CHAPTER 6
SUMMARY AND CONCLUSIONS

Vitamin A deficiency has been recognized as a common public health problem affecting millions of children. Dietary intervention promoting the consumption of provitamin A rich food seems to be a sustainable public health measure for promoting vitamin A status in target population. But the bioavailability of carotenoids is confounded by many factors that affect their absorption, conversion, transport and metabolism. There are different indirect methods to assess the vitamin A status, but none of these methods give the exact picture and it is rather difficult to evaluate intervention programmes. Recent developments in the methodologies particularly stable-isotope dilution methods, provide an accurate determination of vitamin A/provitamin A status in humans where other invasive methods are not possible. Stable isotopically labeled vitamin A precursors such as β-carotene is required for isotope dilution methods. Results of investigations carried out to produce intrinsically labeled β-carotene using the blue-green algae, Spirulina platensis are presented here. The influence of various dietary factors such as protein and fat on the uptake of β-carotene from natural sources has also been studied using different animal models. Studies were also carried out to examine, whether exfoliated colonic epithelial cells can be used as a non-invasive tool to assess the bioavailability of carotenoids in humans. The major observations of these studies are summarized below.

1. Stable isotopically labeled provitamin A carotenoids are required for the bioavailability studies. Spirulina platensis is known to produce high amount of provitamin A carotenoids than any other green leafy vegetables. Experiments were carried out to produce intrinsically labeled β-carotene for bioavailability studies using Spirulina and the results are summarised below.

(a) Culture conditions were standardised for growth of Spirulina cultures in Zarrouk’s medium in normal water to produce optimum amount of biomass and β-carotene using different light intensities, nitrate starvation and inducing copper
stress. Significant increase in the biomass and β-carotene production was observed when high light intensity and low nitrate level in the medium, were used.

(b) Analysis of total carotenoids produced by the algae was done. Seven different individual carotenoids including lutein, zeaxanthin, cryptoxanthin, α-carotene, 13-cis-β-carotene, trans-β-carotene were identified, of which β-carotene constituted about 24%.

(c) Conditions were standardized for metabolic labelling of carotenoids in algal cells with ^{14}C bicarbonate. Flask cultures of *Spirulina* grown in Zarrouk’s medium supplemented with ^{14}C bicarbonate produced ^{14}C labeled carotenoids. Light intensity and nitrate level were optimized for maximum labelling intensity of β-carotene and optimum biomass production.

(d) Optimum conditions for growth of *Spirulina* in Zarrouk’s medium prepared in heavy water (D\(_2\)O) were established so that optimum biomass and maximum amount of intrinsically labeled β-carotene could be produced. *Spirulina* cultures enriched in D\(_2\)O were grown under optimum light and nutrient conditions in heavy water medium in flasks. β-carotene isolated from the algae grown in deuterated cultures were analysed. Although there was reduction in the amount of biomass produced compared to normal water medium, significant amount of carotenoids were produced.

(e) β-carotene isolated from the algae grown in the deuterated cultures were analysed for the \(^2\)H using Mass Spectrometry. MS analysis showed that about 60 to 65 % H atoms in β-carotene were replaced by deuterium as evident from the shift in the m/z ratio from 535 (unlabeled β-carotene) to a range of 545 to 565.

(f) Production of metabolically labeled β-carotene with complete deuteration was possible. About 100%, replacement of H atom with deuterium in the C\(_{40}\)H\(_{56}\) molecule of β-carotene was achieved. This was achieved by suitably controlling the atmospheric exchange of the moisture by growing deuterium enriched cells in flask cultures under closed conditions.

(f) Spent medium was used repeatedly for up to 6th cycle without much reduction in the biomass and β-carotene whereby the cost of production of deuterated compounds can be reduced.

Results of mass spectrometric analysis demonstrate over 60% incorporation of deuterium into the β-carotene, produced by algal cells as evidenced from the shift of m/
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1. The ratio to 565 compared to unlabeled \( \beta \)-carotene. High intensity of the label incorporation is essential for tracer analysis studies in human intervention trials where only very little amount of the \( \beta \)-carotene may be recovered from the blood samples following a feeding experiment.

