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Health safety issues of synthetic food colorants

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ABSTRACT

Increasing attention has been recently paid to the toxicity of additives used in food. The European Parliament and the Council published the REGULATION (EC) No. 1333/2008 on food additives establishing that the toxicity of food additives evaluated before 20th January 2009 must be re-evaluated by European Food Safety Authority (EFSA). The aim of this review is to survey current knowledge specifically on the toxicity issues of synthetic food colorants using official reports published by the EFSA and other available studies published since the respective report. Synthetic colorants described are Tartrazine, Quinoline Yellow, Sunset Yellow, Azorubine, Ponceau 4R, Erythrosine, Allura Red, Patent Blue, Indigo Carmine, Brilliant Blue FCF, Green S, Brilliant Black and Brown HT. Moreover, a summary of evidence on possible detrimental effects of colorant mixes on children's behaviour is provided and future research directions are outlined.

1. Introduction: food colorants

A colour additive, or food colorant, is any dye, pigment, or other substance that imparts colour added to food, drink or any non-food applications including pharmaceuticals. In addition, a colour additive is also any chemical that reacts with another substance and causes formation of a colour (de Boer, 2014; Newsome et al., 2014). The main reasons to use the colour additives in food are:

1) Compensation of colour loss due to exposure to light, air, temperature and storage conditions;
2) Enhancement of natural colours to make the food more attractive and appetizing;
3) Provision of colour to colourless foodstuff; or
4) To allow consumers to identify products on sight, especially drugs (Barrows et al., 2003).

Food colorants can be classified according to several criteria: origin (natural, identical to natural or synthetic; organic and inorganic), solubility (soluble and insoluble) and covering ability (transparent and opaque). However, these categories often overlap. The most widely used classification is the distinction between soluble and insoluble substances.

Soluble colorants can be subdivided into natural, semisynthetic and synthetic ones. Natural dyes are obtained from various food material or other natural materials. They include e.g. riboflavin (E 101), chlorophylls (E140), carotenes (E160a), betalain (E 162) or anthocyans (E 163). The dyes of natural origin are not very stable and can be characterized by their own physiological activity. Colorants are like natural dyes, with the only difference between the two being attributed to the fact that colorants are produced by chemical synthesis. Synthetic dyes are produced also by chemical synthesis but cannot be found naturally. They were originally manufactured from coal tar and now they are obtained from highly purified oil products. The group of synthetic organic colorants consists of azo-dyes, xanthan, chinilin and antrachinon dyes, which have generally more intensive and permanent colour than natural substances, do not impart any flavour to products and they are generally more stable.

Insoluble dyes are called pigments. They are very stable colours exhibiting good cover properties and are also insoluble in common solvents. Pigments can be inorganic with a limited variety of colours available, e.g. white titanium dioxide, calcium carbonate, red iron oxide and black adsorbent carbon, or organic. Organic pigments are usually in the form of lacquers which are insoluble complex salts of water-soluble azo-dyes in a wide colour palette (Golka et al., 2004; Moller and Wallin, 2000).

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1.1. Regulatory measures concerning food dyes in the European Union

The first international collection of food standards and guidelines, called Codex Alimentarius (CA, website: http://www.codexalimentarius.org/), was established early in 1962 by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). In the framework of the CA operates the Codex Committee on Food Additives and Contaminants (CCFAC). The CCFAC specifically focuses on food additives and contaminants and releases recommendations for the permitted maximum levels of use for individual food additives. Codex Alimentarius and WHO/FAO have developed a database, which collects available evidence for biological activity of food additives, so called General Standards for Food Additives (GSFA, Available online: http://www.codexalimentarius.net/gsfaonline/docs/CXS_192e.pdf). The purpose of these standards is to harmonize international rules concerning additives, which are useful in the context of the food world trade.

All member states of the European Union (EU) follow the REGULATION (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority and defining procedures in matters of food safety. These conditions are reflected in all national laws and decrees. In accordance with this regulation, additives used in the EU must be first reviewed by the European Food Safety Authority (EFSA), belonging to the European Commission. EFSA conclusions are based on the recommendations of CCFAC. EFSA publishes, on request from the European Commission, in the EFSA Journal, officially known as "Scientific Opinion." Topics that are covered and discussed include toxicity of food colorants and the method of its application, which is developed by a standard process (detailed information is available on the EFSA website: http://www.efsa.europa.eu). The content of the EFSA Scientific Opinion comprises technical and chemical specifications of the colorant, description of the manufacturing process, analytical methods used for the determination, chemical reactivity with food, summary of the current authorization for use, dose range, toxicokinetic information (absorption, distribution, metabolism and excretion), toxicological data such as acute oral toxicity, sub-chronic and chronic toxicity, carcinogenicity, genotoxicity, developmental and reproductive toxicity and hypersensitivity to the substance.

