5.1 Introduction: Carotenoids are beautiful in colour due to their conjugated double bonds. The visible and near U.V. absorption spectra of linear polyenes (such as carotenoids) arise due to the transitions of electrons from occupied to unoccupied π-orbitals. π-Electron theory that treats the π-orbitals, explicitly involve a series of simplifying assumptions that make it possible to carry out quantum mechanical treatment on large molecules containing π-electron system. Basically one assumes a molecular geometry and then obtains wave-function energy levels etc in terms of set of integrals, that are treated to be fit from experimental data.

The visible absorption spectra of carotenoids are dominated by a single strong electronic transition. The three or more bands usually observed involve transitions to different vibrational levels of the same electronic state. Quantum mechanical calculations produce only a single transition energy difference at the ground state geometry. For a broad structureless band, this should correspond to absorption maxima (λ_max). When vibrational structure is evident, as is the case of many carotenoids, the calculated transition energy corresponds the most intense vibrational components. When two lines are equal in intensity, then comparisons should be made to their mean.

Kakitani et al (1982) carried out calculations on the π-electron systems of the carotenoids. For calculations on neurosporene it was assumed that the π-system is planar. Bond lengths were varied as described previously and bond angles were fixed at 120°. For lutein, which has a conjugated double bond in one of its rings, a torsional ring-chain angle of 40° was assumed. This is a representative value taken from closely
related molecules where steric hindrance distorts the \( \pi \)-system. As mentioned above, the semiempirical parameters used in these calculations were chosen so as to produce reasonable absorption maxima for carotenoids. The calculated \( \lambda_{\text{max}} \) for lutein is 465 nm, which compares well with the experimental value in benzene of 460 nm. For neurosporene, comparison is more difficult because the first two vibrational lines are of approximately equal intensity. Thus, the calculated absorption maximum of 456 nm should most appropriately be compared to the mean of the two lines, which is 452 nm. A more sensitive test of the theory is its ability to reproduce experimentally determined polarizabilities and dipole moments. Reich and Sewe (174) determined for all-trans-\( \beta \)-apo-8'-carotenoic acid that the dipole moment difference between the ground and excited state is 10.7 Debye (D). Kakitani's calculated value of 9.9 D is thus in excellent agreement with the experimental results (175).

A simple energy diagram depicting highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals associated with the ground and excited singlet state configurations of butadiene is an example of a typical \( \pi \)-electron conjugated system and is shown in figure 5.1. The figure shows that the ground and first excited singlet states, \( S_0 \) and \( S_1 \), both have gerade (g) symmetry in the idealized \( C_{2h} \) point group. In the figure, the \( S_1 \) state is formed from a simple HOMO\( \rightarrow \) LUMO electronic transition. However, this is not the case for polyenes and carotenoids, where configuration interaction between more energetic, single HOMO\( \rightarrow \)LUMO + 1 (figure 5.1) and doubly excited HOMO\( \rightarrow \)LUMO electronic configurations of like (g) symmetry cause a state of \( ^1\text{Ag}^- \) symmetry to be formed at lower energy than the low-lying \( ^1\text{Bu}^+ \) state. Figure 5.1 also shows the well-accepted state-ordering diagram for carotenoids and polyenes, and depicts the \( S_0 (^1\text{Ag}^-) \rightarrow S_2 (^1\text{Bu}^+) \) transition associated with the strongly allowed absorption transition.
CHAPTER 5: FOOD COLOURANT

FIGURE 5.1: A molecular orbital diagram (not to scale) depicting the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals and three low-lying electronic states of butadiene. g, gerade; u, ungerade. The bottom part shows an energy level diagram depicting the strongly allowed absorption (a) of carotenoids and polyenes.

The $S_0 \rightarrow S_2$ absorption spectrum of the molecules in DCM red shift with increasing number of conjugated carbon-carbon double bonds is shown in figure 5.2. The absorption spectra of zeaxanthin, β-cryptoxanthin, and β-carotene are very similar due to the three carotenoids having 11 conjugated carbon-carbon double bonds and two terminal rings. Lycopene also has 11 conjugated carbon-carbon double bonds but unlike zeaxanthin, β-cryptoxanthin and β-carotene, it does not have terminal rings.
which causes its absorption spectrum to be red-shifted by ~20 nm relative to β-carotene (figure 5.2). As is typical for these molecules at room temperature, the Franck-Condon maximum corresponds to the \((0-1)\) band of the \(S_0 \rightarrow S_2\) transition. Also, the resolution of the vibronic bands decreases with increasing extent of \(\pi\)-electron conjugation. This is due to spectral inhomogeneity induced by the presence of the terminal rings in the conjugated chain (176).

![Steady-state absorption spectra of the carotenoids](image)

**FIGURE 5.2:** Steady-state absorption spectra of the carotenoids: Neoxanthin (neox), violaxanthin (viol), lutein (lut), zeaxanthin (zeax), and \(\beta\)-cryptoxanthin (\(\beta\)-crypto), and the carotenes, \(\beta\)-carotene (\(\beta\)-car) and lycopene (lyco). The spectra were offset vertically for clarity

The aim of our study was to utilize this beautiful colour of carotenoid as food colourant. It was mentioned earlier that carotenoids are fat-soluble pigments. That is why it is easier to utilize carotenoids as oil based food colourant. But in case of water based food colourant, we had to encapsulate the carotenoids so that it can be dispersed.
into water phase. The encapsulation provides a protection to the carotenoids regarding the exposure to air.

