* fruits during growth and development. *Phytochem.* **14**:3257-2433.

Layrisse, M., Garcia-Casal, M.N., Solano, L., Baron, M.A., Arguello, F., Llovera, D., Ramirez, J., Leets, I. and Tropper, E. (1997). The role of vitamin A on the inhibitors of non-heme iron absorption: Preliminary results. *J.Nutr. Biochem.* **8**:61-67.

Layrisse, M., Gracia-Casal, M.N., Solano, L., Baron, M.A., Arguello, F., Llovera, D., Ramirez, J., Leets, I. and Tropper, E. (1998). Vitamin A reduces the inhibition of iron absorption by phytates and polyphenols. *Food Nutr.Bull.* **19**:3-5.

Lee, C.M., Boileau, A.C., Boileau, T.A., Williams, A.W., Swanson, K.S., Heintz, K.A. and Erdman, J.W. (1999). Review of animal models in carotenoid research. *J.Nutr.* **129**:2271-2277.

Lee, C.M., Lederman, J.D., Hofmann, N.E. and Erdman, J.W. (1998). The Mongolian gerbil (*Meriones unguiculatus*) is an appropriate animal model for evaluation of the conversion of beta-carotene to vitamin A. *J.Nutr.* **128**:280-286.

Lepage, M. and Sims, R.P.A. (1968). Carotenoids of wheat flour: their identification and composition. *Cereal Chem.* **45**:600-611.

Mac Masters, M.M., Hinton, J.J.C. and Bradbury, D. (1971). Microscopic structure and composition of the wheat kernel. In *Wheat: Chemistry and Technology*, Pomeranz, Y. (ed) pp. 51-113. American Association of Cereal Chemists: St Paul, Minnesota, USA.

Mac Phail, P. (1998). Iron. In *Essentials of Human Nutrition*, Mann, J. & Truswell, A.S. (eds) pp. 137-149. Oxford University Press, Oxford, UK.

Mackerras, D. (1995). Antioxidants and health- fruit and vegetables or supplements. *Supp. Food Australia* **47**:S 3-S 23.

Mahant, L. and Eaton, H.D. (1976). Effect of chronic hypovitaminosis-A on water metabolism in weanling rat. *J.Nutr.* **106**:1817-1826.

Mahoney, A.W. and Hendricks, D.G. (1982). Efficiency of hemoglobin regeneration as a method of assessing iron bioavailability in food products. In *Nutritional Bioavailability of Iron* pp. 1-10. American Chemical Society: Washington D.C., USA.

Mahoney, A.W., VanOrden, C.C. and Hendricks, D.G. (1974). Efficiency of converting food iron into hemoglobin by the anemic rat. *Nutr.Metab.* **17**:223-230.

Mailhot, W. and J. C. Patton (1988). Criteria of flour quality. Wheat: Chemistry and Technology. Y. Pomeranz. Minnesota, USA, American Association of Cereal Chemists, Inc. **II:** 69-90.

Malone, W.F. (1991). Studies evaluating antioxidants and β-carotene as chemopreventives. *Am.J.Clin.Nutr.* **53**:305S-313S.

Marenah, C.B., Lewis, B. and Hassal, D. (1983). Hypercholesterolaemia and non-cardiovascular disease: metabolic studies on subjects with low plasma cholesterol concentration. *Brit.Med.J.* **286**:1603-1606.

Mares, D.J., Wang, Y. and Cassidy, C.A. (1997). Separation, identification and tissue location of compounds responsible for the yellow colour of alkaline noodles. In *47th Cereal Chemistry Conference*, Tarr, A.W., Ross, A.S. & Wrigley, C.W. (eds) pp. 114-117: Perth, Australia.

Mathews, C.K. and van-Holde, K.E. (1990). The Visual System. In *Biochemsitry.* Benjamin / Cummings Publishing Company California, USA.

Mathews-Roth, M.M. (1991). Recent progress in the medical application of carotenoids. *Pure Appl.Chem.* **63**:147-156.

Mathis, P. and Schenck, C.C. (1982). The functions of carotenoids in photosynthesis. In *Carotenoid Chemistry and Biochemistry*, G.Britton & Goodwin, T.W. (eds) pp. 339-352. Pergamon Press, Oxford, UK.

Matsuno, T. (1991). Xanthophylls as precursors of retinoids. *Pure Appl.Chem.* **63**:81-88.