Approval of food additives depends on the level of scientific knowledge at a given time; therefore, it is necessary to regularly revise the recommendations and to take into account new scientific information in evaluating the conditions of a specific additive use. For this reason, the monitoring of consumption and use of food additives takes place in each member state. The European Commission prepares a summary report for the EU member states and suggests implications for the next period of monitoring.

1.2. Indicators of food additives' toxicity

Permission to use colorants in the food industry is subjected to a wide range of toxicity tests (such as detection of the acute, sub-chronic and chronic toxicity, carcinogenicity, mutagenicity, teratogenicity, reproductive toxicity, accumulation in the body, bioenergy effects and immune effects) and strict legislative provisions in all developed countries. The available toxicological data shall be assessed and subsequently confirmed in more species. Toxicity is monitored in six species and at least three of them must be mammalian. Most of the tests use small rodents (mouse, rat, guinea-pig, etc.) as well as special breeds of rabbits, dogs, cats or pigs which are particularly close to the human body physiology (Hallagan et al., 1995; Kumar and Madan, 2014; Magnuson et al., 2013). The non-mammalian species used for toxicology studies comprise usually nematodes (e.g. *Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*) or zebrafish (*Danio rerio*) (van Vliet, 2011).

In order to evaluate potential toxicity of food additives, preclinical studies are done in order to determine the **NOAEL (No-Observed-Adverse-Effect Level)**, i.e. "the greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure" (Duffus et al., 2009). For clinical recommendations this value is divided by a safety factor that is usually a value of 100 (in the case of toxic substances with serious effects the safety factor can be increased a thousandfold). Due to this factor, the differences in extrapolating animal models to human and individual differences in human populations in response to the additive are considered. This value is called **Acceptable Daily Intake (ADI)**, expressed as mg per kg of body weight (Duffus et al., 2009). This value indicates the amount of food additive that can be consumed daily throughout life without posing an appreciable risk to consumer health. The specific eating habits of certain groups of consumers (e.g. children, vegetarians, etc.) are also considered to ensure that the ADI will not be exceeded.

The values set by regulatory authorities obviously cannot completely eliminate the risk of possible adverse reactions to a particular substance, especially with regard to vulnerable populations or hypersensitive individuals. However, even in such cases there should be no threat to life, besides perhaps rare anaphylactic reactions unlikely to occur after ingestion of the colorant in food.

Based on provision for food labelling determined by the Codex Alimentarius committee, there was a need to unambiguously identify food additives. CCFAC created an International Numbering System (INS), which allows the identification of food additives on the list of ingredients by a three-digit number. This number replaces the specific name of the additive, which is often long, because it describes a complex chemical structure. Within the European Union, a system of **E numbers** was implemented in order to identify all food additives. E numbers are composed of the letter E (for Europe) followed by the INS three-digit number. Colorants with numeric code E are substances that have proved to have no detrimental effects on human health at expectable exposures.

2. Toxicity evaluation of synthetic food dyes

Increasing attention has been recently paid to the toxicity of additives used in food, namely to azo-dyes. This group of colorants typically consists of bright colours. However, the main concern often limiting their use is potential carcinogenicity occurring after their azoreduction to carcinogenic metabolites by intestinal microbiota (Feng et al., 2012). These metabolites are known to be produced in the human body; however, the clinical importance of this phenomena depends on the ingested amount of the colorant (Golka et al., 2004). Furthermore, given the low rate of absorption, harm to human health is unlikely (see Table 1). However, in light of new findings, is it necessary to regularly assess potential toxicity of food colorants by regulatory authorities and consequently revise guidelines for their use.

The European Parliament and the Council published in 2008 the REGULATION (EC) No. 1333/2008 on food additives (available on the official EU website: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32008R1333). This document established that the toxicity of food additives which were evaluated before 20th January 2009 must be re-evaluated by EFSA. The program was initiated in 25th March 2010 by REGULATION (EC) No. 257/2010 setting up a

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Table 1
Food colorants and their metabolism after oral administration (data from different mammal species including human).