5.2 Emulsions: An emulsion is a mixture of two immiscible (unblendable) substances. One substance (the dispersed phase) is dispersed in the other (the continuous phase). Emulsions tend to have a cloudy appearance, because of the many phase interfaces (the boundary between the phases is called the interface) scatter light that passes through the emulsion. Emulsions are unstable and thus do not form spontaneously. Energy input through shaking, stirring, homogenizers, or spray processes are needed to form an emulsion. The lipid in the emulsion may act as solvent for various other important food components, including oil soluble vitamins, carotenoids, antioxidants, preservatives, and essential oils. Inspection of the most commercially available food emulsions indicates that they contain a wide variety of different constituents such as example, oil, emulsifiers, thickening agents, gelling agents, buffering systems, preservatives, antioxidants, sweetners, salts, colourants, flavours etc. Each of these constituent has its own unique molecular and functional characteristics. To produce a marketable food colourant each ingradient must exhibit its desired functional properties within the food while also being commercially viable, convenient to use, compatible with other ingredients, readily available.

Some of the food additives are:

Antioxidants: Antioxidants such as vitamin C, BHT, ascorbic palmitate act as preservatives by inhibiting the effects of oxygen on food, and can be beneficial to health.
Emulsifiers: Emulsifiers allow water and oils to remain mixed together in an emulsion, as in mayonnaise, ice cream, and homogenized milk. e.g. casein, gum arabic, gelatin.

Flavours: Flavours are additives that give food a particular taste or smell, and may be derived from natural ingredients or created artificially. e.g vanilla.

Flavour enhancers: Flavour enhancers enhance a food's existing flavours. They may be extracted from natural sources (through distillation, solvent extraction, maceration etc among other methods) or created artificially.

Flour treatment agents: Flour treatment agents are added to flour to improve its color or its use in baking.

Humectants: Humectants prevent foods from drying out. e.g. sugar.

Preservatives: Preservatives prevent or inhibit spoilage of food due to fungi, bacteria and other microorganisms e.g. acetic acid.

Encapsulating materials: Microencapsulates protect ingredient from the reaction of the surrounding materials or environment. e.g. agar, gum-arabic etc.

Emulsions are part of a more general class of two-phase systems of matter called colloids. Although the terms colloid and emulsion are sometimes used interchangeably, emulsion tends to imply that both the dispersed and the continuous phase are liquid. Over times, emulsions tend to revert to the stable state of oil separated from water. Surface active substances (surfactants) can increase the kinetic stability of emulsions greatly so that, once formed, the emulsion does not change significantly over years of storage. Homemade oil and vinegar salad dressing is an example of an unstable emulsion that will quickly separate unless shaken continuously. This phenomenon is called coalescence, and happens when small droplets recombine to form bigger ones.
An emulsifier (also known as an emulgent) is a substance which stabilizes an emulsion. In some cases, particles can stabilize emulsions through a mechanism called Pickering stabilization. Both mayonnaise and hollandaise sauce are oil-in-water emulsions that are stabilized with egg yolk lecithin. Detergents are another class of surfactant, and will chemically interact with both oil and water, thus stabilising the interface between oil and water droplets in suspension. This principle is exploited in soap to remove grease for the purpose of cleaning. A wide variety of emulsifiers are used in pharmacy to prepare emulsions such as creams and lotions. Whether an emulsion turns into a water-in-oil emulsion or an oil-in-water emulsion depends on the volume fraction of both phases and on the type of emulsifier.

Encapsulating materials: Encapsulants such as proteins, carbohydrates, lipids, gums, polysaccharides and cellulose materials are commonly used for encapsulation in oil-in-water (table 5.1). They can be used alone or in combination to achieve certain properties.

<table>
<thead>
<tr>
<th>Material class</th>
<th>Types of Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>Albumin, casein, gelatin, peptides, soy protein, whey proteins, vegetable protein</td>
</tr>
<tr>
<td>Sugars</td>
<td>Fructose, dextrose, galactose, glucose, maltose, sucrose, corn syrup solids.</td>
</tr>
<tr>
<td>Starch and starch products</td>
<td>Dextrin, maltodextrin, starches</td>
</tr>
<tr>
<td>Gums</td>
<td>Agar, gum arabic, pectin, alginates.</td>
</tr>
<tr>
<td>Cellulose materials</td>
<td>Acetyl cellulose, carboxyl cellulose, cellulose acetate, butylate phthalate, cellulose acetate phthalate.</td>
</tr>
<tr>
<td>Other carbohydrates</td>
<td>Chitosan, cyclodextin</td>
</tr>
<tr>
<td>Lipids</td>
<td>Acetoglyceride, beeswax, diglyceride,</td>
</tr>
</tbody>
</table>
Emulsion preparation and drying: Preparation of an emulsion involves the dispersion of the oil phase into very fine droplets in water in which the action of the emulsifying agent at the surface of the oil droplets provides protections and functionality to form stable oil-in-water emulsions. High shear process such as homogenization may be used for dispersing the oil into droplets and forming the emulsion. Emulsifiers, antioxidants, and other additives may be incorporated into both the oil phase and the aqueous phase before homogenization. The emulsion formulation and other variables such as temperature, total solids, pH, and storage conditions influence the emulsion properties and stability. Stable emulsion microcapsules can be dried into a powder for more stability (177).

Formulation of food colourant: To get an emulsion we had to prepare two phases of solution: one is oil phase and another is water phase.

In oil phase we dissolved all the oil soluble ingredients like carotenoids, BHT (antioxidant) and Tween 80 (stabilizer).

In water phase we dissolved all the water soluble ingredients like sugar, gum arabic, ascorbic acid to get a matrix for emulsifying the oil based solution.

