

Review

Toxicity of food colours and additives: A review

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Majority of consumer goods are required to be presented with good aesthetics in order to improve acceptability in terms of colours and in some instances taste. When related to food, beverages and drug products, additives are usually added to mask un-inviting colours, obscure offensive odours and increase taste. Food additives therefore include colourants, sweeteners, preservatives and anti-caking agents. Admissible daily intake limits are often recommended for these additives. Being food products, the amount consumed over time may be subject to individual preferences and thus negating the desire to regulate and control the amount consumed cumulatively. There have been several concerns about the safety of food additives and several batteries of tests, and reports are available in literature. This review attempted to give an update on reports that have surfaced in literature over recent past on the use and safety of food colours and other additives. Some safety concerns have been related to three determinations; cytotoxicity, genotoxicity and induction or potential of inducing mutagenicity. In order to accomplish these targeted evaluations, several tests have been prescribed by International conference on harmonization (ICH), organization for economic co-operation and development (OECD) and European food safety authority (EFSA). It is observed that no single test can give a full proof of safety of these food colours and additives, hence minimal tests are recommended to be carried out in order to guarantee safety of these products. Survey of literature, revealed that once some approved additives or colours become a subject of safety concerns, comprehensive evaluations are carried out by researchers and these have often led to the de-classification of some hitherto reported agents as being non-genotoxic or non-carcinogenic. The declassifications of some food colors and additives as human carcinogens are regularly done following the comprehensive evaluation of results of mutagenicity and genotoxicity tests *in vitro* and some *in vivo* tests in mammalian tissues and whole animals. However, such declassifications are often done with caution and the implication is that regular and more comprehensive tests must be carried out. In addition, the requirements of testing for chronic exposures to this and other agents must be emphasized to prevent occurrence of subtle yet terrible side effects resulting from consuming sub-toxic doses of the additives over time.

Key words: Food colour, food additive, cytotoxicity, genotoxicity, mutagenicity, safety concerns.

INTRODUCTION

Food additives are substances that are deliberately added to food substances to impart desired characteristics.

They are used for various purposes including preservation, aesthetics, taste masking and sweetening

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(Yu et al., 2002; Güngörmüş and Kılıç, 2012). However, several epidemiological studies have established an association between environmental exposure to various anthropogenic substances and the development of cancer especially, bladder cancer in later life (Golka et al., 2004).

The ability of drugs and additives to cause genotoxic and/or carcinogenic effects was first reported in the 1970s amongst textile and hair dye applicators (Fraumeni Jr and Miller, 1972; Brambilla and Martelli, 2009). The benzidine-based dyes in use then were reported to cause an increased risk of up to 6.4 times than the expected rate of developing bladder cancer later in life (Golka et al., 2004). Following the sulphanilamide elixir and thalidomide crisis in the 1930s and 60s respectively, researchers like Rall (1975) regarded carcinogenicity of drugs as the next potential drug regulatory crisis and strongly advocated for their continuous evaluation using lifetime rodent carcinogenicity assay. The recommended standard battery of tests for the genotoxicity assessment of new chemical entities includes:

- (1) Salmonella gene mutation test.
- (2) *In vitro* gene mutation test using human lymphocytes.
- (3) A test for chromosomal aberration and DNA damage *in vivo* using rodent bone marrow (FDA, 1993).

The objectives of genotoxicity tests are to detect mutagens and carcinogens as well as study the mechanisms of chemical mutagenesis and carcinogenesis; and assess the mutagenic and carcinogenic hazards of chemicals to humans. Until recently, the end points of the most frequently used methods usually employ the detection of either gene mutation in bacteria or specialized mammalian cells, or the *in vivo* and *in vitro* assessment of chromosomal aberrations in *in vitro* and *in vivo* models or systems (Sasaki et al., 2000).

The current international conference on HARMONIZATION (ICH) guidelines on genotoxicity of small molecules allow for a standard battery of tests at both *in vitro* and *in vivo* interphases (ICH). The tests are designed and aimed at detecting compounds that induce genetic damage by various mechanisms. "These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage or recombination is generally considered to be essential for heritable effects and in the multi-step process of malignancy, a complex process in which genetic changes may play only a part" (ICH S2(R2)). This review attempts to chronicle the various food additives that are currently accepted as 'safe' by either the food and drug administration (FDA or USFDA) or the organization for economic co-operation and development (OECD). The review also covers some independent studies that have been carried out on the food additives

and thereafter presents an overview of current status on the genotoxicity or otherwise of these approved food additives.

Approved food colours and additives

The US FDA and the European Union as well Canadian agency have all equally published a list of approved colourants and additives. The documents from these agencies are comprehensive and there are guidelines to the use of agents meant for use in animal and man. In the FDA Federal register, agents approved for animal use are clearly identified and those allowed for human consumption are also prescribed. The FDA guide stipulates that "color additives listed in 21 CFR Parts 74 and 82 must be analyzed and batch certified by FDA before they can be used in any FDA-regulated product marketed in the U.S. This requirement applies to products imported into this country as well as those manufactured domestically" (FDA, accessed 20 May, 2015). In addition, the document stipulates that "manufacturers of certified color additives must include on the label the name of the certified color additive, a statement indicating general use limitations, any quantitative limitations in products, and the certification lot number assigned to the batch".

The EU document last updated in 2014 also stipulates that "Most additives are only permitted to be used in certain foods and are subject to specific quantitative limits, so it is important to note this list should be used in conjunction with the appropriate European Union legislation" (EU, accessed 20 May, 2015). However, a proviso clearly states that the document is not for legal guidance. Each approved food colour and additives are assigned specific "E" numbers. The following sections of this review provide a comprehensive review and assessment of literature available on approved colourants and other additives. The data available from researchers on the three indices of toxicity (cytotoxicity, genotoxicity and mutagenicity) are reviewed. The chemical structures of the food colours whose toxicity profiles are reviewed are presented in Figure 1.