Colorant	Metabolism	Reference
Tartrazine	A <5% M extensive by GIT microflora to easily absorbed metabolites E predominantly unchanged by urine	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009e)
Quinoline Yellow	A <3–4% E predominantly unchanged by faeces	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009c)
Sunset Yellow	A limited E predominantly unchanged by faeces No human data, inconsistent methodologies.	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009d)
Azorubine	A <10% E 60–75% by faeces, large portion by urine	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009b)
Ponceau 4R	A limited D no marked accumulation in any tissue E 90% by faeces, 25–35% unchanged, majority as products resulting from azo reduction in the GIT	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009f)
Erythrosine	A <1% E 80–100% unchanged by faeces	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2011b)
Allura Red	A limited E predominantly by faeces, 29% unchanged, rest as products resulting from azo reduction in the GIT	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009a)
Patent Blue	A limited M no human hepatic microsomal enzyme metabolism E predominantly by faeces	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2013)
Indigo Carmine	A limited E probably predominantly by faeces as metabolites	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2014)
Brilliant Blue FCF	A not significant M not metabolized in the GIT or liver enzymes E >95% unchanged by faeces	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010c)
Green S	A limited M no metabolism E >95% unchanged by faeces	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010d)
Brown HT	A limited or none E probably by faeces	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010a)
Brilliant Black	A limited E >95% by faeces mainly as poorly absorbed products resulting from azo reduction in the GIT, <5% by urine	(EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010b)

Legend: A – absorption, D – distribution, M – metabolism, E – excretion, GIT – gastrointestinal tract.

programme for the re-evaluation of approved food additives, which called for submission of new data. The azo-dyes used as food colorants have been already revised and the following text summarizes new lines of evidence on their toxicity.

2.1. E 102 tartrazinum, Tartrazine

Description: yellow water-soluble anionic azo-dye.

In 1966, the toxicity of Tartrazine was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Furthermore, in 1975 and 1984, the Scientific Committee for Food (SCF) also evaluated the toxicity of Tartrazine. Both panels have repeatedly concluded that the substance is safe at an ADI dose of 0–7.5 mg/kg of body weight per day. The most recent evaluation of Tartrazine toxicity was published by EFSA in 2009 containing analysis of published data which suggested that the colorant is able to directly influence the nuclear DNA migration assessed by comet assay in an *in vivo* model in mice (Sasaki et al., 2002).

Recently, the risk associated with high consumption of Tartrazine has been evaluated and this colorant was found to be able to act as an activator of oestrogen receptors (xenoestrogen). According to the cholestatic action of oestrogen, it is suspected that the chronic intake of Tartrazine increases the risk of primary biliary cirrhosis in postmenopausal women (Axon et al., 2012). However, this level of exposure is unlikely to be reached after ingestion of food.

Several new studies have assessed the genotoxicity and cytotoxicity of Tartrazine. An *in vitro* study using human peripheral blood lymphocytes assessed cytotoxicity of a few food colouring agents, including Tartrazine. One approach that was used in this study to assess the safety of Tartrazine was a method known as Sister Chromatid Exchanges analysis, which has shown to be a sensitive test for chromosome instability. Furthermore, the effects of Tartrazine on the Proliferation Rate Index as well as the Mitotic Index were studied. The data showed a toxic potential of Tartrazine due to its ability to damage human lymphocytes and bind directly to DNA (cytotoxic effect). No evidence of genotoxicity was seen at doses of 0.02–8 mM (Mpountoukas et al., 2010). However, a different study demonstrated genotoxicity of the dye at a much higher dose, ranging from 0.25 to 64.0 mM. No cytotoxicity was seen (Soares et al., 2015). Collectively, this data demonstrates that Tartrazine might trigger carcinogenesis at a very high dose or cumulative exposure, however this is unlikely to be encountered.

There are also recent reports which show that Tartrazine has the ability to bind to human and bovine serum albumin and form a complex with these proteins, potentially limiting their physiological function (Basu and Kumar 2014b; Masone and Chanforan, 2015; Pan et al., 2011). Tartrazine is poorly absorbed (see Table 1); therefore, this effect probably would not play a major role; however, it might be appreciable in case of larger exposure or in combination with other colorants or drugs able to bind to plasma proteins.

Lastly, Tartrazine at a dose range of 125–500 mg/kg administered for 30 days induced neurotoxicity and deficits in learning and memory, in both mice and rats in a dose dependent manner. Based on histopathological examination, the study concluded that these abilities might be attributed to increased lipid peroxidation and production of reactive oxygen species and inhibition of endogenous antioxidant enzymes (Gao et al., 2011). However, it is necessary to note that the study employed a very high dose which is very

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unlikely to be ingested by people. Maybe this would be better: Taken together, due to the current evidence available, the daily use of Tartrazine as set by the ADI value seems to be reasonably safe.