Mayne, S.T. and Parker, R.S. (1986). Subcellular distribution of dietary beat-carotene in chick liver. *Lipids* **21**:164-169.

Mc Laren, D.S. (1986). *Vitamin A Deficiency and its Control.* Academic Press: New York, USA.

McGuire, R.G. (1992). Reporting of objective color measurements. *Hort.Sci.* **27**:1254-1255.

McGuire, J. (1993). Addressing micronutrient malnutrition. *SCN News* **9**:1-10

Mejia, L. and Arroyave, G. (1982). The effect of vitamin A fortification of sugar on iron metabolism in preschool children in Guatemala. *Am.J.Clin.Nutr.* **36**:87-93.

Mejia, L.A. (1985). Vitamin A deficiency as a factor in nutritional anemia. In *International Journal for Vitamin and Nutrition Research.* In *Vitamins: Nutrients and Therapeutic Agents*, Hank, A. & Hornig, D. (eds), Suppl. 27. pp. 75-84. Hans Huber Publishers: Bern, Stuttgart, Toronto.

Mejia, L.A. and Chew, F. (1988). Hematological effect of supplementing anemic children with vitamin-A alone and in combination with iron. *Am.J.Clin.Nutr.* **48**:595-598.

Mejia, L.A., Hodges, R.E. and Rucker, R.B. (1979). Role of vitamin-A in the absorption, retention and distribution of iron in the rat. *J.Nutr.* **109**:129-137.

Miki, W. (1991). Biological functions and activities of animal carotenoids. *Pure Appl.Chem.* **63**:141-146.

Miller, N.J., Simpson, J., Candeias, L.P., Bramley, P.M. and Rice-Evans, C.A. (1996). Antioxidant activities of carotenoids and xanthophylls. *FEBS Letters* **384**:240-242.

Miskelly, D.M. (1984). Flour components affecting pasta and noodle colour. *J.Sci.Food Agric.* **35**:463-465.

Mohannah, C. and Nys, Y. (1998). Influence of age, sex and cross on body concentrations of trace elements (zinc, iron, copper and manganese) in chickens. *Brit.Poult.Sci.* **39**:536-543.

Mohanram, M., Kulkarni, K.A. and Reddy, V. (1977). Hematological studies in vitamin A deficient children. *Int.J.Vit.Nutr.Res.* **47**:389-393.

Monma, M., Terao, J., Ito, M., Saito, M. and Chikuni, K. (1994). Carotenoid components in soybean seeds varying with seed colour and maturation stage. *Biosci.Biotech.Biochem.* **58**:926-930.

Moon, R.C. (1989). Comparative aspects of carotenoids and retinoids as chemopreventive agents for cancer. *J.Nutr.* **119**:127-134.

Moore, T. (1957). *Vitamin A*. Elsevier: Amsterdam.

Morris, D.L.M. and Kritchevesky, S.B. (1994). Serum carotenoids and coronary heart disease: the lipid research clinics coronary primary prevention trial and follow-up study. *JAMA* **272**:1439-1441.

Mosca, L., Rubenfire, M., Mandel, C., Rock, C., Tarshis, T., Tsai, A. and Pearson, T. (1997). Antioxidant nutrient supplementation reduces the susceptibility of low density lipoprotein to oxidation in patients with coronary artery disease. *J.Am.College Cardiol.* **30**:392-399.

Muhilal, Permeisih, D., Idjradinata, Y.R., Muherdiyantiingsih and Karyadi, D. (1998). Vitamin A fortified monosodium glutamate and health, growth and survival of children: a controlled field trial. *Am.J.Clin.Nutr.* **48**:1271-1276.

Murakoshi, M., Takayasu, J. and Kimura, O. (1989). Inhibitory effects of alpha-carotene on proliferation of the human neuroblastoma cell line GOTO. *J.Natl.Cancer Inst.* **81**:1649-1652.

Mwanri, L., Worsley, A., Ryan, P. and Masika, J. (2000). Supplemental vitamin A improves anemia and growth in anemic school children in Tanzania. *J.Nutr.* **130**:2691-2696.

Nam, H.S., Cho, S.Y. and Rhee, J.S. (1988). High performance liquid chromatography analysis of carotenoids from *Rhodotorula glutinis. J. Chrom.* **448**:445-447.

