Chapter 6

SUMMARY

Food dyes are added to various food commodities to enhance their aesthetic appeal (Gautam et al., 2010). The addition of dyes or colorants to food materials expectedly dates back to 1500 BC (Chequer et al., 2012). The food colorants then used were extracted mainly from natural sources viz., plants (indigo and saffron), insects (colchineal beetles and lac insects), animals (some species of molluscs and shellfish) and minerals (ferrous sulfate and clay) (Kadolph, 2008; Tayade et al., 2013). However, due to the industrial revolution in late 19th century, there occurred a tremendous rise in synthetic food dyes. Since then, many food industrialists have been using artificial dyes to color various food items (Kanetkar, 2010). The reasons to use synthetic food colorants are to produce high tinctorial power and wide array of shades as well as to correct natural variations in food colors that are lost during storage or processing of food (Babu and Shenoliker, 1995; Sharma et al., 2008; Abdelmigid, 2009; Al-Shinnawy, 2009). Apart from this, some food industrialists use synthetic food dye to hide the low quality and stale food products. The uses of red lead (Pb₃O₄) and vermillion (HgS) to color cheese as well as confectionery items and copper arsenate (Cu₃(AsO₄)₂) to recolor the used tea leaves are well documented (Downham and Collins, 1995; Almela et al., 2002; Dias and Cardoso, 2006).

Many synthetic food dyes like sudan I-IV, amaranth, erythrosine etc. were suspected of being toxic and were banned (Jadhav et al., 2013). The toxicological data also indicated that the indiscriminate use of synthetic food colors could pose serious health problems (Bhatia, 2000; Marmion, 1991; Gomes et al., 2013). Hence presently, the use of synthetic food dye is strictly regulated and each food dye requires Food and Drug Administration (FDA) approval prior to its inclusion to any food product (Abdelmigid, 2009). The maximum permissible level of all food colors that can be added either individually or as blends to different food items has been recommended as 100 ppm (PFA, 2003). In India, Prevention of Food Adulteration has recommended only 8 synthetic colors, viz., tartrazine, sunset yellow, ponceau 4R, fast green FCF, carmoisine, brilliant blue, indigo carmine and erythrosine as food additives (PFA,
However, many confectioners use very high concentrations of these synthetic food dyes without knowing their toxic effects (Bhattacharjee, 2014). Some studies have reported the use of textile and non permitted dyes in food items which caused serious health problems (Khanna and Singh, 1975; Tripathi et al., 2007).

There are number of reports on toxic effects of synthetic food dyes and their blends in various test systems viz., bacteria (Das and Mukherji, 2004; Kaur et al., 2010; Ferraz et al., 2011; Kaur et al., 2012; Zanoni et al., 2013; Atri et al., 2014), plant (Macioszek and Knononowicz, 2004; Kumar and Srivastava, 2011; Gomes et al., 2013; Bhattacharjee, 2014) and mammalian (Hassan, 2010; Himri et al., 2011; Visweswaran and Krishnamoorthy, 2012; Chequer et al., 2012; Divya and Devika, 2012; Dixit and Goyal, 2013; Saxena and Sharma, 2014).


Apart from the toxicological assays, various analytical methods have been used to determine the chemical composition of synthetic food dyes and their concentration in soft drinks, juices, fruit jellies, candies, spices, edible animal tissues, packed and other processed food commodities. Such methods included capillary electrophoresis (Huang et al., 2002; Pardo et al., 2006), thin layer chromatography (Oka et al., 1987; Oka et al., 1994), electrochemistry (Ni et al., 1996; Combeau et al., 2002), spectrophotometry (dos Santos et al., 2010; Soylak et al., 2011; Unsal et al., 2012), ion-pair chromatography (Fuh and Chia, 2002), high performance liquid chromatography (HPLC) with ultraviolet/visible (UV/Vis) and diode-array detectors (DAD) (Minioti et al., 2007; Yoshioka and Ichihashi, 2008; de Andrade et al., 2014) and liquid chromatography–mass spectrometry (LC–MS) (Feng et al., 2011; Zou et al., 2013).

Considering the harmful effects of synthetic food dyes, the present work was planned with following objectives.

- To estimate mutagenic effects of six most commonly used synthetic food dyes viz., bright green, chocolate brown, respebay red, blue, pink and orange employing Ames assay.
- To estimate genotoxicity of the food dyes using Allium sativum root chromosomal aberration assay and Rattus micronuclei assay.
- To carry out the chemical analysis of synthetic food dyes using various analytical techniques.