SUMMARY OF LITERATURE ON THE GENOTOXICITY ASSESSMENT OF SOME FOOD ADDITIVES

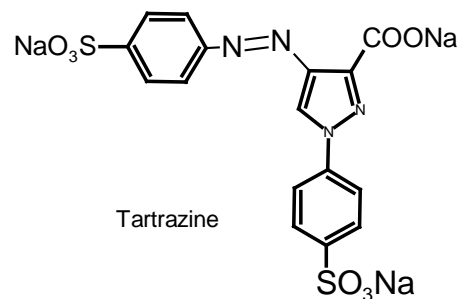
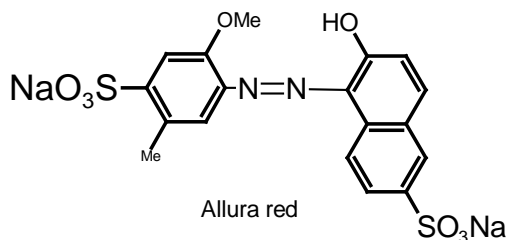
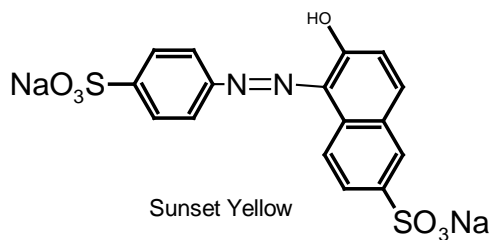
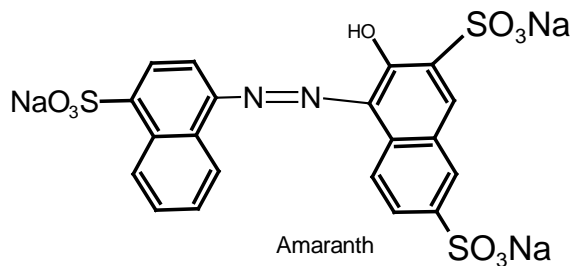
Amaranth

Food Red No.2, CAS No. 915-67-3.
Chemical name: Trisodium 2-hydroxy-1-(4-sulphonato-1-naphthylazo)naphthalene-3,6-disulphonate.

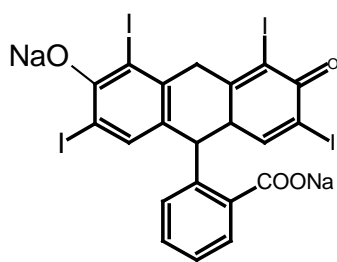
Cytogenetic evaluation

Amaranth in an *in vitro* assay increased the frequency of

(a) Azo dyes



(b) Xanthene dyes



(c) Triphenylmethane dyes

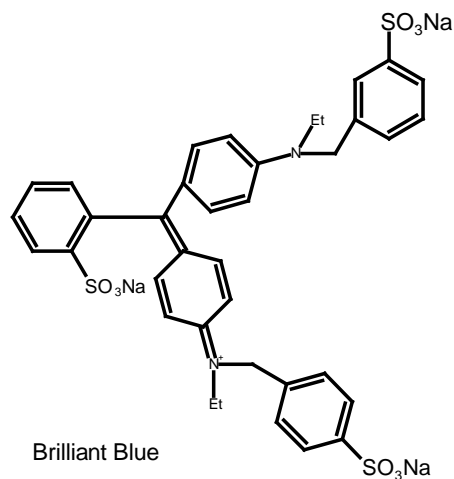


Figure 1. Some representative food colours.

sister chromatid exchange in treated cells compared to control. It also demonstrated cell cytostatic potential (Mpountoukas et al., 2010). There is an abundance of old literature on amaranth because of its long history of use in the food and drug Industry (Combes and Haveland-Smith, 1982; Leconinte and Lesca, 1978). There are many reports documenting the lack of genotoxicity of

amaranth on bacteria (Brown et al., 1978; Haveland-Smith and Combes, 1980; Chung et al., 1981; Das and Mukherjee, 2004).

However, there is still a large amount of uncertainty and discrepancies on the reported *in vivo* follow up tests. Sasaki et al. (2002) reported the genotoxicity of amaranth using comet assay on eight mouse organs. Following a

24 h single dose treatment, amaranth at doses lower than the admissible daily intake (2000 mg/kg body weight, (b.w.)) showed genotoxicity damage in three different organs. These included tests aimed to expose specific colon DNA damage at doses as low as 10 mg/kg b.w. after a 3 h sampling time (Yu et al., 2002). Results from a similar study are corroborative but seem to suggest inter-species differences in toxicity towards mice and rats (Shimada et al., 2010).

On the other hand, the dye as assessed by the micronucleus gut assay of mice following double dosing at 24 h interval was reported to be non-mutagenic and did not show any significant difference in the frequency of micronucleated cells relative to control. The authors went on to suggest that the toxicity observed in Sasaki et al. (2000) report might be due to acute cell toxicity and death following treatment with the dye (Poul et al., 2009). Similar results were obtained in another study that involved the micronucleus assay of the stomach, colon and bone marrow of rats (Okada et al., 2013). The inability of the colourant to induce chromosomal aberrations in *in vivo* bone marrow assay has also been reported (Das and Mukherjee, 2004).

There is insufficient evidence to confirm carcinogenicity of amaranth in humans and it is presently unclassified as a human carcinogen. Such declassifications for food colours and additives are, however, often done with caution and the implication is that regular and more comprehensive tests must be carried out with very large sample size in order to be able to draw unequivocal conclusions of toxicity or otherwise. In addition, the requirements of testing for chronic exposures to these agents must be emphasized to prevent occurrence of subtle yet terrible side effects resulting from consuming chronic and sub-toxic doses of the additives over time.

Tartrazine

FD&C Yellow No. 5, Food Yellow No.4, E 102, CAS 1934-21-0.

Chemical name: 3-carboxy-5-hydroxy-1-(4'-sulphophenyl)-4-(4'-sulphophenylazo) pyrazole trisodium salt.