Niki, E., Yamamoto, Y., Komuro, E. and Sato, K. (1991). Membrane damage due to lipid oxidation. *Am.J. Clin.Nutr.* **53**:201S-205S.

Nockles, C.F. and Kienholz, E.W. (1967). Influence of vitamin A deficiency on testes, bursa fabricius, adrenal and hematocrit in cockerels. *J.Nutr.* **92**:384-388.

NRC. (1989). *Recommended Dietary Allowances*. National Academy Press: Washington DC, USA.

O'Brien, T.P. (1979). Opening address to seed physiology symposium. In *Seed Physiology Symposium*: Canberra. May 1979, Monash University, Australia.

O'Dell, B.L., Burpo, C.E. and Svage, J.E. (1972). Evaluation of zinc availability in foodstuffs of plant and animal origin. *J.Nutr.* **102**:653-660.

Olson, J.A. (1984). Vitamin A. In *Handbook of Vitamins: Nutritional, Biochemical and Clinical Aspects*, Machlin., L.J. (ed). Marcel Dekker Inc.: New York, USA.

Olson, J.A. (1987). Recommended dietary intakes (RDI) of vitamin A in humans. *Am.J.Clin.Nutr.* **45**:704-716.

Olson, J.A. (1996). Benefits and liabilities of vitamin A and carotenoids. *J.Nutr.* **126**:S 1208-S 1212.

Olson, J.A. and Krinsky, N.I. (1995). Introduction to carotenoids. *FASEB J* **9**:1547-1558.

Orth, R.A. and Shellenberger, J.A. (1988). Origin, production and utilization of wheat. In *Wheat Chemistry and Technology*, Pomeranz, Y. (ed), Vol. 1. pp. 11. American Asoociation of Cereal Chemists: St Paul, Minnesota, USA.

Pagnini-Hill, A., Chao, A., Ross, R.K. and Henderson, B.E. (1987). Vitamin A, β-carotene, and the risk of cancer: a prospective study. *J.Natl.Cancer Inst.* **79**:443-449.

Palozza, P. and Krinsky, N.I. (1992). Antioxidant effects of carotenoids *in vivo* and *in vitro* : An overview. *Methods in Enzymology* **213**:403-452.

Panasenko, O. M., Panasenko,O. O. *et al.* (1997). Hypochlorite destroys carotenoids in low density lipoproteins thus decreasing their resistance to peroxidative modification. *Biochem.-Moscow* **62**(10): 1140-1145.

Parker, R.S. (1989). Carotenoids in human blood and tissues. *J.Nutr.* **119**:101-104.

Passwater, R. (1993). Alpha carotenes and other carotenoids. *http://www.solgar.com/nutrition-library/articles/murakoshi-interview.html.*

Patton, J. and Dishaw, M. (1968). Flour colour evaluation with the green Agtron. *Cereal Sci. Today* **13**:163-188.

Paul, H.E. and Paul, M.F. (1946). The relation of vitamin A intake to length of life, growth, tooth structure and eye condition. *J.Nutr.* **31**:67-70.

Peiser, G.D. and Yang, S.F. (1979). Sulfite-mediated destruction of β-carotene. *J.Agric.Food Chem.* **27**:446-449.

Pennell, M.D., Davies, M.I., Rasper, J. and Motzok, I. (1976). Biological availability of iron supplements for rats, chicks and humans. *J.Nutr.* **106**:265-274.

Peto, R., Doll, R., Buckley, J.D. and Sporn, M.D. (1981). Can dietary β-carotene materially reduce cancer rates? *Nature* **290**:201-208.

Phillip, D. and Young, A.J. (1995). Occurrence of the carotenoid lactucaxanthin in higher plant LHC II. *Photosynth.Res.* **43**:273-282.

Pinzino, C. (1999). Aging, free radicals, and antioxidants in wheat seeds. *J.Agric.Food Chem.* **47**:1333-1339.

Pla, G.W. and Fritz, J.C. (1970). Vitamins and other nutrients: availability of iron. *JAOAC* **53**:791-800.

Pla, G.W. and Fritz, J.C. (1971). Collaborative study of the hemoglobin repletion test in chicks and rats for measuring availability of iron. *JAOAC* **54**:13-17.

Pla, G.W., Harrison, B.N. and Fritz, J.C. (1973). Comparison of chicks and rats as test animals for studying bioavailability of iron, with special reference to use of reduced iron in enriched bread. *JAOAC* **56**:1369-1373.