For experimentation, six synthetic food dye samples viz., bright green, chocolate brown, respebay red (powder form) and blue, orange, pink (liquid form) were purchased from local market of Amritsar, Punjab (India). As the liquid dyes contained two preservatives viz., propylene glycol and sodium benzoate, these chemicals were purchased from Merck and Qualigens, respectively. Five food dye standards viz., brilliant blue (E133), carmoisine (E122), erythrosine (E127), sunset yellow (E110) and tartrazine (E102) were procured from Sigma Aldrich for chemical analysis. All the
other chemicals used were of analytical grade. Different concentrations *viz.*, 25, 50, 75, 100, 250, 500, 750, 1000 µg/l of food dye samples in powder form and 25, 50, 75, 100, 250, 500, 750, 1000 µl/l of liquid food dye samples were prepared by using the double distilled water. Similar concentrations were prepared for sodium benzoate and propylene glycol.

At first, synthetic food dyes *viz.*, bright green, chocolate brown, respebary red (powder form) and blue, orange, pink (liquid form) and two preservatives *viz.*, propylene glycol and sodium benzoate were evaluated for their mutagenic responses employing Ames assay. For estimation of mutagenicity, two tester strains of *Salmonella typhimurium* *viz.*, TA98 (strain with frame shift mutation) and TA100 (strain with base pair substitution mutation) were used. It was observed that all the dyes induced moderate to high mutagenic responses in TA100 tester strain of *Salmonella typhimurium*. Except orange and blue food dyes, all other food dyes were observed to be non mutagenic in tester strain TA98 of *Salmonella typhimurium*. The order of the induction of revertant colonies in different strains at the maximum dose *i.e.* 1000 µg/l for food dyes (powder form) was observed as follows:

i. **TA98 without S9 mix:** respebary red (49) > bright green (42.33) > chocolate brown (37.33).

ii. **TA98 with S9 mix:** respebary red (48.33) > chocolate brown (41.66) > bright green (38.66).

iii. **TA100 without S9 mix:** respebary red (277.33) > bright green (231.33) > chocolate brown (185.00).

iv. **TA100 with S9 mix:** respebary red (272.66) > chocolate brown (247.33) > bright green (229.33).

The order of the induction of revertant colonies at the maximum doses *i.e.* 1000 µl/l for liquid food dyes was observed as:

i. **TA98 without S9 mix:** orange (81.33) > blue (57.66) > pink (35.00).

ii. **TA98 with S9 mix:** orange (83.66) > blue (77.33) > pink (64.66).

iii. **TA100 without S9 mix:** orange (332.66) > blue (322.33) > pink (240.66).
iv. **TA100 with S9 mix:** pink (239) > blue (237.66) > orange (207.66).

In case of preservatives, propylene glycol and sodium benzoate induced moderate to high mutagenic effects in TA100 tester strain and non mutagenic in TA98 tester strain of *Salmonella typhimurium*. Propylene glycol was found to be more mutagenic as compared to sodium benzoate. The data was statistically analyzed using one way ANOVA. It was observed that the number of revertant colonies for both TA98 and TA100 tester strains varied significantly at \( p < 0.05 \) with respect to control for all the dyes at higher concentrations.

*Allium sativum* root chromosomal aberration assay was performed using root dip treatment method following 3 h treatment. Different chromosomal abnormalities were observed in the root tip cells of *Allium sativum* which were apportioned to physiological aberrations (delayed anaphase, vagrants, c-mitosis, stickiness, abnormal anaphase and abnormal metaphase) and clastogenic aberrations (chromosomal breaks, chromatin bridges and chromatin rings).

The order of the genotoxic effect of food dyes was orange > blue > respebay red > bright green > pink > chocolate brown. It was observed that in all the food dyes delayed anaphase dominated among physiological aberrations except for orange food dye where the c-mitosis dominated. Among the clastogenic aberrations, frequency of chromosomal break was found to be the maximum. In case of preservatives, propylene glycol induced the higher number of total chromosomal aberrations as compared to sodium benzoate. The data obtained was analyzed statistically using Chi-square test. It was observed that percent total aberrant cell at all concentrations of food dyes varied significantly at three values of \( p < 0.05, p < 0.01 \) and \( p < 0.001 \) as compared to control values.

As orange food dye induced maximum mutagenic response in both the tester strains *i.e.* TA98 and TA100 of *Salmonella typhimurium* in Ames test as well as maximum genotoxicity in *Allium sativum* root chromosomal aberration assay, this dye was further explored for its genotoxicity in *Rattus* micronuclei assay. The rats were divided in to four groups depending upon the dose of dye given like Control: 0 mg/Kg b.w./day, Group I: 50 mg/Kg b.w./day, Group II: 100 mg/Kg b.w./day and Group III:
150 mg/Kg b.w./day. It was observed that rats of all the groups were found to have micronuclei in their polychromatic erythrocytes after the treatment of 30 days. The frequency of micronuclei was observed to be 2.45, 3.65, 3.61 and 4.61 % for Control, Group I, Group II and Group III, respectively. Results obtained were further analyzed using Chi-square test. Although, the frequency of the micronucleated polychromatic erythrocytes found in Group I, Group II and Group III was not significantly higher than the control group. Yet, the present study clearly indicate that exposure of food dyes can induce the formation of micronuclei.