Cytogenetic evaluation

In a recent study, the interaction of tartrazine and endogenous material like bovine haemoglobin was described as spontaneously involving Van der Waal's forces and hydrogen bonds between the oxygen atoms at position 31 and 15 in the dye (Li et al., 2014). In a similar study, tartrazine showed extensive DNA binding, cytostatic potential and reduced mitotic index (Mpountoukas et al., 2010).

Gene mutation assays

Several reports have indicated the non-mutagenicity of tartrazine using *Salmonella typhimurium* and *Escherichia coli* (Chung et al., 1981; Das and Mukherjee, 2004; Elhkim et al., 2007; EFSA, 2009). The addition of rat liver microsome preparation S9 have also not shown the colourant as genotoxic (EFSA, 2009). In a *Salmonella* mutagenicity assay without metabolic activation, Das and Mukherjee (2004) reported the inability of the dye (at 10 to 1000 µg/plate concentration) to induce point mutation in Tester strain T97a and base pair mutation in T100. However, there was a significant positive result with the strain T98 but this was not dose-related.

In vivo assays

Sasaki et al. (2002) reported the results of comet assay on eight mouse organs following oral administration of tartrazine up to doses of 2000 mg/kg b.w. After only 3 h post-administration, DNA damage was reported at dose levels of 10 mg/kg b.w in the colon and the glandular stomach at doses higher than 10 mg/kg b.w. (Sasaki et al., 2002). Poul et al. (2009) demonstrated the non-mutagenicity of tartrazine when administered as oral gavage up to doses of 2000 mg/kg b.w. The dye at any of the tested doses did not produce an increase in the number of micronucleated colonic cells when compared with water and olive oil control groups. The food dye however produced an increase in mitotic index at all tested doses although this effect was not dose related. The authors thereafter concluded that the extensive DNA damage observed by Sasaki et al. (2002) at the same dose level could be due to the acute cytotoxicity of the dye. It could also be due to insufficient DNA repair at the 3 h sampling time (Poul et al., 2009).

Chronic toxicity

Long term carcinogenicity tests

In order to assess the effects of tartrazine on chronic administration, some reports are currently available in the literature. A 90-day oral sub-chronic toxicity involving daily dosing of Wistar rats has been reported. The rats were divided into groups of six and fed a diet containing 5, 7.5 and 10 mg/kg b.w. The haematological, clinical chemistry and histopathology parameters of the treated animals were then compared with a control group that received only distilled water. The results showed significant dose-related increase in blood chemistry parameters like glucose, creatinine, blood urea nitrogen, total cholesterol, total serum protein, triglycerides and aspartate aminotransferase when compared with the control group (Himri et al., 2011). A lack of carcinogenicity

in F334 rats have been reported when tartrazine was administered in doses of 0, 1 and 2% in the drinking water of groups of 50 male and female rats for 2 years (Maekawa et al., 1987). No toxic lesions were reported in the treated groups at any of the dye doses. All of the tumours observed, both in the treated and control groups, were characteristically spontaneous in this strain of rats and the authors did not find any difference in the organ distribution and the histology of the affected organs when they compared the treated groups with the control. Similarly, when compared with the incidences of spontaneous mesothelioma (amongst males) and endometrial stromal polyps (in females) derived from historical controls; the authors concluded that the increase in the incidences of these tumours that was observed in the 1% treated group is not related to the dye administration.

Reproductive and developmental toxicology

The teratogenic potential of dose levels of 0, 60, 100, 200, 400 and 600 mg/kg b.w of tartrazine when administered to pregnant Osborne-Mendel within the first 19 days of gestation has been reported. Tartrazine when presented either as oral gavages or in drinking water did not produce any significant toxic effect in the treatment groups when compared with the control groups. The primary outcomes used to assess teratogenicity included maternal clinical findings, rate/success of implantation, foetal size and development, foetal bone and visceral organ development (Collins et al., 1990, 1992).

Mehedi et al. (2009) however, reported the toxicity of tartrazine on the reproductive organs of male Swiss albino mice which were administered doses of 0, 0.1, 1.0 and 2.5% in drinking water for 13 weeks. The results showed that although there was no significant difference in the weights of the seminal vesicle and epididymis of the treated animals, a reduced sperm count and increased incidence of sperm abnormalities were observed in the treatment group that received 2.5% dose. They also reported a reduction in reproductive performance as estimated by mating index in the groups that received 1 and 2.5% doses.

Hyperactivity, anxiety and depression effects

Kamel and El-Iethy (2011) reported that tartrazine caused an increased activity, anxiety and anti-social behaviour among male Wistar rats. The rats were put in three groups of 15 and given tartrazine doses of 0, 1 and 2.5% in their drinking water. The treated animals showed an increase in anxiety as measured by the different animal models of open field, elevated plus-maze and the dark-light transition tests. Hyperactivity, as measured with the open maze model, depression and a decreased

frequency of social contacts in the treated groups was also reported.

Allura Red

FD & C Red 40, CAS No. 25956-17-6.

Chemical Name: disodium 2-hydroxy-1-(2-methoxy-5-methyl-4-sulphonatophenylazo) naphthalene-6-sulphonate.

Allura red was first introduced in the USA as a food colourant in 1971. It is non-genotoxic in many gene mutation tests involving prokaryotic and eukaryotic cells with or without activation (Chung et al., 1981; Combes and Haveland-Smith, 1982). However, allura red was reported to show direct genotoxic effect when different concentrations of the dye ranging from 9.76 to 5000 µg/mL was incubated with a culture of *Saccharomyces cerevisiae* at 37°C. Comet assay revealed dose-related DNA damage starting at concentration of 1250 µg/ml, though no positive correlation could be established with exposure time (Jabeen et al., 2013).

In vivo assays

The non-teratogenicity of allura red has been reported after groups of 11 day old pregnant rats were fed up to 2000 mg/kg b.w. of single oral doses of allura red (Tsuda et al., 2001). Comet assay was used to assess DNA damage in the embryo at 3, 6 and 24 h sampling times. When assessed by comet assay, colon-specific DNA damage was reported in mice at doses of 10 mg/kg b.w. three hour post-administration but no damage was observed in rats at any of the tested dye doses or exposure times (Shimada et al., 2010).

