General discussion and Summary

Malnutrition among vulnerable groups is a great health problem in the world, which includes both under- and over-nutrition. The major under-nutrition problems are protein-energy malnutrition and deficiencies of the micronutrients—vitamin A, iron and iodine. Among the micronutrient malnutrition, vitamin A deficiency (VAD) is considered to be major in the world. Vitamin A is obtained from the diet as provitamin A carotenoids from fruits and vegetables or as preformed retinyl esters from liver, eggs, dairy products or fortified foods. Excess of vitamin A is stored as retinyl esters predominantly in the liver, although other tissues too contain some stores. Vitamin A is released from the liver as retinol and transported in plasma by retinol binding protein. The plasma concentration of retinol in the nutritionally adequate individual is tightly regulated by homeostatic mechanisms.

VAD is a major public health problem in many parts of the world: over 131 million children of preschool age are vitamin A deficient and 4.4 million preschool children are afflicted with xerophthalmia (West, 2002). The basis for the VAD in developing countries stem from low intake of vitamin A rich foods, however, vegetables and fruits are usually consumed less with insufficient amounts of fat in the diet. Children, pregnant and lactating women are often vitamin A deficient and in addition, parasitic and other infections increase the vitamin A requirements. Miller et al. (2002) reported that the diets of children in developing countries without vitamin A supplementation do not meet the vitamin A requirements. The situation is mainly due to low concentrations of vitamin A in mothers’ milk. Inadequate dietary intake of vitamin A during and after weaning and the high prevalence of childhood illnesses are other added causes for VAD among children. According to the WHO criteria, VAD is a significant health problem in India (WHO/UNICEF, 1996).

Control of VAD depends to a large degree on an adequate supply of vitamin A. The strategies involved in prevention and control of VAD may be defined as short, medium and long term based on the action taken. Short-term or an emergency measure includes the administration of single, large doses of vitamin A to vulnerable groups on a periodic basis. The Government of India has a vitamin A policy and an operational plan
to address VAD nation-wide, to give 5 mega-doses of vitamin A to each child between 9 months and 5 years of age. The first dose is 100,000 IU, given along with measles immunization. The second to fifth doses are 200,000 IU, were given every six months. The program targets children with access to Government’s services, either through primary health care or the Integrated Child Development Services (Ministry of Health and Family Welfare, Government of India, 1991). However, in 1998, the number of children benefited by the program was estimated to be one quarter of the total eligible population (UNICEF, 2000). Problems include workers not being clear on the protocol for vitamin A distribution and irregularities in supply (Vijayaraghavan, 1995). Based on the recommendations of a review committee, the program being modified to improve the outreach to the target population (ACC/SCN, 1992). The issue of providing vitamin A supplements to lactating mothers will be explored (Government of India, 1996).

In the medium-term, the fortification of a dietary vehicle (sugar or monosodium glutamate) with vitamin A was initiated. Effort has also been made to find a suitable vehicle for fortification with vitamin A. Several foods are currently being fortified, including milk and margarine. However, they do not reach most of the population in need due to its cost effectiveness.

Long-term strategy involves the consumption of provitamin A carotenoids rich foods like dark green leafy vegetables, vegetables and fruits. Increased dietary intake of vitamin A through home gardening and nutrition education programs comprises the long-term solution to this problem. Home gardening and nutrition education programs are strategies, which have been recommended (Government of India, 1996). Increased production of vitamin A rich foods through home gardens, school gardens and community gardens can result in increased consumption of vitamin A rich foods and lead to a reduction in the incidence of VAD.