Pomeranz, Y. (1960). Determination of bread crumb color as related to the color of flour used to bake the bread. *Cereal Chem.* **37**:765-770.

Poor, C.L., Miller, S.D., Fahey, J.C.J., Easter, R.A. and Erdman, J.W. (1987). Animal models for carotenoid utilisation studies: Evaluation of the chick and pig. *Nutr.Rep.Inter.* **36**:229-234.

Potrykus, I., Burkhardt, P.K., Datta, S.K., Fütterer, J., Ghosh-Biswas, G.C., Klöti, A., Spangenberg, G. and Wunn, J. (1995). Genetic engineering of Indica rice in support of sustained production of affordable and high quality food in developing countries. *Euphytica* **85**:441-449.

Racia, N., Scott, J., Lowry, L. and Sauberlich, H.E. (1972). Vitamin A concentration in human tissues collected from five areas of the USA. *Am.J.Clin.Nutr.* **25**:291-296.

Ramakrishnan, T. and Francis, F. (1973). Color and carotenoid changes in heated paprika. *J.Food Sci.* **39**:25-28.

Ranger, C.R. and Neale, R.J. (1984). Iron availability from soy, meat and soy/meat samples in anemic rats with and without prevention of coprophagy. *J.Food Sci.* **49**:585-589.

Raymundo, L.C., Griffiths, A.E. and Simpson, K.L. (1967). Effect of DMSO on the biosynthesis of carotenoids in detached tomatoes. *Phytochem.* **6**:1527-1532.

Rechcigl, M., Berger, S., Loosli, J.K. and Williams, H.H. (1962). Dietary protein and utilization of vitamin A. *J.Nutr.*:435-440.

Reddy, M.B. and Cook, J.D. (1991). Assessment of dietary determinants of nonheme-iron absorption in humans and rats. *Am.J.Clin.Nutr.* **54**:723-728.

Ringer, T.V., Deloof, M.J., Winterrowd, G.E., Francom, S.F., Gaylor, S.K., Ryan, J.A., Sanders, M.E. and Hughes, G.S. (1991). Beta-carotene's effects on serum lipoproteins and immunologic indicies in humans. *Am.J.Clin.Nutr.* **53**:688-694.

Robinson, I.M., Dockerty, J.F. and Fearn, T. (1984). The conditioning of wheat: the influence of conditioning temperatures on flour yield, moisture content and colour. *J.Sci.Food Agric.* **35**:1340-11350.

Rock, C.L. (1997). Carotenoids: Biology and treatment. *Pharmacol.Therap.* **75**:185.

Roodenberg, A.J.C., West, C.E. and Beynen, A.C. (1996). Vitamin A status affects the efficacy of iron repletion in rats with mild iron deficiency. *J.Nutr. Biochem.* **7**:99-105.

Roodenburg, A.J.C., West, C.E., Beguin, Y., Van Dijk, J.E., Van Eijk, H.G., Marx, J.J.M. and Benyen, A.C. (2000). Indicators of erythrocyte formation and degradation in rats with either vitamin A or iron deficiency. *J.Nutr.Biochem.* **11**:223-230.

Roodenburg, A.J.C., West, C.E., Yu, S. and Beynen, A.C. (1994). Comparison between time-dependent changes in iron metabolism in rats as induced by marginal deficiency of either vitamin A or iron. *Brit.J.Nutr.* **71**:687-699.

Rosser, J.M. (1997). Extraction and Identification of Carotenoids in Bread Wheat. Honours Thesis. Department of Plant Science. The University of Adelaide, Adelaide, Australia.

Saiz, M.P., Marti, M.T. and Planas, J. (1990). Sexual and age variation of organ iron content in shaver chickens. *Brit.Poultry Sci.* **31**:339-349.

Sander, L.C. and Craft, N.E. (1990). Device for subambient temperature control in liquid chromatography. *Analytical Chem.* **62**:1545.

Sander, L.C. and Wise, S.A. (1989). Subambient temperature modification of selectivity in reverse phase HPLC. *Analytical Chem.* **61**:1749.

Sato, N., Gleave, M.E., Burchovsky, M., Rennie, P.S., Beralde, E. and Sullivan, L.D. (1997). A metastatic and androgen-sensitive human prostate cancer model using intrapostatic innoculation of LNCaP cells in SCID mice. *Cancer Res.* **57**:1584-1589.