After evaluation of mutagenicity and genotoxicity of the food dyes, the chemical analysis of food dyes was done using various analytical techniques viz., ultra high performance liquid chromatography (UHPLC), mass spectrometry (MS), scanning electron microscope -energy dispersive X-ray (SEM - EDX) and atomic absorption spectrophotometry (AAS).

During the chromatographic analysis of food dyes using UHPLC, it was observed that the components of all the food dye samples were separated in less than 30 min. Four synthetic food dyes viz., brilliant blue (E133), sunset yellow (E110), tartrazine (E102) and erythrosine (E127) were separated at the retention time (min) of 9.96, 15.00, 21.43 and 27.00, respectively for bright green food dye sample, whereas synthetic food dyes viz., brilliant blue (E133), sunset yellow (E110), tartrazine (E102) and erythrosine (E127) were observed at the retention time (min) of 9.346, 14.895, 21.445, 27.037, respectively for chocolate brown. In case of respebary red, three chromatographic peaks corresponding to sunset yellow (E110), brilliant blue (E133) and erythrosine (E127) were separated at the retention time (min) of 15.017, 21.468 and 27.174, respectively. The component of blue food dye viz., E110, E133 and E127 were separated at the retention time (min) of 14.914, 22.037 and 26.784, respectively. In case of orange food dye, the retention time ($t_R$) for three peaks of sunset yellow (E110), brilliant blue (E133) and erythrosine (E127) were 14.717, 21.424, 26.953, respectively. During the chromatographic analysis of pink food dye, the retention time (min) for three peaks of sunset yellow (E110), brilliant blue (E133) and erythrosine (E127) were 14.915, 21.445, 26.930, respectively.
Mass spectra for six synthetic food dyes were recorded in the electrospray ionization (ESI) positive mode. In the mass spectrum of bright green food dye, the peak corresponding to ion [M+H]+ at m/z = 792.346 was obtained which represented the main components of the dye *i.e* brilliant blue FCF (E133). The mass spectrum of chocolate brown dye, exhibited one major peak at [M+Na]+ m/z = 478.95 which indicated the of presence of tartrazine. The mass spectrum of respebary red resulted in a peak at [M+Na]+ m/z = 478.96 which represented sunset yellow (E110). The mass spectrum of blue dye was shown a major peak at m/z = 771.13 which indicated the presence of brilliant blue FCF (E133). Orange food dye, resulted in one major peak at m/z = 474.95 corresponding to sunset yellow (E110). The other peaks detected in this spectra at m/z = 700.94 and m/z = 861.76 corresponded to the dimmers and trimmers of sunset yellow, respectively. The mass spectrum of pink dye has shown a major peak at m/z = 902.59 that indicated the presence of erythrosine (E127). After the chemical analysis of the synthetic food dyes, elemental analysis of the food dyes was carried out.

The elemental analysis of all synthetic food dyes was done by means of scanning electron microscope-energy dispersive X-ray (Model No. SUPRA-55; Make: Zeiss- Model No. X-Max; Make: Oxford) using Inca software. Various elements *viz.*, carbon (C), oxygen (O), sodium (Na), chlorine (Cl), copper (Cu), iron (Fe) and manganese (Mn) were detected in all the six synthetic food dyes. Apart from this, small traces Nd (Wt % 0.42) was found in respebary red. Iodine was found in the blue and pink food dye by the weight percentages of 4.79 % and 35.98 %, respectively.

The food dye samples were also analyzed for presence of various heavy metals using atomic absorption spectrophotometer (Model: 240 FS; Make: Agilent). It was observed that all synthetic food dyes contained heavy metals *viz.*, cobalt, copper, iron, manganese, lead and zinc which were found in the range of 0.013 mg/g - 0.014 mg/g, 0.004 - 0.007 mg/g, 0.273 - 0.497 mg/g, 0.004 - 0.014 mg/g, 0.042 - 0.044 mg/g and 0.010 - 0.035 mg/g, respectively. It was observed that the maximum content (0.014 mg/g) of cobalt was found in chocolate brown. Maximum content (0.007 mg/g) of copper was found in bright green and respebary red. Iron was found to be maximum (0.497 mg/g) in orange food dye. Pink dye contained the maximum content (0.014 mg/g) of manganese. Lead was found to be the maximum (0.044 mg/g) in two synthetic
food dyes *viz.*, bright green and chocolate brown. The maximum content of zinc (0.035 mg/g) was observed in pink food dye.

The present study revealed the mutagenic as well as genotoxic effects of the food dyes in three bioassays. The presence of heavy metals like Co, Cu, Fe, Mn, Pb and Zn in synthetic food dyes is also a matter of concern. It is evident that prolonged consumption of food products containing these dyes can cause the adverse health effects in human beings. Therefore, the chemical as well as toxicological studies for the food products containing different food additives including the food dyes should be strictly implemented.