Carotenoids possessing provitamin A activity are generally regarded as an important dietary source of vitamin A for humans. Among these, \( \beta \)-carotene has the highest provitamin A activity. In addition, a potential beneficial health effect of \( \beta \)-carotene unrelated to its role as provitamin A has attracted more attention in the past few decades. In Asia and Africa where VAD is a public health concern, provitamin A carotenoids contribute over 80% of the food supply. Rural communities depend primarily on foods rich in provitamin A carotenoids such as dark green leafy vegetables, yellow
and orange vegetables and fruits for their vitamin A supply. In recent years, several indigenous foods rich in \( \beta \)-carotene have emerged as potential sources to meet vitamin A requirements. To persuade increased consumption of vitamin A rich foods, it is essential to determine the vitamin A value of agric/ horticultural produce, which in turn helps rural community to select right source of provitamin A rich foods to combat VAD (Suber et al. 1995; USDA-NCC, 1998). Hence, the vitamin A activity of plant foods is very much essential as it varies depending on the geographical region. We have reported the carotenoid composition and vitamin A activity of GLVs, which are commonly consumed in southern part of India (Lakshminarayana et al. 2005; Raju et al. 2007). The data on carotenoid composition may help in identifying specific carotenoids rich GLVs for supplementation purposes and for providing information to consumers and public health workers to assess the dietary carotenoid intake and their relationship between health and diseases. However, the supply of vitamin A from plant sources is not only determined by the actual carotenoid content of a food, but also the bioavailability/bioefficacy of these carotenoids, especially \( \beta \)-carotene to retinol. Thus, the food matrix in which \( \beta \)-carotene is embedded is important. Release of \( \beta \)-carotene from the food matrix is more difficult than releasing it from a lipid droplet. It has been shown that \( \beta \)-carotene fed in fat or a simple matrix is more bioavailable than \( \beta \)-carotene from vegetables (Brown et al. 1989; de Pee et al. 1998; Van het Hof et al. 1999; Huang et al. 2000).

Several studies have suggested that carotenoid bioavailability is enhanced by the addition of dietary fat. Dietary fat is the vehicle for transport of both vitamin A and carotenoids. Fat facilitates the absorption of \( \beta \)-carotene by increasing the bile-flow, which in turn facilitates the transport of \( \beta \)-carotene into the mucosal cells (El-Gorab et al. 1975; Jayarajan et al. 1980). Plasma and serum \( \beta \)-carotene responses after a meal are increased dramatically by the presence of dietary fat (Prince and Frisoli, 1993; Shiau et al. 1994). When dietary fat is absent or intake is too low, absorption of \( \beta \)-carotene was reduced (Jialal et al. 1991; Prince and Frisoli 1993). Nevertheless, addition of even a small amount of fat to the diet improves the absorption of carotenoids from vegetables (Roels et al. 1958) and optimal absorption may require an intake of as little as 3-5 g of fat per meal (Jayarajan et al. 1980; Jalal et al. 1998; Roodenburg et al. 2000; Van het Hof et al. 2000). The type of fat in the meal ingested with \( \beta \)-carotene also reported to
influence the degree of absorption; beef tallow resulted in a greater absorption when compared with sunflower oil (Hu et al. 2000) and long-chain triglycerides were better than medium-chain triglycerides, which primarily are absorbed via the portal route (Borel et al. 1998b).

The bioavailability of pure β-carotene is not representative of the β-carotene in foods as several factors such as the species of carotenoids, matrix in which it is incorporated and absorption modifiers may influence bioavailability greatly (de Pee et al. 1995; Torronen et al. 1996; Castenmiller et al. 1999). The β-carotene absorption in vivo involves sequential steps starting from its release from food matrix, dispersion in emulsion, solubilization into mixed micelles and permeation to intestinal mucosal cells and incorporation into lymphatic lipoproteins (Dimitrov et al. 1988; Olson, 1994; Furr and Clark, 1997). Thus, the carotenoids must be solubilized in mixed micelles before cellular uptake. These processes are dependent mostly on the physicochemical properties of the food matrix (Jalal et al. 1998). Moreover, de Pee et al. (1998) demonstrated that β-carotene is less available from dark green leafy vegetables than from fruit. Studies have indicated that β-carotene from raw vegetables is less available than that from cooked or processed vegetables (Torronen et al. 1996; Rock et al. 1998). While the bioavailability of β-carotene from dark green leafy vegetables have been questioned (de Pee et al. 1995), Takyi (1999) showed the value of β-carotene from dark green leafy vegetables in improving vitamin A status.