The results of the latter study are partly responsible for the somewhat surprising European Food Safety Authority (EFSA) classification of allura red as prohibited in animal feeds though it is still regarded as safe for human use (EFSA, 2009, 2012). Abramsson and Ilbäck (2013) using a flow-cytometric based micronucleus assay *in vivo* reported that intra-peritoneal administration of allura red does not cause a depression in cell proliferation or an increase in the frequency of micronuclei in polychromated erythrocytic cells.

The authors tested the dyes up to doses of 2000 mg/kg b.w. as suggested by the preliminary acute toxicity carried out by Sasaki et al. (2002). They also justified the use of the intra-peritoneal route on the poor uptake of allura red from the intestine and suggested that this coupled with the possibility of microbial formation of substances with local cellular action might partly explain the toxicity seen in the report by Shimada et al. (2010).

Sunset yellow FCF

Food Yellow No.5, CAS No. 2783-94-0.

Chemical name: Disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate.

Early reports demonstrated the non-mutagenicity of sunset yellow on *E. coli*, four tester strains of *S. typhimurium* TA 1538, 1535, 100 and 98 strains with or without metabolic activation (Chung et al., 1981; JECFA, 1982; Wever et al., 1989). Haveland-Smith and Combes (1980), also tested the ability of twenty five dyes to induce mutations in a tryptophan-requiring *E. coli* strain (sensitive to base substitutions) and a histidine auxotroph of *S. typhimurium* strain TA1538 (specific for frameshifts). Sunset yellow was demonstrated to be non-mutagenic with or without metabolic activation.

In vivo testing

Sunset Yellow FCF did not induce DNA damage in any of the eight mouse organs-glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, bone marrow-assessed by comet assay following a single oral dosing of the dye up to 2000 mg/kg (Sasaki et al., 2002). Similarly, Poul et al. (2009) did not find any statistical difference in the gut micronuclei assay of groups of mice that had received the dye up to 2000 mg/kg b.w and a control group. The metabolites were monitored in the faeces using HPLC and reported to be non-genotoxic as well. The results of these studies agrees with that of Wever et al. (1989) who did not find any mutagenicity or clastogenicity effects when the dye was administered to rodents by gavage.

Chronic/long-term carcinogenicity studies

A number of studies describing the administration of sunset yellow in doses of 0 to 2% in mice for periods of 52 to 80 weeks have reported no significant difference in the incidence of tumours when compared with appropriate control groups (Bonser et al., 1956; Gaunt et al., 1974; JECFA, 1982; EFSA, 2009). Similar long-term studies in rats, hamster and dogs have not detected any carcinogenic effects associated with the dye when administered up to 5% doses (JECFA, 1982; EFSA, 2009).

Erythrosine

Food Red No. 3, CAS No. 16423-68-0

Chemical Name: disodium 2-(2,4,5,7-tetraiodo-6-oxido-3-oxoxanthin-9-yl) benzoate

Gene mutation assay

The inability of erythrosine to induce gene mutation in *S.*

typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 at concentrations of up to 10,000 µg/plate have been demonstrated. The dye or its metabolites are reportedly non-mutagenic (Auletta et al., 1977; Bonin and Baker, 1980; Ishidate et al., 1984; JECFA, 1986; EFSA, 2011). In another report, erythrosine was not only non-mutagenic in *S. typhimurium* strains TA 97a, TA 98, TA 100, TA 102 and TA 104 but also reduced the anti-mutagenic potential of benzopyrene, sodium azide and ethidium bromide (Lakdawalla and Netrawali, 1988).

However, the mutagenicity of erythrosine has been reported in the *Bacillus subtilis* multigene sporulation assay (Lakdawalla and Netrawali, 1988) and *S. cerevisiae* strains D7 and XV185-14C (Matula and Downie, 1984). The *in vitro* genotoxicity of erythrosine using the Comet and Cytokinesis-block micronucleus cytome (CBMN-Cyt) assays have been investigated (Chequer et al., 2012). HepG-2 cells, which have the ability to mimic phase I and II metabolic activities, were incubated with dye concentrations of 0.1, 0.2, 2.0, 10, 25, 50, or 70 µg/mL. Results of Comet assay after 4 h of incubation showed significant DNA damage at 50 and 70 µg/mL when compared with the control group. Similarly, in the CBMN-Cyt assay, erythrosine caused an increase in micronuclei frequency at all doses greater than 0.1 µg/ml.

In another study to determine the cytotoxicity and genotoxicity of erythrosine using V79 hamster lung cells, the dye was cytostatic at 200 µg/ml and toxic to at least 90% of the cells at 400 µg/ml. In excess of 300 µg/ml genotoxicity was reported although this was not dose-related (Rogers et al., 1988). Using the Comet assay to monitor 8 mouse organs, Sasaki et al. (2012), also reported that the dye, after only 3 h post administration, produced dose-related DNA damage in the glandular stomach, colon and urinary bladder in mice that had received a single oral dose of 10, 100 or 2000 mg/kg b. w. The lowest dose that caused significant damage in the stomach and colon was 100 mg/kg bw.

Erythrosine (at doses of 50, 100 and 200 mg/kg b.w. repeated after 24 h) did not increase the frequency of sister chromatid exchanges in peripheral blood lymphocytes in male B6C3F1 mice. No increase in the frequency of micronuclei in the bone marrow polychromatic erythrocytes or peripheral blood reticulocytes was also observed. The authors suggested that the lack of a clastogenic potential provides support for the non-genotoxic mechanism of the carcinogenicity of erythrosine (Zuno et al., 1994).

Cytogenetic evaluation

A high degree of cytotoxicity and cytostaticity has been reported at erythrosine doses of 2,4 and 8 mM when tested on human peripheral blood cells *in vitro* (Mpountoukas et al., 2010). In the same study, extensive

direct binding of the dye to calf thymus DNA was reported.