The emulsion can be prepared by homogenizing the above two solutions.
Emulsion can be dried into a powder: The emulsion we had produced was in aqueous medium therefore we could not dry it by rotavapor. We adopted two technologies to dry the emulsion:

(a) Lyophilization: Freeze-Drying

(b) Spray drying

Lyophilization: Freeze-Drying: Biological materials often must be dried to stabilize them for storage or distribution. Drying is always associated with some loss of activity or other damage. Lyophilization, also called freeze-drying, is a method of drying that significantly reduces such damage. As lyophilization is the most complex and expensive form of drying, its use is usually restricted to delicate, heat-sensitive materials of high value.
Many microorganisms and proteins survive in the process of lyophilization intact, and therefore, it is a favoured method of drying vaccines, pharmaceuticals, blood fractions, and diagnostics. Some specialist food products are also lyophilized. They rehydrate easily and quickly because of the porous structure left after the ice have sublimed.

Thus, the principal advantages of lyophilization as a drying process are:

- Minimum damage and loss of activity in delicate materials
- Speed and completeness of dehydration
- Porous, friable structure

The principal disadvantages of lyophilization are:

- High capital cost of equipment (about three times more than other methods)
- High energy costs (2-3 times more than other methods)
- Long process time (typically 24 hour drying cycle)

**Principles of lyophilization equipment:** There are three stages in the complete freeze-drying process: Freezing, primary drying, and secondary drying.

**Freezing and Primary Drying:** The freezing process consists of freezing the material. In a lab, this is often done by placing the material in a freeze-drying flask and rotating the flask in a bath, called a shell freezer, which is cooled by mechanical refrigeration, dry ice and methanol, or liquid nitrogen. On a larger-scale, freezing is usually done using a freeze-drying machine. In this step, it is important to cool the material below its freezing point, the lowest temperature at which the solid and liquid
phase of the material can coexist. This ensures that sublimation rather than melting will occur in the following steps. Larger crystals are easier to freeze dry. To produce larger crystals the product should be frozen slowly or can be cycled up and down in temperature. This process is called annealing.

One mL of ice produces more than 1,000,000 mL of water vapour at typical lyophilization cycle pressures. The more energy-efficient vacuum pumps cannot handle large quantities of water vapour. For this reason it is usual to fit a refrigerated trap (called the ice condenser) between the lyophilization chamber and the vacuum pump.

**Secondary freeze drying:** The secondary drying phase aims to sublimate the water molecules that are adsorbed during the freezing process, since the mobile water molecules were sublimated in the primary drying phase. This part of the freeze-drying process is governed by the material's adsorption isotherms. In this phase, the temperature is raised even higher than in the primary drying phase to break any physico-chemical interactions that have formed between the water molecules and the frozen material. Usually the pressure is also lowered in this stage to encourage sublimation.

**Spray drying:** Spray drying is a very widely applied, technical method used to dry aqueous or organic solutions, emulsions etc., in industrial chemistry and food industry. Dry milk powder, detergents and dyes are just a few spray-dried products currently available. Spray drying can be used to preserve food or simply as a quick drying method. It also provides the advantage of weight and volume reduction. It is the transformation of feed from a fluid state into a dried particle form by spraying the feed into a hot drying medium. Intensive research and development during the last
two decades has resulted spray drying as a highly competitive means of drying a wide variety of products.

**Procedure of Manufacture of “Beadlet” Products**

**FIGURE 5.4:** Schematic diagram of the spray drying.

**Principle of Spray drying:** Spray drying involves evaporation of moisture from an atomized feed by mixing the spray and the drying medium. The drying medium is typically air. The drying proceeds until the desired moisture content is reached in the sprayed particles and the product is then separated from the air. The mixture being sprayed can be a solvent, emulsion, suspension or dispersion.

The complete process of spray drying basically consists of a sequence of four processes:
(a) Dispersion of the feed solution in small droplets: The dispersion can be achieved with a pressure nozzle, a two fluid nozzle, a rotary disk atomiser or an ultrasonic nozzle. So, different kinds of energy can be used to disperse the liquid body into fine particles. The selection upon the atomiser type depends upon the nature and amount of feed and the desired characteristics of the dried product. The higher the energy for the dispersion, the smaller are the generated droplets.

(b) Mixing of spray and drying medium (air) with heat and mass transfer: The manner in which spray contacts the drying air is an important factor in spray dryer design, as this has great bearing on the properties of the dried product by influencing droplet behaviour during drying.

Co-Current flow: The material is sprayed in the same direction as the flow of hot air through the apparatus. The droplets come into contact with the hot drying air when they are the most moist. The product is treated with care due to the sudden vapourization.

Counter-Current flow: The material is sprayed in the opposite direction of the flow of hot air. The hot air flows upwards and the product falls through increasingly hot air into the collection tray. The residual moisture is eliminated, and the product becomes very hot. This method is suitable only for thermally stable products.

Combined: The advantages of both spraying methods are combined. The product is sprayed upwards and only remains in the hot zone for a short time to eliminate the residual moisture. Gravity then pulls the product into the cooler zone. Due to the fact that the product is only in the hot zone for a short time and the product is treated with care.
(c) Disk atomizer (rotary wheel): The material to be sprayed flows onto a rapidly rotating atomizing disk and is converted to a fine mist. The drying air flows in the same direction. The product is treated with care, just as in the co-current flow method.

Open-cycle and closed cycle system: The most common type of spray dryer is the open-cycle, co-current spray dryer. In such a design, the atomised feed and the drying air is simultaneously injected into a spray-drying chamber from the same direction.