2. Flask cultures of *Spirulina* in heavy water medium produced biomass. In order to scale-up the production, a transparent vertical column mini bioreactor with 1 L capacity was designed and standardised the conditions for the growth of *Spirulina platensis* in heavy water as indicated below.

   (a) Suspension cultures of *Spirulina* enriched in heavy water was inoculated into bioreactor and grown in heavy water. Further optimization of biomass production was achieved by bubbling \( \text{CO}_2 \)-air mixture. Productivity of the bioreactor was tested by growing the algae up to 40 days and compared it with growth in flasks. Comparatively greater amount of biomass was obtained from the bioreactor culture. The difference in biomass production may be due to the high rate of photosynthesis, uniform distribution of algal cell and temperature stability in the bioreactor. This indicates that the proposed bioreactor is more productive than the conventional cultures in flasks.

   (b) Growth of *Spirulina* in bioreactor using spent medium showed that the same medium can be used repeatedly for five cycles without loss in biomass and labeled carotenoid production, whereby the cost of production of the deuterated carotenoids can be further reduced.

   (c) MS analysis of the carotenoids showed that about 60\% of the H atoms in the \( \beta \)-carotene was replaced by \( ^2\text{H} \).

   The relatively good yield of deuterated provitamin A carotenoid particularly \( \beta \)-carotene from the *Spirulina* cultures produced in mini bioreactor makes it a useful system for producing deuterated \( \beta \)-carotene for bioavailability studies. The deuterated-retinol-dilution (DRD) technique provides a quantitative estimate of total body stores of vitamin A.

3. The bioavailability of \( \beta \)-carotene from *Spirulina*, Agathi, and carrot was studied under different dietary conditions using rat, rabbit and monkey animal models. In the present study change in the level of \( \beta \)-carotene in the plasma after a single dose of supplement, was taken as a measure of the bioavailability in experimental animals.
such as rabbits, rats, and monkeys. These animals were supplemented with *Spirulina*, as a source of carotenoids and the plasma response was studied under different dietary conditions involving protein and fat and the following results were obtained.

(a) While the plasma of animals receiving fat containing diet showed maximum level of β-carotene in 6 hours after ingestion of *Spirulina*, in animals receiving fat free diet the maximum level of β-carotene in the plasma appeared in 12 hours suggesting a delay in the absorption of carotenoids in the absence of fat. Similarly in the presence of protein in the diet, maximum plasma response was seen after 6 hours while in protein free diet it took 12 hours. This suggests that fat and protein content in the diet modulated plasma bioavailability of β-carotene. Products of fat hydrolysis increase the solubility of carotenoids in the gut lumen and stimulate bile secretion, which may enhance absorption. The absence of fat in the diet might have delayed absorption.

(b) The retinol level remained unchanged and no significant increase was noted when compared to the basal values in both fat free and fat containing diet. This may be because there was adequate store of vitamin A in tissues, particularly of liver which is the source of plasma retinol.

(c) Comparative bioavailability of β-carotene from different carotenoid rich preparations such as Agathi, carrot and *Spirulina* was studied using female lactating rabbits. Rabbits kept as control, showed no significant change in β-carotene content in the milk, whereas for other supplements the β-carotene level in the milk recorded maximum at 12th hour after ingestion. Rabbits supplemented with *Spirulina* showed significantly greater level of β-carotene in milk when compared to carrots and Agathi leaves. These result indicate that carotenoid bioavailability depends on food matrix in which carotenoids are incorporated.

(d) Milk response was comparatively high when *Spirulina* was fed to the rabbits. However, the retinol level in the milk, did not show any significant change on feeding of different carotenoid supplements when compared to the basal controls.

(e) The bioavailability of β-carotene from *Spirulina* was studied in other model systems like rats and monkeys. While in rats fed fat containing diet, the peek plasma level of β-carotene was attained at 6th hour, it took 12 hours to attain maximum plasma levels in rats fed fat free diet. Similarly in rats fed protein
containing diet peak plasma level of β-carotene was attained at 6th hour while it took 12 hours to attain maximum level in rats fed protein free diet. But there was no significant increase in the retinol content during 24 hours period after supplementing carotenoid containing supplements. These results indicate that presence of fat and protein in the diet cause a faster rate of absorption of β-carotene from the supplements. Supplementation of *Spirulina* also caused an increase in plasma levels of β-carotene which attained a maximum level in six hours in monkeys as well.