Long-term carcinogenicity

Long term carcinogenicity studies have demonstrated that there was no significant difference in the incidence of non-neoplastic lesions or malignant tumours observed in the control and treated groups of Charles River CD weanling rats of both genders that have been exposed *in utero* to the dye and subsequently fed erythrosine doses of 0.1, 0.5, or 1.0% for 30 months. In the high dose part of the same study, in which groups of mice were fed 0 or 4% erythrosine in their diet for 29 months following *in utero* exposure, no dose-related effects were found in blood chemistry parameters, physical behaviour, urinalysis or mortality. However, there was an average of 100% gain in thyroid weights among the treated males as well as a significant increase in the incidence of follicular adenoma of the thyroid in the treated males compared to the control (Borzelleca et al., 1987; EFSA, 2011). A similar study in mice has been conducted (Borzelleca and Hallagan, 1987).

In another study, no tumour was observed in groups of Osborne Mendel rats that received oral gavages (0, 0.5, 1.0, 2.0 or 5.0%) or sub-cutaneous injections (12 mg/rat) for 24 months (Hansen et al., 1973). No mortality or dose-related adverse effects were reported in a similar study where groups of three female and three male beagle dogs were fed 0, 0.5, 1.0, 2.0 or 5.0% for 2 years (Hansen et al., 1973).

Reproductive and developmental toxicity

Erythrosine does not cause damage to the foetus following maternal exposure to dye (Collins et al., 1993a, b; EFSA, 2011). A number of studies have also failed to establish significant dose-related adverse effects in the reproductive toxicity and post-partum development of F0 and F1 generation of rats that received erythrosine (Vorhees et al., 1983; JECFA, 1986; Borzelleca et al., 1987; Tanaka, 2001). Erythrosine has also been discovered to possess no psychotoxic potential in developing rats (Vorhees et al., 1983). Erythrosine, however, was adjudged to have potential toxic effects on the reproductive process in male adult mice causing a decrease in the epididymal sperm count and motility as well as an increased frequency in sperm abnormalities (Abdel-Aziz et al., 1997; Vivekanandhi et al., 2006).

Brilliant Blue FCF

CAS No. 3844-45-9, Food Blue No. 1, FD & C Blue. No.1

Chemical Name: Ethyl - [4 - [[4 - [ethyl -[(3 - sulfophenyl) methyl] amino] phenyl] - (2 - sulfophenyl) methylidene] - 1 - cyclohexa - 2, 5 - dienyldiene] - [(3 - sulfophenyl) methyl] azanium.

Gene mutation assay

The non-mutagenicity of brilliant blue in various *Salmonella* strains with or without metabolic activation has been demonstrated in many studies (Brown et al., 1978; Bonin and Baker, 1980; Haveland-Smith and Combes, 1980; Ishidate et al., 1984).

In vivo assays

Brilliant Blue did not increase the frequency of micronuclei in the bone marrow of groups of mice that received intra-peritoneal doses of 0, 500, 1000 or 2000 mg/kg b.w. (EFSA, 2010). In another study that was not considered by the last EFSA re-evaluation of brilliant blue because of the inconsistent osmolality of cell culture medium used, the dye was reportedly genotoxic in the chromosome aberration assay of over 190 additives using Chinese hamster fibroblast cell line (Ishidate et al., 1984; EFSA, 2010). The colourant at doses up to 2000 mg/kg b.w. gave negative results in a genotoxicity assessment of 39 food additives by comet assay on eight mouse organs (Sasaki et al., 2002).

Long-term/carcinogenicity studies

In a lifetime/carcinogenicity study that involve F0 and F1 generation, dye doses of 0, 0.1, 1.0 or 2.0% were fed to groups of Charles River CD rats for a period of 116 weeks for the males and 111 weeks for the females. A no-observed-adverse-effect-level (NOAEL) of 2 and 1% was established for the male and female groups respectively. In the same study with CD mice fed up to 5% dye, no adverse effects was observed and the NOAEL was established at 5% for both genders (Borzelleca et al., 1990). In a study conducted before the OECD guidelines were formulated and publicized, dietary concentrations of brilliant Blue, 0.03, 0.3 or 3% when fed to groups of 30 rats for 75 weeks did not produce any dose-related adverse effects on the growth or mortality (Mannell et al, 1962). Similar results were seen with the sub-cutaneous injection (Mannell and Grice, 1964).

Preservatives

In addition to the use of colourants, other agents are also included in food and other consumer goods. The inclusions of preservatives in such products are meant to

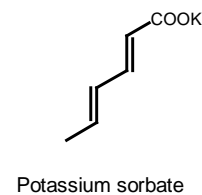
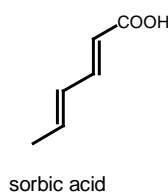
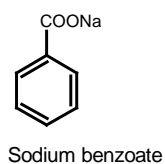
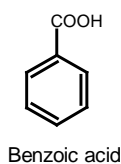
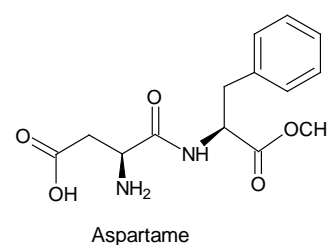
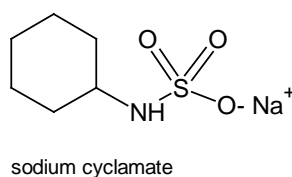
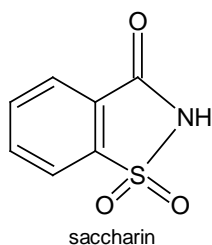
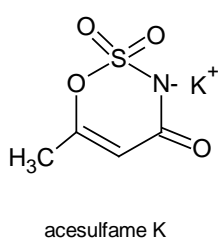
Preservatives**Sweeteners**

Figure 2. Some common food preservatives and additives.

serve the purpose of “warding off” microbial spoilage of such products. Safety concerns are also equally important in the use of these preservatives. The sections below give a review of some commonly used approved preservatives and the tests that have been carried out to determine their toxicity or otherwise. Figure 2 shows the chemical structures of the preservatives and sweeteners reviewed in this report.