Data on the physicochemical properties of carotenoids in biological emulsions are essential to know the pathway of carotenoid metabolism. Better understanding of the physicochemical properties of carotenoids, their interactions with various phospholipids, is needed to clarify their functions in biological membranes. Carotenoids are in the immediate vicinity of their constituent molecules such as lipids and proteins in organized structures in biological membranes and must be able to fit into this complex system with the correct position and orientation (Britton, 1995). We have studied the role of dietary factors (phospholipids and fatty acids) on various physicochemical properties like pH and viscosity of the micellar solution, particle size and structure of the mixed micelles. The extent of β-carotene incorporated to the micelles was determined by image processing technique. From the results it is concluded that, the factor that determines
the incorporation of \( \beta \)-carotene into the micelles is the type of phospholipid, fatty acid, carotenoid and pH of the micellar solution.

We have followed the postprandial appearance of \( \beta \)-carotene and its major cleavage product, retinyl palmitate in plasma as a measure of intestinal \( \beta \)-carotene absorption and cleavage after a single dose of micellar \( \beta \)-carotene with phospholipids in rats (Raju et al. 2005). \( \beta \)-Carotene levels in the plasma and liver after an oral administration were not significantly different between the NoPL and PC groups, but were significantly lower compared with LPC group. The higher mean level of \( \beta \)-carotene in the liver and its AUC value for the LPC group compared with those in the other two groups indicated an enhanced accumulation of \( \beta \)-carotene in the liver of LPC group. Similarly, plasma retinyl palmitate in the LPC group increased significantly higher than the base line level after \( \beta \)-carotene administration. The increase in retinyl palmitate might be due to the enhanced uptake of \( \beta \)-carotene and its metabolism in the intestinal cells (Olson, 1994). The higher level of plasma retinyl palmitate in LPC group might be due to conversion of \( \beta \)-carotene into retinyl palmitate in the cells by influencing the activity of enzymes \( \beta \)-carotene dioxygenase and PLA\(_2\) than the other two groups (Sugawara et al. 2001).

The results of the study with fatty acids in rats demonstrate that the influence of micellar oleic and eicosapentaenoic acids on the plasma \( \beta \)-carotene level was higher than that of linoleic acid. Plasma response of newly absorbed \( \beta \)-carotene was significantly higher in OA group compared with LA and EPA groups (Raju et al. 2006). A significantly larger particle size of the mixed micelles containing linoleic acid could be one of the reasons for the lower level of plasma \( \beta \)-carotene compared with smaller particle size of micelles containing oleic acid. The elevated plasma retinyl palmitate after single dose of \( \beta \)-carotene solubilized in OA and EPA micelles than LA micelles suggests that these fatty acids may influence the cleavage of more \( \beta \)-carotene molecules to retinol, which may be further esterified with palmitic acid. Results of repeated dose study with phospholipids and fatty acids showed significantly higher level of plasma \( \beta \)-carotene, its accumulation in the liver and its conversion into vitamin A than normal rats. Plasma \( \beta \)-carotene level in OAR and LPCR groups were lower whereas, the retinyl palmitate level in plasma and its accumulation in liver of the same groups were
significantly higher than other groups. This may be due to increased activity of \( \beta \)-carotene cleavage enzyme in those groups as determined by \textit{in vitro}. The results of the present study and the previous \textit{in vitro} and \textit{in vivo} studies using rat and Caco-2 cells (Rahman, 2000; Jackson, 2002), suggest that the lipolysis or hydrolysis of triglycerides and release of fatty acids in the intestinal tract by lipase is vital for the efficient uptake or esterification of carotenoids. These fatty acids play a significant role in the solubilization of carotenoids in lipid micelles and their efficient uptake by the enterocytes (Borel et al. 1996).