(d) Drying of spray (removal of moisture): As soon as droplets of the spray come into contact with the drying air, evaporation takes place from the saturated vapour film, which is quickly established at the droplet surface. The evaporation leads to a cooling of the droplet and thus to a small thermal load. Drying chamber design and air flow rate provide a droplet residence time in the chamber, so that the desired droplet moisture removal is completed and product removed from the dryer before product temperatures can rise to the outlet drying air temperature. Hence, there is little likelihood of heat damage to the product.

5.3 Purification of carotenoids: Carotenoids were extracted from the algae following the procedure of section 2.2 and the organic solvents were evaporated to dryness. The residue contained carotenoids, xanthophyll esters, and chlorophylls. Due to the presence of green coloured chlorophyll and red coloured carotenoids the residue looks black in colour. We carried out the following steps to purify the algal carotenoids.

- Hydrolysis: Hydrolysis is the most obvious method for removing chlorophylls. As we followed the procedure of section 2.5 where we keep the
carotenoid-alkali reaction mixture overnight at 4°C; some amount of carotenoid is lost during hydrolysis. But even then this is the most suitable method for hydrolysis of large amount of carotenoids. As the esters of xanthophylls are more stable than their free form, hence in case of *Euglena sp.* (II) we did not hydrolyze the algal extract. We separated the chlorophyll by chromatographic techniques.

- **Column chromatography:** The carotenoids were purified by column chromatography for the preparation of food colourant as detailed in section 2.5.1.

In case of *Euglena sp.* (I) the saponified algal extract was dissolved in a minimum amount of petroleum ether (40-60°C). The column was eluted with a mobile phase starting with 0% ethyl acetate in petroleum ether and slowly increasing the ratio of ethyl acetate to 30%. The first fraction eluted with mobile phase 0% ethyl acetate in petroleum ether, and showed visible spectra (434 472 504 nm in hexane) similar to lycopene and showed the same R<sub>f</sub> value (0.89 in 10% ethyl acetate in petroleum ether) that of standard lycopene in the TLC plate.

The second fraction eluted with mobile phase 30% ethyl acetate in petroleum ether, and showed visible spectra (421 451 474 nm in hexane) similar to zeaxanthin and showed the same R<sub>f</sub> value (0.23 in 10% ethyl acetate in petroleum ether) that of standard zeaxanthin.

In case of *Euglena sp.* (II) the non-saponified algal extract was dissolved in petroleum ether (40-60°C). The extract was separated slowly through the column with the mobile phase starting with 0% ethyl acetate in petroleum ether and slowly increasing the ratio of ethyl acetate to 30%. The first fraction separated out with mobile phase 5% ethyl acetate in petroleum ether, and showed visible spectra similar to astaxanthin (469 nm). As already mentioned in chapter-3 that astaxanthin is very sensitive towards alkali. If astaxanthin is hydrolyzed by the general hydrolysis
procedure it gets denatured. So, to identify the fraction (column chromatographically separated carotenoid) we hydrolyzed a small amount of carotenoid component following the procedure of section 3.11. It showed the same $R_f$ value (0.46 in 20% ethyl acetate in petroleum ether) as that of standard astaxanthin on the silica gel TLC plate.

Another fraction was separated with mobile phase 30% ethyl acetate in petroleum ether, but this fraction has almost similar $R_f$ value to that of chlorophyll. So, this fraction of carotenoid could not be separated from chlorophyll by column chromatography. This fraction was collected and hydrolyzed by the procedure of section 2.3. On hydrolysis chlorophyll got extracted into aqueous phase in separating funnel while the hexane layer extracted the carotenoids. This fraction of carotenoid showed visible spectra (421 451 474 nm in hexane) similar to zeaxanthin and showed the same $R_f$ value (0.23 in 10% ethyl acetate in petroleum ether) as that of standard zeaxanthin.

5.4 Preparation of oil based food colourant: To prepare oil based food colourant we had to dissolve the carotenoids in oil. Carotenoids are not easily soluble in oil. So, it is difficult to prepare a highly concentrated solution of carotenoid in oil. We extracted carotenoids from algal source exactly in a similar way of section 2.2 and evaporated to dryness. The carotenoids were purified by column chromatography [and hydrolysis in case of the algae Euglena sp. (I)] as detailed in section 5.3. We utilized these carotenoids to prepare oil based food colourant by adopting the two following methods.
• **Method I: Preparation of oil based food colourant in presence of organic solvent (ethanol):** In the first method, we dissolved 1.5 mg of purified algal carotenoid in the minimum amount of ethanol. This carotenoid solution was poured to 10 mL of groundnut oil. The mixture was stirred to get a homogenized solution. The organic solvent (ethanol) was evaporated in a rotavapour. The last trace amount of ethanol was evaporated completely in vacuum under reduced pressure using a vacuum pump.

• **Method II: Preparation of oil-based food colourant in absence of organic solvent:** In the above method the trace amount of ethanol may remain in the food colourant. So, to avoid the presence of any organic solvent we adopted a second procedure. In the second method, we added 10 mL of groundnut oil in purified algal carotenoid. As the solubility increases at elevated temperature in order to dissolve the carotenoids in oil the suspension of carotenoid in oil was heated (to about 80°C). There remains always a chance of loss of carotenoid in higher temperature. So, edible oil soluble antioxidant must be used e.g. ascorbic palmitate is a good oil soluble edible antioxidant. In this method the concentration could be increased to about 4 mg of carotenoid in 10 mL of groundnut oil.