Benzoic acid and sodium benzoate

The preservative could not induce umu gene expression in *S. typhimurium* TA 1535/pSK1002 which was defined as a 2-fold increase in the background activity of beta-galactosidase (Nakamura et al., 1987). Similar negative results were obtained in other studies with or without metabolic activation (Ishidate et al., 1984; Zeiger et al., 1988).

Cytogenetic assay

In a cytogenetic assay in which 50, 100, 200 and 500 µg/mL concentrations of the preservative were incubated with human lymphocytes, the additive, when compared with a negative control, caused dose-dependent increase in the frequency of micronuclei, sister chromatid exchange and chromosomal aberrations at all tested doses after 24 and 48 hours (Yilmaz et al., 2009). Chromatid breaks and sister chromatid unions were the

most frequently reported aberrations in the study. After 48 h, 100, 200 and 500 µg/mL doses significantly reduce the mitotic index in a dose-related manner. This result agrees with another that concluded that sodium benzoate, potassium benzoate are clastogenic, mutagenic and cytotoxic to human lymphocytes *in vitro* (Zengin et al., 2011).

Benzoic acid was also found to increase chromosomal aberrations and decrease mitotic index in the root tips of *Allium sativum* after 24 and 48 h treatment with doses up to 500 mg/L (Yilmaz et al., 2008). Similar results were obtained with sodium benzoate (Njagi and Gopalan, 1982; Onyemaobi et al., 2012). Sodium benzoate was also positive in producing toxic effects using an *in vitro* screening with Chinese hamster cell line (Ishidate and Odashima, 1977; Ishidate et al., 1984).

In vivo assays

In a genotoxicity assessment of 39 food additives using comet assay on eight mouse organs, benzoic acid and sodium benzoate did not produce DNA damage in any of the organ at all tested doses (Sasaki et al., 2002).

Long-term carcinogenicity studies

The available studies on long-term carcinogenicity tests were conducted before the OECD guidelines and they are uniform in categorizing sodium benzoate as non-

carcinogens (Sodemoto and Enomoto, 1980; Toth, 1984). Despite some equivocal results obtained with *in vitro* chromosomal aberration assays, no positive genotoxic effects have been reported in an *in vivo* assay. In the light of this and the non-carcinogenicity in long-term assays, benzoic acid and sodium benzoate are classified as non-carcinogens (OECDs; SCCP, 2005).

Sorbic acid and sorbates

Oral administration of sorbic acid at doses up to 5000 mg/kg b. w. did not induce sister chromatid exchanges or increase in micronuclei in the bone marrow of mice. No DNA damage was observed when sorbic acid was incubated with human A549 cells or following intraperitoneal treatment of rats with 500 to 1200 mg potassium sorbate/kg b. w. (Jung et al., 1992).

Other studies have reported the lack of mutagenic and carcinogenic potential in both *in vitro* and *in vivo* models (Ishidate et al., 1984; Münzner et al., 1990; Walker, 1990; Schiffmann and Schlatter, 1992; Mamur et al., 2010). However, prolonged storage of sodium sorbate has reportedly yielded an oxidative degradation product, 4, 5-oxohexanoate which is mutagenic in the Ames test (Jung et al., 1992; Schiffmann and Schlatter, 1992) and may be partly responsible for the weak genotoxic results reported in cultured Chinese hamster V79 cells and wing spot test of *Drosophila* (Hasegawa et al., 1984; Schlatter et al., 1992).

The latter result emphasizes the need to consider the effect of metabolism on toxicities of the food colors and other additives. Metabolism of chemical compounds can either activate or deactivate a molecule. The end-results of exposure of a latent functional group in a chemical compound can lead potential toxicity.

Sweeteners

The development and use of sweeteners have been dogged by controversy and opinions are still divided as to the role they play in cancers especially of the brain and bladder, leukemia, lymphoma (Magnuson et al., 2007; Whitehouse et al., 2008; EFSA, 2013).

The use of artificial high-intensity sweetening agents in the food industry is increasingly becoming more popular. Their increased use is often tied to the promoted benefits of the products which include tooth friendliness, improved quality of life for patients of diabetes of various types and the allure of consumers enjoying their favourite 'unhealthy' drinks or snacks without the attendant problem of weight gain. This aggressive promotion has however not quietened many consumers who still maintain that artificial sweeteners are not safe and that their preference over beet sugar or cane sugar by the food industry has been solely for economic reasons. It is therefore necessary for the food industry to ensure

consumer confidence by controlling the content of sweeteners in processed food and ensuring that the limit amount or concentration permissible in various types of food are not exceeded. Artificial sweeteners include those classified as the first generation-saccharin, cyclamate, aspartame and the new generation including acesulfame-K, sucralose, neotame. Different blends of the sweeteners are also used extensively in soft drinks (Weihrauch and Diehl, 2004). Another classification of artificial sweeteners grouped them into synthetic, semi-synthetic and natural. They comprise of a wide variety of organic molecules (example, carbohydrate derivatives, salts of organic acids, terpenoids and even proteins (Capitan-Vallvey et al., 2006).

Gene mutation assay

Aspartame, acesulfame-K and saccharin were found to be non-mutagenic in *S. typhimurium* strains T 97a and T 100a study (Bandyopadhyay et al., 2008). This study however is not compliant with the OECD guideline that requires full testing with five strains including: *S. typhimurium* TA1535, *S. typhimurium* TA1537 or TA97 or TA97a, and *S. typhimurium* TA98, *S. typhimurium* TA100 and *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101), or *S. typhimurium* TA102 (OECD, 1997). Another study did not find any genotoxic potential when aspartame, acesulfame K, cyclamate, saccharin or sucralose were tested on hepatocytes from F344 and Sprague-Dawley male rats (Jeffrey and Williams, 2000).