The absorption of carotenoids is also dependent on the type and amount of other carotenoids present in the food matrix. In the present study, an equimolar amount of \( \beta \)-carotene and lutein solubilized in the mixed micelles was given to rats with phospholipids. Results show higher level of \( \beta \)-carotene and retinyl palmitate in plasma and liver \( \beta \)-carotene than lutein. There was no evidence that absorption of provitamin A is affected by either carotene or vitamin A status, because absorption occurs through passive diffusion. However, the serum retinol level influences the conversion of provitamin A to retinol. A low-vitamin A status appears to increase \( \beta \)-carotene cleavage (Villard and Bates, 1986; Van Vliet et al. 1996). In addition, intra individual variability in the conversion of \( \beta \)-carotene to retinol may contribute to the variable response to consumption of \( \beta \)-carotene (Lin et al. 2000; Van Lieshout et al. 2001). An improved \( \beta \)-carotene absorption and its conversion into vitamin A in VAD rats was observed in the present study after a single oral dose of micellar \( \beta \)-carotene containing phospholipids and fatty acids than vitamin A sufficient rats. Studies in rats have shown that conversion of \( \beta \)-carotene to retinol is higher if the rats are vitamin A deficient and lower if they are protein deficient (Parvin and Sivakumar, 2000).

The bioavailability of pure \( \beta \)-carotene is not representative of the \( \beta \)-carotene in foods, as several factors such as the species and amount of carotenoids, matrix in which it is incorporated and absorption modifiers, amount and type of fat and nutrient status of the individual may influence availability greatly. The type of fat may also affect carotenoid absorption. In this study, we used the \( \beta \)-carotene rich green leafy vegetable (\textit{Chenopodium album} L.) with specific fatty acid rich vegetable oils (as fat source) \textit{viz.}, groundnut, sunflower, olive and soybean oils and soy PC (phospholipids source) to
Chapter 7. General discussion and Summary

evaluate their effect on the bioefficacy of β-carotene from GLV. The results show that, improved vitamin A status in VAD rats fed GLV with olive and soybean oil than other oils used in this study. The growth rate of VAD rats improved significantly after feeding the GLV supplemented diet. This is in agreement with Clark et al. (2000) who found more efficient absorption of carotenoids by rats when the carotenoids (lycopene and astaxanthin) were administered in olive oil than in corn oil. The possible role of olive oil may be due to its increased synthesis of triacylglycerol rich chylomicron, which helps in better incorporation of β-carotene and thereby reflects in increased plasma level. Although, the exact mechanism of olive oil effect on the intestinal uptake of β-carotene is not clear, the possible role may be on secretion of bile, which in turn facilitates the formation of micelles and incorporation of β-carotene and its intestinal uptake. Phospholipids, especially PC, are present in the diet either as a natural component of the food matrix or as an emulsifier/ stabilizer in processed foods (Arzt, 1990; Zeisel, 2003). Soy PC influences increased absorption of dietary β-carotene and its cleavage in the present study. Feeding rats with diet supplemented with soybean lecithin can stimulate bile formation and secretion rate of bile acids, phospholipids and cholesterol (LeBlanc et al. 1998). Additionally, the bile constitutes a large physiological pool of phospholipids (mainly PC) (Tso, 1991). Although both dietary and biliary PC are important for the emulsification of dietary lipids in the digestive tract, the presence of PC in mixed micelles inhibits the absorption of carotenoids by human intestinal Caco-2 cells (Sugawara et al. 2001; Yonekura, 2006) and mice (Baskaran et al. 2003), most likely by shifting the carotenoid partition into the micellar phase. However, during the normal digestive process, most of the dietary and biliary PC are hydrolyzed by phospholipases, producing lysophosphatidylcholine (LPC) which restore or even enhance β-carotene and lutein absorption by Caco-2 cells and experimental animals (Sugawara et al. 2001; Baskaran et al. 2003; Raju et al. 2005).