5.4.1 Use of oil based food colourant in food items:

**Preparation of cake dough and baking the cake:** In a mixing glass bowl 50g of sugar, 1 egg white, and 100 mL of groundnut oil were mixed with a hand whisker. In a separate bowl 1 g of baking powder and 50 g wheat were mixed thoroughly. The baking powder and wheat mixture was then added to the first mixture slowly with constant stirring until a homogeneous mixture was achieved. To this dough 50 mL of oil based food colourant (prepared by Method II) using the algal carotenoids of
Euglena sp. (I), containing 20 mg of carotenoid was added. The mixture was stirred vigorously. The dough was placed in a baking pan and baked in an oven at medium temperature for 30 minutes. The product appeared yellow in colour. The photograph of the cake thus prepared is shown in figure 5.5. 20g of the cake was weighed and grounded with sodium sulfate. The mass was stirred with water. To that mixture MeOH: hexane (50:50) was added and the carotenoid was extracted in the hexane layer. The extraction was continued till the extraction was complete. The visible spectrum of the organic layer was recorded. The spectra showed absorption at 434 472 504 nm in hexane. Thus, it was found that the carotenoid in the cake was not denatured during the cooking process.

5.4.2 Stability determination of carotenoid in oil-based food colourant:

(a) Carotenoids were extracted from Euglena sp. (I) and purified by saponification as well as column chromatography as detailed in section 5.3. The pure carotenoid was estimated by visible spectroscopy and it was found to be 0.892 mg. This amount of
carotenoid was dissolved in 100 mL groundnut oil and kept at room temperature at
diffused light. The coloured oil was estimated at regular intervals of time.

**Estimation of the carotenoid in oil based food colourant during the stability
determination:** The total amount of carotenoid was estimated at a time interval of 40
to 60 days up to 165 days. For this purpose 1mL of oil solution was withdrawn and
the carotenoid present in the oil was estimated spectrophotometrically as detailed
under the section of 2.4. The estimation was repeated keeping a time interval of 40-
60 days to check the change in concentration in course of time in the food colourant.
The change in concentration of carotenoid is enlisted in table 5.2.

**TABLE 5.2:** The change in carotenoid concentration in the oil based food colourant
in 165 days

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Days</th>
<th>Carotenoid in mg 100 mL of oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.892</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>0.863</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>0.787</td>
</tr>
<tr>
<td>4</td>
<td>105</td>
<td>0.702</td>
</tr>
<tr>
<td>5</td>
<td>165</td>
<td>0.681</td>
</tr>
</tbody>
</table>

Graph for the Spectrophotometric study of decrease in concentration of
carotenoid in Carotenoid in oil kept at room temperature.
Statistical analysis: Using M S Excel software the tabulated data were analysed by Pearson’s Co-efficient of correlation method. This method was used for measuring the linear relationship between the two variables viz Days & Concentration of carotenoids. The Pearson’s Co-efficient of correlation was found to be -0.955. The negative sign of the correlation coefficient indicates the direction of relation is negative i.e. as the time progresses concentration of carotenoid decreases. As the value is almost near -1, this suggests that there is a strong negative correlation.

5.5 Preparation of water based food colourant: The solubility of carotenoids in water is virtually zero. Nature does not restrict the occurrence of carotenoids to lipid systems. Large amounts of carotenoids occur in the form of very fine dispersions and as such are capable of colouring aqueous media. Common examples of this are to be seen in the carotenoid colouring of the juices of the tomato, orange and carrot. The submicroscopic structure may also be a factor in their outstanding stability.

Methods of application of carotenoids to aqueous systems include the formation of emulsions, colloidal suspensions and dispersions in suitable colloids, particularly when a surface active agent is present.

5.5.1 Preparation of sugar and gum-arabic encapsulated water soluble crystals of carotenoids: Carotenoid from Euglena sp. (I) was extracted and purified as detailed in section 5.3. 20.8mg of carotenoid (containing lycopene) was dissolved in minimum amount of organic-solvent. To the concentrated solution of carotenoid 2.78g of sugar (anhydrous dextrose) was added. The mixture was stirred for a few
minutes so that the organic solvent gets evaporated completely. The product remains as a dry coloured powder. The coloured powder was then dissolved in concentrated solution of gum-arabic (1g in 25mL) and stirred at 60°C to get a paste. To prevent the loss of carotenoid, 0.02g of ascorbic acid was added as an antioxidant. To prevent the crystals from absorbing moisture a small amount of starch was also added. The paste was kept in desiccators at 4°C for 2 days. The paste gets hardened and by grinding the hard mass we get water-soluble crystals. The photograph of the product is shown in figure 5.7.

FIGURE 5.6: Schematic diagram of the steps of synthesis of water-soluble crystal of carotenoids
5.5.1.1 Utilization of the food colourant (Sugar and Gum-arabic encapsulated carotenoid) in food items:

- **Preparation of Coconut sweet:** Carotenoids were extracted from *Euglena sp.*(I) and purified by following the procedure detailed in section 5.3. The pure lycopene was estimated (spectrophotometrically) to be 2.743mg. This amount of carotenoid was encapsulated in the same way as in section 5.5.1. 200g of coconut and 250g sugar were mixed and stirred over gas oven. When a paste was formed, to that paste 2g of sugar and gum-arabic encapsulated carotenoid was added. 0.2g of ascorbic acid was also added as an antioxidant. The paste was continuously stirred and allowed to cool at room temperature. At room temperature the paste became harder. Then some balls of the sweet dish were prepared. The photograph is shown in figure 5.8.

The stability of the carotenoid was checked as follows: 20g of the sweet was weighed and grounded with sodium sulfate. The mass was stirred with water. To that
mixture MeOH: hexane (50:50) was added and carotenoid was extracted. The extraction was continued till carotenoid was extracted in the organic layer completely. The visible spectrum of the organic layer was recorded. The spectra showed absorption at 434 472 504 nm in hexane i.e. similar to lycopene. Thus, the denaturation in the carotenoid during food processing was negligible.