In vivo assays

Chromosomal aberration assays in bone marrow of Swiss albino mice that had been fed blends of aspartame and acesulfame-K did not show any genotoxic activity (Mukhopadhyay et al., 2000). Contradictorily, acesulfame K at dose below the recommended no-toxic-effects amounts showed positive clastogenic potentials in a similar study (Mukherjee and Chakrabarti, 1997). Comet assay in eight organs of mice that have been fed the sweeteners showed that aspartame and acesulfame K lacked genotoxic potentials. However, sodium cyclamate produced significant DNA damage in the glandular stomach, colon, kidney and urinary bladder. Saccharin and sucralose were also genotoxic (Sasaki et al., 2002).

Long-term carcinogenicity studies

Contrary to public suspicions, there is no credible link between aspartame, saccharin or cyclamate and cancer. A number of long-term animal studies and epidemiological studies in human population have not shown that the use of these additives increases the risk of developing cancer in later life (Weihrauch and Diehl,

2004; Magnuson et al., 2007; EFSA, 2013).

However, two studies that reported tumourigenic potential generated a lot of media interest and ultimately led to withdrawal of saccharin in Canada and a label warning on all food containing saccharin (Squire, 1985; Taylor et al., 1980). Taylor et al. (1980), reported a significant increase in the incidence of neoplasms in F1 generation of male Charles River CD rats that received a 7.5% dietary concentration of saccharin. A subsequent report by Squire (1985) also found sodium saccharin related increase in the incidence of urinary bladder hyperplasia.

Saccharin would later be de-listed as a carcinogen following a study that showed that sodium ascorbate like most sodium salts (sodium saccharin inclusive) produces cytotoxic calcium phosphate containing crystals in the male rats. This important step in carcinogenicity is specific in rats because of a combined effect of high urine protein levels and the resultant high urine osmolality at levels that are not observed in humans (Cohen et al., 1998).

Role of metabolism in carcinogenicity

Mutagens that cause damage by interacting directly with DNA are categorized as genotoxic. The theoretical considerations that have been considered in explaining their mechanism of toxicity include DNA intercalation in between base pairs (Hendry et al., 2007) as well as metabolic activation following one or a combination of any of intestinal azo reduction, hepatic azo reduction, hepatic oxidation, liver microsomal enzymes activation (Moller and Wallin, 2000).

Metabolism plays important roles in the activity of xenobiotics in the body by producing highly reactive electrophiles that covalently bind to DNA and proteins (Guengerich, 2001). The reduction of the azo linkage in azo dyes by the intestinal bacteria produces aromatic amines which are responsible for the toxicity, mutagenicity and carcinogenicity of these dyes (Chung et al., 1992; Chequer et al., 2011). Over 45 intestinal bacteria express azoreductases that metabolize 2-hydroxylazo naphthols to aromatic amines (Zimmermann et al., 1982; Rafii and Cerniglia, 1995; Moller and Wallin, 2000). Microsomal hepatic azoreduction have also been reported with the food/drug additive amaranth. In a study, the activity of the azoreductase was greatly enhanced in mice by their pretreatment with the cytochrome P-450 inducers phenobarbital and 3-methylcholanthrene (Moller and Wallin, 2000).

Hepatic oxidation is mediated by cytochrome P-450 and the widely distributed peroxidase enzymes. Oxidation often produces highly unstable electrophilic free radicals that readily stabilize themselves by alkylating DNA, thereby causing disruption. Inhibition of Human CYP3A4 and P-glycoprotein by superoxide

radicals generated from xanthene colourants have been reported (Mizutani, 2009). Many genotoxic substances have also been reported to act *via* direct DNA and/or protein adducts formation (Adegoke et al., 2012a; Adegoke et al., 2012b).

However, a large number of mutagens that do not directly interfere with DNA integrity have also been implicated in human carcinogenicity. Such substances, classified as non-genotoxic, act by effecting epigenetic changes. Epigenetics is defined as "the heritable changes in the activity of gene expression without alteration of DNA sequences, which has been linked to many human diseases, including cancer. DNA methylation and histone modifications are well-known epigenetic changes that can lead to gene activation or inactivation" (Kawasaki and Abe, 2012). Such changes are transmitted through mitosis and meiosis processes and always occur with the preservation of the original DNA sequence (Brait and Sidransky, 2011; Kawasaki and Abe, 2012).

A number of mechanisms have been proposed for the toxicity of non-genotoxic carcinogens and these include peroxisome proliferation, aryl hydrocarbon receptor (AhR) binding, inhibition of gap junctional intercellular communication, oxidative stress, alteration of DNA methylation, endocrine disruption and generative cell proliferation (Kristensen et al., 2009; Serafimova et al., 2010).

The categorization of mutagens is not always distinct since they can act by a combination of mechanisms. Also, gene mutations are only partly responsible for tumour development as cancer is caused by a combination of factors (Sarasin, 2003; Sugimura et al., 1992). For example, *p*-chloroaniline is regarded as a genotoxic non-carcinogen that produces detectable genotoxic responses but not in sufficient levels as to initiate tumourigenesis in a long term bioassay (Kirkland and Speit, 2008). Similarly, a number of cellular damages like DNA strand breakage are repaired by the cells while some genotoxic processes do not occur with sufficient degree to lead to inheritable changes. This therefore requires that genotoxicity tests must not only be sensitive (able to give positive results with carcinogens) but also specific, that is, able to give negative results with non-carcinogens.