Schiedt, K., Leuenberger, F.J., Vecchi, M. and Glinz, E. (1985). Absorption, retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken. *Pure Appl.Chem.* **57**:685-692.

Schwartz, J. and Shklar, G. (1987). Regression of experimental hamster cancer by beta-carotene and algae extracts. *J.Oral Maxillofac.Surg.* **45**:510-515.

Scita, G. (1992). Stability of β-carotene under different laboratory conditions. *Methods in Enzymology* **213**:175-185.

Scott, M.L., Nesheim, M.C. and Young, R.J. (1982). Nutrition of the Chicken. M.L. Scott and Associates, Ithaca, New York, USA.

Seddon, J.M., Ajani, U.A., Sperduto, R.D., Hillier, R., Blair, N., Burton, T.C., Farber, M.D., Gragoudas, E.S., Haller, J., Miller, D.T., Yannuzzi, L.A. and Willett, W. (1994). Dietary carotenoids, vitamin A, C, and E, and advanced age-related macular degeneration. *J.Am.Med.Assoc.* **272**:1413-1420.

Shah, B.G. and Belonje, B. (1985). Rat bioassay of hydrogen-reduced iron powder in flour and bread. *Nutr.Rep.Int.* **32**:1171-1178.

Shatrugna, V., Raman, L., Uma, K. and Sujatha, T. (1997). Interaction between vitamin A and iron: Effects of supplements in pregnancy. *Int..J.Vit.Nutr.Res.* **67**:145-148.

Shewfelt, R., Thai, C. and Davis, J. (1988). Prediction of changes in colour in raw, baked and smoked wild and pen-reared Atlantic salmon. *J.Food Sci.* **53**:1433-1437.

Shuey, W.C. (1976). Influence of wheat cultivars and environment on Agtron values and flour ash. *Cereal Chem* **53**:429-440.

Shuey, W.C., Maneval, R.D. and Dick, J.W. (1977). Eighty percent extraction of flour by tail-end regrinding and redressing. *Cereal Chem.* **54**:42-55.

Siefermann-Harms, D., Hertzberg, S., Borch, G. and Liaaen-Jensen, S. (1981). Lactucaxanthin, an ε,ε-carotene-3,3'-diol from *Lactuca sativa*. *Phytochem.* **20**:85-88.

Simpson, K., Zakana, M., Brown, P. and Krstulovic, A. (1979). Use of reverse phase high performance liquid chromatographs for the determination of provitamin A contents in tomato. *J.Chrom.* **176**:109-117.

Sims, R.P.A. and Lepage, M. (1968). A basis for measuring the intensity of wheat flour pigments. *Cereal Chem.* **45**:605-611.

Skarsaune, S.K. and Shuey, W.C. (1975). The effect of several variables on instrumental flour colour. *Cereal Foods World* **20**:286-288.

Sklan, D., Harlevy, O. and Donoghue, S. (1986). The effect of different dietary levels of vitamin A on metabolism of copper, iron and zinc in the chick. *Int.J.Vit.Nutr.Res.* **57**:11-18.

Slifka, K.A., Bowen, P.E., Stacewicz-Sapontzakis, M. and Chrissy, S.D. (1999). A survey of carotenoids in captive wild animals. *J.Nutr.* **129**:380-390.

Smirnoff, N. (1993). Tansley review 52. The role of active O2 in the response of plants to water deficit and desiccation. *New Phytol.* **125**:27-58.

Smith, J.C.J. (1980). The vitamin A - zinc connection: a review. *Ann NY Acad. Sci.* **355**:62-74.

Snodderly, D.M., Auran, J.D. and Delori, F.C. (1984). The macular pigment II. Spatial distribution in primate retinas. *Invest. Opthamol. Vis.Sci.* **25**:674-685.

Snodderly, M. (1995). Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am.J.Clin.Nutr.* **62**.

Solomons, N.W. and Russell, R.M. (1980). The interaction of vitamin A and zinc: implications for human nutrition. *Am.J.Clin.Nutr.* **33**:2031-2040.

Solon, M.A. (1986). Control of vitamin A deficiency by education and the public health approach. In *Vitamin A Deficiency and its Control*, Bauerfeind, J.C. (ed) pp. 285. Academic Press: New York, USA.

Sommerburg, O., Siems, W.G. and van Kuijk, F.J.G.M. (2000). Localisation of carotenoids in different eye tissues. *BioFactors* **11**:3-6.