○ Preparation of Payes (an Indian sweet dish): 25g of rice was soaked in water for 15 minutes. Then the water was filtered off. In a gas oven 100 mL of milk was boiled. To that boiling milk the rice was added with continuous stirring. When the mass became a concentrated paste, another 100 mL of milk was added to the paste with continuous stirring. When the mass got concentrated, again 50 mL of milk was added. To the mixture 70g of sugar was added with constant stirring and allowed to concentrate. When the sweet dish was prepared we put off the gas oven. To the paste the food colourant (3mg of algal carotenoid extracted from Euglena sp. (I)) prepared as detailed in section 5.5.1 was mixed to get a nice orange colour. Photograph of the sweet dish is shown in figure 5.9.
Use of Sugar and Gum-arabic encapsulated carotenoid as food colourant

FIGURE 5.8: Use of food colourant (prepared in section 5.5.1) in coconut sweet

FIGURE 5.9: Use of food colourant (prepared in section 5.5.1) in payes (Indian sweet dish)
5.5.2 Preparation of oil-in-water emulsion: To prepare oil-in-water emulsion we prepared the following two stock solutions:

- **Oil based solution:** As already discussed, carotenoids have a poor solubility in oil. As our aim was to dry the emulsion by means of spray drying or freeze-drying we could not afford to increase the oil percentage in oil-in-water emulsion beyond 2%. Hence, we needed to prepare a very concentrated carotenoid solution. This concentrated solution was prepared by the procedure detailed in section 5.4. To the 2 mL of such a solution 0.5 mL of Tween 80 was added. Tween 80 acts as emulsion stabilizer. 0.5 mL of 0.02% BHT in ethanol (which is an oil soluble antioxidant), was added. In place of BHT it is better to use ascorbyl palmitate and tocopherol.

- **Water based solution:** The aqueous phase comprised a solution of a film-forming, hydrophilic colloid with a plasticiser and humectant. Water-soluble antioxidants (ascorbates) and other substances were added in this phase. 0.5 g of gelatin or gum arabic was dissolved in 98 mL water. Gelatin or gum-arabic was used as emulsifier while preparing the oil-in-water emulsion. After stirring by mechanical stirrer (Remi’s motor) for five hours the gum arabic got dissolved and produced a colloidal solution. The gum-arabic acted as emulsifier as well as encapsulant. To the colloidal solution 7 g of sugar was added. Sugar acted as binder and humectants during drying process. To the solution 0.02% ascorbic acid was added. Ascorbic acid acted as an antioxidant. Thus, water based solution was produced.

These two solutions were stirred vigorously by a mechanical stirrer for five hours and then homogenized by glass homogenizer for next 2 hours at 2000 rpm to get a stable emulsion. We prepared three emulsions with three different carotenoids.
The photograph (figure 5.12) showed three different colours these emulsions. The stable emulsions were obtained when the oil particle size became 1-10 μm in diameter (measured under microscope). (figure 5.11)

**FIGURE 5.10:** Schematic diagram to produce oil in water emulsion
FIGURE 5.11: Oil–in–water emulsion containing lycopene under the microscope

FIGURE 5.12: Three carotenoids in oil in water emulsion. Zeaxanthin 0.826 mg, astaxanthin 1.526 mg, lycopene 2.324 mg in 100 mL emulsion

5.5.2.1 Stability determination of carotenoid in oil-in-water: The emulsions were stored in airtight bottle and kept in 4°C. In a test tube, 1 mL of carotenoid in oil-in-water emulsion was taken. To the emulsion 1 mL of MeOH was added and vortexed. Then, to the solution 1 mL hexane and 2 mL water were added and vortexed for five minutes. Then the mixture was centrifuged for five minutes. Hexane layer containing carotenoid was pipetted out. The procedure was continued till the complete extraction of carotenoid. The total carotenoid was estimated
spectrophotometrically after recording the O.D at maximum absorption as detailed in section 2.4. The procedure was carried out repeatedly after a regular interval of times to check the amount of carotenoid, thereby checking the stability of carotenoid in oil-in-water emulsion. The amount of carotenoid in 100mL oil estimated at a regular interval of time is shown in Table 5.3.

**TABLE 5.3:** The change in concentration of carotenoids in emulsion with time.

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Days</th>
<th>Carotenoid (mg) in 100 mL of emulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1.105</td>
</tr>
<tr>
<td>2</td>
<td>206</td>
<td>0.902</td>
</tr>
<tr>
<td>3</td>
<td>245</td>
<td>0.873</td>
</tr>
<tr>
<td>4</td>
<td>270</td>
<td>0.628</td>
</tr>
<tr>
<td>5</td>
<td>330</td>
<td>0.518</td>
</tr>
</tbody>
</table>

Graph for decrease in carotenoid concentration in ‘Carotenoid in oil-in-water emulsion’
**Statistical analysis:** Using Microsoft Excel software the tabulated data was analyzed by Pearson’s Coefficient of correlation method and it was found to be -0.903. The negative sign indicates that there is an inverse relationship between the progress time and the concentration of carotenoid. As the value is near -1, this suggests that there is a strong negative relationship between the two variables.