Limitations and challenges of genotoxicity assessment

Appropriate In vivo follow-up assay of genotoxicity

In addition to the recommended genotoxicity tests (FDA, 1993), there has been increasing calls for the inclusion of an appropriate *in vivo* genotoxicity test following a positive result in the *in vitro* assay. Although, the micronucleus assay has been conventionally used (Heddle et al., 1983; Heddle et al., 1991; Kirsch-Volders

et al., 2011), other tests such as those based on DNA adduct formation (Dybing et al., 1984), *in vivo* chromosomal aberration (You et al., 1993), transgenic mutation (Heddle et al., 2000; Lambert et al., 2005) and comet assay (Tice et al., 2000; Kumaravel and Jha, 2006; Olive and Banáth, 2006) have also been routinely employed in the past as *in vivo* follow up assay when equivocal or inconclusive results are obtained in the *in vitro* assay. Kawaguchi et al. (2010), reported an identical sensitivity of the micronucleus test and the comet assay in detecting the studied mutagens but were quick to point out that the power of the comet assay to detect a low level of genotoxic potential can be superior to that of micronucleus test by the inclusion of using DNA re-synthesis inhibitors.

In another study to compare the ability of these follow up tests to detect 67 known carcinogens that were missed in the traditional micronucleus test, transgenic assay detected about 50%, UDS about 20%, while the comet assay detected up to 90%. The comet assay also showed a high specificity giving negative results with non-carcinogen with an accuracy of up to 78% (Kirkland and Speit, 2008). When compared to other genotoxicity assessment methods, the comet assay is flexible, requiring only a small amount of sample and cells (Tice et al., 2000). It has also been most adaptable for the detection of various forms of DNA damage including double strand breaks, cross DNA damage and apoptosis (Olive and Banáth, 2006; Speit and Hartmann, 2006).

The comet assay has also been employed in multi-end point assay (Bowen et al., 2011) as it can be conducted using cells from virtually all organs (Kirkland and Speit, 2008). This is very useful as some chemicals may not require liver activation and may produce tumours in the tissue of first contact. It may therefore be necessary to assess DNA damage on the gastrointestinal tract for an oral drug, the rectum for a suppository or the skin for a topical preparation. However, the comet assay has some limitations as well with the most important of these being the overestimation of genotoxicity as DNA damages like strand damage, may ultimately be repaired or lead to cell death and not necessarily tumour (Kirkland and Speit, 2008).

There is also the need for the standardization of the methodologies of the comet assay to make it acceptable to all international regulatory agencies. Similarly, more studies are required to populate and validate comet assay data especially those obtained with organs other than the liver (Burlinson et al., 2007; Lovell and Pa, 2008).

Specificity

While the battery of tests described above remain the current recommendations for the genotoxicity testing of pharmaceuticals (ICH, 1997), their specificity has been questioned (Kirkland and Speit, 2008; Mahadevan et al.,

2011). The test sets are particularly poor in detecting chlorinated substances and produce false negative results with a large number of carcinogens with non-genotoxic mechanisms (Mahadevan et al., 2011). Quantitative Structure-Activity Relationships (QSARs) predictive toxicology in recent times has been used not only in molecular design but also to clarify contradicting genotoxicity results (Benz et al., 2007; Contrera et al., 2007; Keshavarz et al., 2012).

Cytotoxicity

Meaningful assessment of genotoxicity can only be made after taking account for any confounding effects of cytotoxicity. A number of methods have been used to assess metabolically viable cells and these include dye exclusion technique using trypan blue (Adegoke et al., 2012), dual dye viability assay, histopathology and neutral diffusion assay (Tice et al., 2000). Although, there is no consensus yet as to the extent of cell viability that is acceptable in the comet assay, 70 to 80% cell viability is generally acceptable. In recent times, however, the abilities of the living cells to take up some dyes to the exclusion of dead cells have been used as index of cytotoxicity. In this regards, tests such as Evans blue staining, acridine orange and similar tests are currently used. Some other tests that measure metabolic activities are also prominently utilized to determine cell viability and detect if agents are cytotoxic or otherwise.

Overview of current toxicity status

This review has attempted to chronicle the various tests and reports that are available in literature on the toxicity profiles of approved food colours and additives. The literature is increasing daily on the assessment of the status of safety (or otherwise) of these agents. The fact that the agents are consumer goods gives them the tendency to be exposed to public scrutiny and assessments.

One concern about regulations available is that there are no uniform guidelines across the regional and worldwide agencies on the exact status of the agents. Food colourants and additives that are acceptable in the USA through FDA legislation may not be particularly acceptable in the EU countries or their uses are stringently controlled. Similar scenario of lack of uniformity also occurs in Asian countries. These call for the design of appropriate harmonization on the list of approved colourants and additives. One would imagine that if there is harmonization of test requirements for the assessment of safety status then there should also be uniform agreement on the number and type of these agents acceptable all over the world. One other concern that will require urgent attention is that of having internationally acceptable *admissible daily intake* (ADI).

The ADIs of most agents vary across the different regions of the world and this is a great concern. A worldwide acceptable tolerance limits in terms of consumption should be advised. There is a need to go beyond daily allowable intake. The argument that majority of these additives are water soluble, and hence readily excreted may not necessarily account for the lack of information on effects of cumulative consumption of these agents.

Another major consideration that would need urgent attention is the extrapolation of *in vitro* toxicological data from isolated human and mammalian cells to real practical use settings. There are so many limitations to the use of isolated cells and handling of these compounds may not sufficiently correlate with *in vivo* outcomes. Herein is the real concern in getting to know the appropriate tests that would adequately mimic the conditions *in vivo*. The use of whole animals and their body organs merely approximates to the biological outcomes that are anticipated upon ingestion of these additives on long term use. Indeed, many long-term use experiments in lower animals cannot provide adequate data for meaningful extrapolation to humans. The effects of carrying over residuals of approved colours and additives from animal sources upon consumption of animals and their products are other areas that will also require adequate attention in the near future.

CONCLUSION

The possibility of toxicity amongst such varied chemical structures means all new chemical entities are to be regarded as toxic until their safety is demonstrated. The choice of a particular method for evaluating a compound can therefore not be based on its chemistry alone or expected mechanism of action. Also, because of the often contradicting results and insufficient data to conclusively categorize many routinely used substances as safe or carcinogenic, there is need for constant evaluation of new chemical entities and existing ones as well.

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Conflicts of interest

The authors have none to declare.

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