Spady, D.K., Turley, S.D. and Dietschy, J.M. (1985). Receptor independent low density lipoprotein transport in the rat *in vivo*. *J.Clin.Invest.* **76**:1113-1122.

Stähelin, H.B., Gey, H.B., Eichholzer, M. and Ludin, E. (1991). Carotene and cancer prevention : the Basel study. *Am.J.Clin.Nutr.* **53**:265 S-269 S.

Stahl, W., Junghans,A. *et al.* (1998). Carotenoid Mixtures Protect Multilamellar Liposomes Against Oxidative Damage - Synergistic Effects of Lycopene and Lutein. *FEBS Letters* **427**(2): 305-308.

Suharno, D., West., C.E., Muhilal, Karyadi, D. and Hautvast, J.G.A.J. (1993). Supplementation with vitamin A and iron for nutritional anaemia in pregnant women in West Java, Indonesia. *Lancet* **342**:1325-1328.

Sure, B., Kik, M.C. and Waters, D.J. (1929). The effect of avitaminosis on hematopoietic function. I Vitamin A deficiency. *J.Biol.Chem.* **83**:375-379.

Takahata, Y., Noda, T. and Nagata, T. (1993). HPLC determination of β-carotene content of sweet potato cultivars and its relationship with colour values. *Japan J. Breed.* **43**:421-427.

Tang, G., Gu, X.-F., Hu, S.-M., Xu, Q.-M., Qin, J., Dolnikowski, G.G., Fjeld, C.R., Gao, X., Russell, R.M. and Yin, S.-A. (1999). Green and yellow vegetables can maintain body stores of vitamin A in Chinese children. *Am.J.Clin.Nutr.* **70**:1069-1076.

Taylor, R.F. and Little, A.D. (1983). Chromatography of carotenoids and retinoids. *Adv. Chrom.* **22**:157-206.

Terao, J., Nagano, A., Song, J.H. and Parks, D.K. (1990). In *9th International Symposium on Carotenoids* pp. 153.

The Eye Diseases Case-Control Study Group (1992). Risk factors for neovascular age-related macular degeneration. *Arch.Opthamol.* **110**:1701-1708.

The Eye Disease Case-Control Study Group (1993). Antioxidant status and neovascular age-related macular degeneration. *Arch.Opthamol.* **111**:104-109.

Thiele, A. and Krause, G.H. (1994). Xanthophyll cycle and thermal energy dissipation in photosystem II: relationship between zeaxanthin formation, energy dependent fluorescence quenching and photoinhibition. *J.Plant Physiol.* **144**:324-332 □.

Thommen, H. (1979). The role of carotenoids in plant physiology. *Pure Appl.Chem.* **51**:867-869.

Tyczkowski, J.K. and Hamilton, P.B. (1986). Evidence for differential absorption of zeta-carotene, cryptoxanthin, and lutein in young broiler chickens. *Poultry Sci.* **65**:1137-1140.

Udomkesmalee, E., Dhanamitta, S., Sirisinha, S., Charoenkiakul, S., Tuntipopipat, S., Banjong, O., Rojroongwasinkul, N., Kramer, T. and Smith, J.C.J. (1992). Effect of vitamin A and zinc supplementation on the nutriture of children in Northeast Thailand. *Am.J.Clin.Nutr.* **56**:50-57.

UNICEF. (2000). *www.unicef.org/vitamina/.*

US Institute of Medicine, F.N.B., Committee on the Scientific Evaluation of Dietary Reference Intakes. (2000). *Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc.* National Academy Press: Washington DC., USA.

Vallee, B.L. and Hoch, F.L. (1957). Zinc in horse liver alcohol dehydrogenase. *J.Biol.Chem.* **225**:622-626.

van het Hof, K.H., Brouwer, I.A., West, C.E., Haddeman, E., Steegers-Theunissen, R., van Dusseldorp, M., Weststrate, J.A., Eskes, T.K.A.B. and Hautvast, J.G.A.J. (1999). Bioavailabilty of lutein from vegetables is 5 times higher than that of β-carotene. *Am.J.Clin.Nutr.* **70**:261-268.

van het Hof, K.H., West, C.E., Weststrate, J.A. and Hautvast, J.G.A.J. (2000). Dietary factors that affect the bioavailability of carotenoids. *J.Nutr.* **130**:503-506.

van Lieshout, M., West, C.E., Permaesih, D., Muhilal, Wang, Y., Sun, Y., van Breeman, R.B., Creemers, A.F.L., Verhoeven, M.A. and Lugtenburg, J. (2001). Bioefficacy of β-carotene dissolved in oil studied in children in Indonesia. *Am.J.Clin.Nutr.* **73**:949-958.

van Poppel, G. (1993). Carotenoids and cancer. *Eur.J.Cancer* **29A**:1335-1344.