5.5.3 Preparation of water soluble food colourant by freeze-drying: Water-soluble crystals were successfully prepared by freeze-drying. The emulsion containing lycopene prepared from the procedure detailed in section 5.5.2, was cooled at -20°C. The water was removed under reduced pressure by vacuum pump. 25 mL of water was removed by the machine in 8 hours. The resultant product was a crystalline solid but it was found that these coloured solids easily absorb moisture. To prevent the crystals from absorbing moisture 0.5 g of starch was added to the emulsion before freeze-drying. This product is cold water dispersible and produces an emulsion when dissolved in cold water. The photograph of the product was shown in figure 5.13.
FIGURE 5.13: Freeze dried water dispersible crystals of emulsion containing lycopene.

The product cannot be dissolved in organic solvents. Rather, it forms a stable emulsion in cold water. To extract the carotenoids in organic solvents we had to dissolve the product in cold water. An emulsion was formed. To that emulsion a solution of hexane: MeOH (50:50) was added. The mixture was vortexed and centrifuged. The carotenoids got extracted by hexane layer. The visible spectrum of the hexane layer was recorded and it showed similar absorption to that of lycopene (434 472 504 nm in hexane).

5.5.4 Preparation of water-soluble beadlet by spray drying: The oil-in-water emulsion containing lycopene (prepared following the procedure as detailed in section 5.5.2) was taken in a beaker. From that beaker the emulsion was pumped up to the spray tower (figure 5.4).

The glass chamber of the spray drier was at 100°C. From the spray tower the emulsion was sprayed out through the spray nozzle. The pressure was adjusted to
The atomizer of the nozzle was adjusted to moderate size. The emulsion was sprayed to the heat chamber. From the heat chamber the sprayed out emulsion droplets reached the cyclone condenser. From the bottom of the cyclone condenser cold air and starch were passed. The starch particles caught the emulsion drop to form beadlets. These beadlets were collected at the bottom of the cyclone condenser.

The photograph of the beadlet prepared in this manner containing lycopene (extracted from *Euglena sp.* (I) is shown in figure 5.14. During the spray drying the carotenoid was passed through the hot chamber at 100°C. So, to check whether the carotenoids in the beadlets remained intact we extracted the carotenoids following the procedure of 5.5.3. We recorded the visible spectra of the carotenoid extracted from the beadlets and we found that in was similar to lycopene (434 472 504 nm in hexane).

Thus, we can conclude that the lycopene in beadlets was not denatured during processes like spray drying or freeze-drying.

**FIGURE 5.14:** Photograph of Spray dried product: water-soluble beadlet of carotenoid (lycopene) in emulsion.
5.6 Preparation of salad dressing: Salad dressing is a dense emulsion (looks like cream). This is generally used for the decoration of salad. We prepared salad dressing based on the study of Khachatryan (178).

The carotenoid was extracted from alga *Euglena sp.* (II). The carotenoids were purified by column chromatography as detailed in section 5.3. The pure carotenoid was estimated to be 1.155mg. As the carotenoids were extracted from *Euglena sp.* (II) the main carotenoid was astaxanthin. This amount of carotenoids was dissolved in 15 mL of oil. The oil solution was homogenized in 30 mL of water. This emulsion was quite unstable. So, we continued homogenizing by Remi’s motor.

Another solution was prepared by dissolving casein in water simultaneously. Casein is the milk protein which sparingly soluble in water. 1g of casein was dissolved in 50 mL of water by stirring vigorously.

The casein in water solution was added to the unstable emulsion with continuous stirring. Then 5 mL of Tween 80 was added with stirring vigorously, and homogenized by Remi -motor. A gel started forming. Gradually, the viscosity of the emulsion increases to form a gel. To this gel 0.2g ascorbic acid was added. The pH of the gel was found to be 5-6. This was then poured over the salad to be dressed. The photograph of the salad dressing is shown in figure 5.15.
5.7 Discussion: The beneficial roles of different carotenoids are already discussed in the 1st Chapter. Both lycopene and astaxanthin are found to play significant roles in human body due to their antioxidant capacity. For example, lycopene can act as anticancer agent in case of prostate cancer and oral cancer. High content of serum lycopene is associated with lower risk of C.V.D. Pretreatment with lycopene offers protection against cellular damage, DNA damage, lipid peroxidation and can be developed as an effective radioprotector during radiotherapy. Astaxanthin can also act as an anticarcinogenic agent. It is also responsible for healthy heart, skin and eye conditions. Astaxanthin can also protect the lung and kidney from damage.

That is why now a days such food which is rich in carotenoids is prescribed by the dieticians. Generally, carrot and tomato are the most obvious choice for carotenoid
intake. Spinach is also a good source for lutein. Many studies are still going on to investigate how this carotenoids are beneficial to human health.

However, because of the presence of unsaturated bonds in its structure, lycopene is susceptible to oxidants. Thus, light and heat, are the cause of deterioration of lycopene when exposed to such factors (179,180). Therefore, the free lycopene must be protected in some forms from oxidative damage before its application.

In food processing, encapsulation technique has been widely used to protect the food ingredient against deterioration, volatile losses and premature interaction with other ingredients. Microencapsulation is the science of packaging components within a second material and delivering them in small particles i.e. microencapsules. Microencapsulation is used as a means of isolating ingredient from the reaction of the surrounding materials or environment (181). Thus, a component like carotenoid, which is sensitive to air, can be preserved by Microencapsulation. It may be used for stabilizing a sensitive ingredient, masking flavours, and for controlled delivery of active components. An associated benefit is the improved ease of handling when microencapsulated ingredients are delivered in the powder format.

The protective mechanism therein is to form a membrane (wall system) to enclose droplets or particles of the encapsulated material (core). So far, various kinds of microencapsulation techniques such as solvent dispersion/evaporation, phase separation, co-crystallization, interfacial polymerization etc., have been developed, amongst which, spray-drying is the most commonly used technique in the food industry due to its continuous production and easiness of industrialization (182-185).