The activity of lipases (pancreatic lipase and PLA2) was determined to study the level of fatty acids released in the intestinal mucosa for the formation of micelles and the extent to which the release of β-carotene from the food, its incorporation into the mixed micelles and transportation to the target tissues through intestinal mucosa. Lipid profile in plasma and intestinal mucosa was determined to study the possible role of dietary
modulators used in this study on the transport of absorbed β-carotene into target organs. β-Carotene cleavage enzyme activity in vitro was found to be higher in the intestinal mucosa of normal rats fed micellar β-carotene with phospholipids. Hence, the increased plasma level of retinyl palmitate was observed in those groups compared with the NoPL groups (Raju et al. 2005). There have been few studies in which enzyme activity in vitro was compared with in vivo. Van Vliet et al. (1996) correlated the in vitro intestinal β-carotene dioxygenase activity with the in vivo β-carotene conversion to vitamin A. They concluded that the activity of the intestinal enzyme is an adequate indicator of in vivo cleavage activity.

There were significant changes in the plasma lipid profile after single and repeated dose micelles containing β-carotene with phospholipids and fatty acids. The plasma triglycerides level was found to increase in the rats fed with micellar β-carotene containing phospholipids and fatty acids. Similarly, the level of phospholipids and total cholesterol also showed significant change in the plasma of rats fed micellar β-carotene with phospholipids and fatty acids. The plasma fatty acid composition clearly demonstrated the possible mechanism of β-carotene uptake from the mixed micelles containing phospholipids and fatty acids. After the gavage of micellar β-carotene with phospholipids and fatty acids (single and repeated dose), there was a significant change in the plasma fatty acid profile compared to control group. VAD is found to alter the lipid composition significantly compared with the control rats. During VAD there was a decreased level of plasma triglycerides, total cholesterol and phospholipids than control group. It is evidenced that there was a significantly higher level of β-carotene in the plasma after a single dose of micellar β-carotene along with phospholipids. Changes in the phospholipids would compromise the integrity and function of cell membranes as they depend on the lipid balance, especially on the cholesterol/phospholipid ratio. There was an altered fatty acids level in the plasma of VAD rats compared with the normal rats. The fatty acid level in the mixed micelles was reflected in the plasma of VAD rats fed micellar β-carotene with phospholipids. There was an increased level of other fatty acids apart from the micellar fatty acids, which clearly shows the re-secretion of the fatty acids from the enterocytes into the circulation.
In conclusion, the present study shows that the green leafy vegetables are very good sources of provitamin A carotenoids. They are rich sources of $\beta$-carotene, possessing high vitamin A activity as determined by HPLC. Physiochemical properties of the mixed micelles containing $\beta$-carotene with phospholipids and fatty acids determine the extent of $\beta$-carotene, which incorporated into the mixed micelles as determined by the image processing technique. This gives an idea about the process of intestinal $\beta$-carotene uptake and its conversion into retinol. The dietary factors (phospholipids and fatty acids) used in the present study were found to improve bioavailability and bioconversion $\beta$-carotene into vitamin A after a single, repeated dose of micellar and dietary $\beta$-carotene in normal and vitamin A deficient rats. Feeding micellar $\beta$-carotene with phospholipids and fatty acids to VAD rats improved its absorption and cleavage in the intestinal mucosa, which resulted in higher plasma $\beta$-carotene and its cleavage product than the vitamin A sufficient rats. Dietary studies with VAD rats showed increased gain in body weight and vitamin A status after feeding GLV supplemented diet with different vegetable oils (fatty acids source) and soy PC (phospholipid source). The activity of $\beta$-carotene and retinol metabolizing enzymes were improved under VAD, which was comparatively higher than normal rats. Feeding GLV supplemented diet to the VAD rats improved the effects of VAD by normalizing the altered lipid profile and activity of enzymes very close to the control (vitamin A sufficient) rats.

Summary

1. This study gives a complete picture on carotenoid composition of commonly and less commonly known GLVs. This is the first report showing HPLC data on carotenoids and their vitamin A activity of locally available GLVs.