Varma, S.D. (1991). Scientific basis for medical therapy of cataracts by antioxidants. *Am.J.Clin.Nutr.* **53**:335S-245S.

Wald, G. (1945). Human vision and the spectrum. *Science* **101**:653-658.

Wald, G. (1968). The molecular basis of visual excitation. *Nature* **219**:800-807.

Watson, C.A. and Shuey, W.C. (1977). Effect of storage time on Agtron reflectance values of flour. *Cereal Chem.* **54**:64-69.

Weedon, B.C.L. and Moss, G.P. (1995). Structure and nomenclature. In *Carotenoids: Isolation and Analysis*, G. Britton, S. Liaaen-Jensen & H. Pfander (eds), Vol. 1A. pp. 27-70. Birkhauser Verlag: Basel.

Welch, R.M. and Graham, R.D. (1999) A new paradigm for world agriculture: meeting human needs. Productive, sustainable, nutritious. *Field Crops Res.* **60**:1-10

Welch, R.M., Combs, G.F. and Duxbury, J.M. (1997). Toward a 'Greener' revolution. *Issue.Sci.Technol.* **Fall 1997**:50-58.

West, C.E. and Eilander, A. (2001). Bioefficacy of carotenoids. *Sight and Life* **2001**:6-10.

West, C.E., Sijtsma, S.R., Peters, H.P.F., Rombout, J.H.W.M. and van der Zipp, A.J. (1992). Production of chickens with marginal vitamin A deficiency. *Brit.J.Nutr.* **68**:283-291.

Whittaker, P. and Vanderveen, J.E. (1990). Effect of EDTA on the bioavailability to rats of fortification iron used in Egyptian balady bread. *Brit.J.Nutr.* **63**:587-595.

WHO. (1992). National strategies for overcoming micronutrient malnutrition: Geneva.

Wildfeuer, L. and Acker, L. (1968). Uber die Bestimmung von Carotinoiden in Teigwaren und deren Robstoffen. *Mitt.Geb.Lebensmittelunters Hyg.* **59**:392-395.

Williams, W.P., Davies, R.E. and Couch, J.R. (1962). The utilization of carotenoids by the hen and chick. *Poultry Sci.* **42**:691-699.

Woodall, A.A., Lee, S.W.M., Weesie, R.J., Jackson, M.J. and Britton, G. (1997a). Oxidation of carotenoids by free radicals - Relationship between structure and reactivity. *Biochimica et Biophysica Acta - General Subjects* **1336**:33-42.

Yap, S.C., Choo, Y.M., Hew, N.F. and Goh, S.H. (1997). Distribution of dietary palm carotenoids and their metabolites in the rabbit. *Nutr.Res.* **17**:1721-1731.

Yeum, K.J., Taylor, A., Tang, G. and Russell, R.M. (1995). Measurement of carotenoids, retinoids, and tocopherols in human lenses. *Inves.Opthamol. Vis. Sci.* **36**:2576.

Zakaria, M. and Simpson, K. (1979). Use of HPLC for the determination of provitamin A carotenes in tomatoes. *J.Chrom.* **176**:109-117.

Zamora, R., Hidalgo, F.J. and A.L., T. (1983). Comparative antioxidant effectiveness of dietary β-carotene, vitamin E and co-enzyme Q in rat erythrocytes and plasma. *J.Nutr.* **121**:50-54.

Zeleny, L. (1954). *Storage of cereals and their products*. American Association of Cereal Chemists: St Paul, Minnesota, USA.

Zhang, D., Hendricks, D.G. and Mahoney, A.W. (1992). Effect of coprophagy on bioavailability of iron from plant foods fed to anemic rats. *Plant Foods Hum.Nutr.* **42**:97-108.

Ziegler, R.G. (1991). Vegetables, fruits and carotenoids and the risk of cancer. *Am.J.Clin.Nutr.* **53**:251S-259S.