During the literature survey, we found that a research note by Zhao et al where they reported that microencapsulation of lycopene using spray drying had not been reported before (186). But in their work they prepared the emulsion for spray drying
described as Zhu et al. (187). Gelatin and sucrose were dissolved in hot distilled water, being stirred, to form an aqueous solution containing different ratios of gelatin and sucrose. Lycopene sample with certain purity, preheated to dissolve in acetone, was dripped into the aqueous solution by stirring to form a coarse emulsion. The coarse emulsion was then homogenized in a Model SLS-60-7 High Pressure Homogenizer (Shenglu Homogeneous Machine Ltd. Shanghai, China) to form a homogenous emulsion under a certain homogenization pressure. In their method they produced the emulsion in presence of the organic solvent acetone. And homogenization procedure also involved very sophisticated instrument. But in the process of preparing oil-in-water emulsion we did not use any organic solvent and instruments used were very cheap and easy available. The spray drying instrument was costly but it gives very stable water dispersible microencapsulate. Zhao et al has studied the stability of the microencapsulate and found that these encapsulates are resistant to light and air. Chin et al. extracted lycopene from tomato waste and encapsulated by gelatin and polymer of γ-glutamic acid. They found that the thermal stability of the coating is upto 120°C (188).

Moris et al spray dried the algal cell of Dunaliella salina to produce β-carotene rich powder. According to this article all-trans -β-carotene has been chemically synthesized by Hoffmann La Roche since 1950s, however the cis forms are not produced synthetically. So, to use algal system finds its advantage because in algal carotenoid both the cis-trans isomers are present. As the effect of Euglena sp. (I) and Euglena sp. (II) on animals has not been examined so far, so it is not wise to use these algae directly as the food colourant in the food items. Though we studied the toxicity of the algae and we found that the algae are free from algal toxins but at this stage we cannot claim that the two species of Euglena can be directly used in the food
items as a source of colour. Preparation of food colourant by extracting the algal carotenoids is a new step towards food science.

Astaxanthin is very sensitive to light and air. It can easily degrade by thermal and oxidative process. Generally, instability of such pigments is minimized by encapsulating with a polymer matrix. (189). Synthetic astaxanthin was microencapsulated by Higuera-Ciapara et al. in a chitosan matrix cross-linked with glutaraldehyde by using the method of multiple emulsion/solvent evaporation. A powdered product containing microcapsules with a diameter of 5–50 μm was obtained by this process. The stability of the pigment in the microcapsules was studied by them under storage at 25, 35 and 45 °C for 8 weeks by measuring isomerization and loss of concentration of pigment with the help of HPLC. They found that their product was quite stable (190). Vernon-Carter investigated by spectroscopy that the microencapsulated pigments underwent zero order degradation kinetics when microencapsulated by mesquite gum. Mesquite gum provided the pigments a better protection than gum-arabic. The superior protection provided by mesquite gum is attributed to the thicker adsorbed polymer layers of the microcapsules due to its higher molecular mass as compared to that of gum arabic. They reported that Trouts fed diets containing the microcapsules for three weeks acquired muscle pigmentation similar to the colour exhibited by the natural grown fish, establishing the feasibility of employing these microcapsules in fish pigmentation (191). Prachanart et al. also protected astaxanthin and its biological activity against oxidative environmental conditions by encapsulating the homogenized algal cells in chitosan. They transformed *Haematococcus pluvialis* into beads, which were then coated with 5 layers of chitosan film, resulting in chitosan-algae capsules that have a mean diameter of 0.43 cm and the total film thickness of approximately 100 μm. They found no
significant loss in the amount of astaxanthin content in *H. pluvialis* due to the process of encapsulation. However, approximately 3% loss of antioxidant activity of the *H. pluvialis* was observed after encapsulation. The results of stability under different storage conditions showed that although encapsulation caused 3% loss of antioxidant activity, the longer term stability of the dried algal biomass, beads, and capsules indicated that encapsulation of *H. pluvialis* in chitosan film was capable of protecting the algae cells from oxidative stresses (189). During our study we did not try to encapsulate the algae. We extracted the carotenoid from the algae and then tried to encapsulate by producing oil-in-water emulsion. This emulsion showed stability for a long time. We studied the stability upto 330 days and found that though amount of astaxanthin decreased but the spectrophotometric character remained intact. Further, we should try to solidify the emulsion containing astaxanthin and check the extent of encapsulation by scanning electron microscope as studied by Prachanart et al. We prepared salad dressing using astaxanthin extracted from *Euglena sp.* (II). The procedure we followed is quite similar to Khachatryan 2001. But in his work he used lutein for the colouration of salad dressing. Instead of using Whey protein (as in case of Khachatryan) we used casein. The gel was quite stable and worthy for decorating salad.

Sugar and gum-arabic encapsulated carotenoid which is detailed in section 5.5.1 is a new method for carotenoid encapsulation where we do not need any sophisticated instrument like high pressure homogenizer or spray drier but it is a useful method for the preparation of water-based food colourant. This food colourant is a quite stable in of heat and air even during the cooking process.
Further work may be carried out:

- To increase the amount of carotenoid in oil-based food colourant by using the orange oil as the solute.
- The emulsion containing astaxanthin can be transformed into solids by spray-drying or freeze-drying.
- The stability of carotenoids in emulsion can be checked at elevated temperature.
- The shape and extent of microencapsulation of the spray-dried powder can be studied by SEM and XRD of the beadlets.
- Toxicity study of the beadlets can be carried out by examining the effect of these food colourants in animals.