Results of these investigations in rat, rabbits and monkeys indicate that the rate of uptake and bioavailability of provitamin A carotenoids depend on (a) the nature of food matrix in which carotenoids are present and (b) the presence of major nutrients like fat and protein. It therefore appears that pure provitamin A supplements in interventions without adequate amount of major nutrients like fat may not yield the desired results and may not correct vitamin A deficiency, because of poor bioavailability of provitamin A carotenoids. These results thus highlight the necessity to ensure that carotenoid supplements for interventions are fortified with suitable other nutrients particularly fat. The possibility of synergistic and possible beneficial effects of nutrients of plant origin are not clear and needs further research.

4. Human stools contain viable exfoliated colonic epithelial cells and now it is possible to obtain pure exfoliated viable cells from the stool. The possibility of using human exfoliated colonic epithelial cells as a noninvasive tool, to assess provitamin A/vitamin A bioavailability was examined and the results are presented.

(a) The colonic epithelial cells isolated from different stool samples were analysed for their β-carotene and vitamin A content and the results showed that β-carotene and retinol were present in both interphase and pellet fraction of the cells. However, the cells in the interphase fraction contained significantly higher amounts of β-carotene

(b) The β-carotene content was significantly lower in cells from stool samples of subjects on β-carotene poor diet than those receiving β-carotene rich diet.

(c) Colonic epithelial cells isolated from stool samples collected daily during a wash out period when the subjects were put on a β-carotene poor diet showed a steady decrease in β-carotene content.
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(d) Kinetic analysis showed that a single spell of β-carotene rich diet in the form of *Spirulina* and Agathi supplements after the wash out periods caused an increase in the β-carotene content after a lag period of 5-7 days but the vitamin A level during these periods were not significantly affected.

(e) Analysis of the β-carotene content in the plasma also showed similar changes, which correlated with that of exfoliated colonic epithelial cells.

(f) β-carotene content increased steadily from a basal level on 6th day and then decreased to basal level on 10th day while there was no significant change in the retinol level in the plasma during this period. These results suggest that the exfoliated colonic cells may be loaded with β-carotene provided by the blood and not by any direct transfer from carotenoid supplements.

The relationship between the β-carotene content in the diet and that of the colonic epithelial cells suggests that analysis of β-carotene content in exfoliated human colonic epithelial cells is a useful non-invasive method to assess the bioavailability of provitamin A β-carotene.

5. Colonic epithelial cells resemble the human intestinal epithelial cells that absorb nutrients from the diet. Therefore the present study was also aimed at to evaluate the *in vitro* uptake of β-carotene by the colonic epithelial cells.

(a) Supplementing 14C-β-carotene to colonic epithelial cells in culture caused uptake of radioactivity by cells indicating the ability to take up β-carotene by colonic epithelial cells in culture.

(b) Addition of deoxy cholic acid, a primary bile acid increased uptake of β-carotene by the colonic cells from the medium in a concentration dependent manner. Maximum absorption was obtained in presence of 12 µM deoxy cholic acid when compared to the control where no bile salts were present in the medium. These *in vitro* studies indicate that bile acids promote uptake of carotenoids and suggest that secretion of bile salts through the enterohepatic recirculation is a critical factor in determining the uptake/bioavailability of carotenoids.

(c) Addition of phospholipids like lecithin and tri acyl glycerol like triolein, increased uptake of β-carotene by colonic epithelial cells in culture. These results support the role of fat in the diet on the absorption of carotenoids by experimental animals described earlier.
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Usual methods for the bioavailability studies of vitamin A involve invasive sampling of blood or tissues which may not be practical in the case of a large target population particularly children. Experiments using exfoliated colonic epithelial cells demonstrate that the micronutrient analysis, particularly β-carotene of colonic epithelial cell is a useful non-invasive method to assess the bioavailability of provitamin A carotenoids and increase the efficacy of intervention studies involving dietary supplementation. It requires further validation using large number of samples particularly children at different stages of growth.