2. The procedure adapted for the extraction, purification and HPLC analysis of carotenoids is relatively simple, reliable and accurate for the determination of carotenoids and vitamin A activity of the plant materials.

3. Interestingly, most of the less commonly consumed GLVs are the richest source of lutein, $\beta$-carotene and RE. Hence, these GLVs could be exploited as good sources of vitamin A and lutein to overcome VAD and AMD.
4. The nutritional significance of these findings is clear since these GLVs are important sources of vitamin A for a majority of the communities in the country. The data on the carotenoid composition could be helpful to create nutritional awareness among various communities on the importance of these GLVs.

5. Effect of specific phospholipids and fatty acids on particle size, structure, pH and viscosity of the mixed micelles was determined in vitro. Results revealed that these physicochemical properties interfere with solubilization of carotenoids in the mixed micelles and their bioavailability.

6. Addition of linoleic acid to the mixed micelles resulted in increased particle size of the micelles, pH and viscosity with lower level of β-carotene incorporation into the micelles than oleic acid, which may be due the level of unsaturation in fatty acid. Similarly addition of phosphatidylcholine to the micelles resulted in lower intensity of incorporated β-carotene than lysophosphatidylcholine. The possible reason for the above result may be due to the orientation of β-carotene in micelles containing phospholipids and fatty acids.

7. Application of image processing technique for analyzing β-carotene incorporation into the micelles is an important and newer method, which gives direct indication on the amount of β-carotene incorporated. The techniques used in the present study for determining various physicochemical properties of mixed micelles will provide an insight to understand the mechanism of formation of micelles and their characteristic features and their quantitative capacity to incorporate provitamin A carotenoids.

8. Enhanced β-carotene bioavailability and its conversion into vitamin A from micelles containing β-carotene with specific phospholipids and fatty acids was observed in normal and vitamin A deficient rats. The effect was higher in VAD rats compared with the normal rats.

9. The mechanism by which these dietary factors improved the β-carotene bioavailability in rats fed mixed carotenoids dispersed in mixed micelles showed better result on the carotenoids interactions during their intestinal uptake and conversion.
10. The extent to which the intestinal $\beta$-carotene uptake and its cleavage into retinol was dependent on the physicochemical properties of mixed micelles.

11. The supplementation of diet with $\beta$-carotene rich leafy greens containing phospholipids or fatty acids show promising results in the alleviation of VAD in rats and this strengthens the efficacy of food-based strategy as preventive measure in humans.

12. Further, $\beta$-carotene supplementation with physiological emulsions containing phospholipids (PC or LPC) and fatty acids (OA or EPA) can improve the plasma retinol status, which in turn help in overcoming vitamin A deficiency disorders.

13. The present study reports the effect of feeding a single and repeated oral dose of micellar $\beta$-carotene with phospholipids and fatty acids on the activity of $\beta$-carotene cleavage enzyme in the intestinal mucosa of rats.

14. The activity of $\beta$-carotene cleavage enzyme was higher in groups fed above dietary factors (phospholipids and fatty acids) than the groups that did not receive any fat.

15. The plasma lipid profile also showed positive response to the micellar $\beta$-carotene containing phospholipids and fatty acids. Similar response was observed after an equimolar dose of micellar $\beta$-carotene and under VAD condition.

16. Further, to assess the possible role of these dietary factors on the plasma lipid profile and also on the $\beta$-carotene and retinol metabolizing enzymes, the rats were fed with GLV supplemented diet with different vegetable oils (fatty acid source) and soy PC (phospholipid source) and the above parameters were determined in normal and vitamin A deficient rats.

17. From the results it is concluded that, the type of fat and nutritional status of the individual plays a major role in the metabolism of $\beta$-carotene. Further, it also stimulates various retinoid-metabolizing enzymes as observed in the present study.

18. The type of fat play a significant role in stimulating the lipolytic enzymes to release the fatty acids which in turn helps in the formation of mixed micelles and incorporation of $\beta$-carotene thereby improving the vitamin A status in rats.