2.2. E 104 flavum quinolini, Quinoline Yellow

Description: yellow water-soluble anionic quinophthalone dye.

Toxicity of Quinoline Yellow has been repeatedly evaluated by JECFA in 1975, 1978 and 1984, and the SCF in 1984. All assessments concluded that the substance is safe at ADI of 0–10 mg/kg of body weight per day. In 2009, the EFSA assessed new evidence, noticing that Quinoline Yellow exhibits mild genotoxic properties in two *in vitro* cell models, human lymphocytes and Vicia faba root tip meristems, assessed by the micronucleus and comet assays (Macioszek and Kononowicz, 2004). However, it should be noted that the cells were exposed to very high concentrations which are impossible to be ingested by food consumption in people. Furthermore, it has been proven that this dye is able to inhibit cholinesterase in erythrocytes and plasma pseudocholinesterase, but this finding is of concern in non-alimentary intoxications. The binding of enzyme was dialyzable, which indicates the reversibility of the process (Osman et al., 2002). All these data were included in a reassessment of the toxicity and ADI of this dye by the EFSA Panel, where a significant reduction of ADI to 0.5 mg/kg of body weight per day was recommended. In addition, EFSA Scientific Opinion warned that even these lower dosages may provoke hypersensitive reactions in susceptible individuals, e.g. contact dermatitis which was already reported (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009c; Leleu et al., 2013).

Furthermore, a new study has assessed the DNA damaging effect of this colorant at doses ranging from low 0.5–20 μg mL(−1) reporting dose dependent genotoxic effect (Chequer et al., 2015). Reports on binding to human and bovine serum albumin are also emerging (Masone and Chanforan, 2015; Shahabadi et al., 2012). Overall, it seems that the evidence regarding potential toxicity of Quinoline Yellow remains controversial and may soon require a re-assessment by EFSA panel.

2.3. E 110 flavum orangeatum, Sunset Yellow

Description: orange water-soluble anionic monoazo-dye.

Toxicity of Sunset Yellow was evaluated by JECFA in 1982 and by the SCF in 1984. It was concluded that the substance is safe at an ADI of 0–2.5 mg/kg of body weight per day. Recent experimental data shows that the dye at high doses has xenoestrogenic activity in a similar manner as Tartrazine (Axon et al., 2012). Sunset Yellow is able to reversibly inhibit cholinesterase in erythrocytes and plasma pseudocholinesterase in a similar fashion as was observed with Quinoline Yellow (Osman et al., 2002). However, as already mentioned, the relevance of this finding for the food industry is questionable.

The toxicity of Sunset Yellow has been re-evaluated by EFSA in 2009. The Panel stated low absorption from the intestine (see Table 1) and a probable destruction of molecules by azo-reduction to sulfonated aromatic amines that do not show genotoxic activity. An experimental *in vivo* model in mice did not observe any effect on DNA damage, assessed by comet assay, even at doses up to 2000 mg/kg (Sasaki et al., 2002). However, this effect was recently proven in a study on actively dividing root tip cells of *Brassica campestris* L. (Dwivedi and Kumar 2015) and human blood lymphocytes (Kus and Eroglu, 2015).

Interestingly, studies in laboratory rodents have reported that Sunset Yellow reduced the size of testes (Mathur et al., 2005b) and distorted the lipid profile of the animals (Mathur et al., 2005a). Both experiments employed lower doses than those used in earlier trials, however, their main limitation is the quality of the substance used, as Sunset Yellow was obtained in the Indian market and its purity was not properly defined. Considering these new lines of evidence, the Panel decided to reduce the ADI to 1 mg/kg of body weight per day, and limit the validity period to two years (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009d). At the end of this two year period, a study evaluated the real consumption of Sunset Yellow as a food ingredient. This study was conducted on basis of new data, collected in collaboration with the Swiss supermarket chain Migros. It was found that 99% of consumers had an intake lower than 1 mg/kg of body weight per day of the dye (Sardi et al., 2010). This data confirmed the expected low population exposure to Sunset Yellow, thus the reduced ADI as set by the Panel remained valid (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2011a).

Recently, Sunset Yellow was also reported to bind human and bovine serum albumin and thus lead to complex formation (Masone and Chanforan, 2015). It is unlikely that this colorant will harm human health due to the expected low intake in the general population. However, Tartrazine, Quinoline Yellow and Sunset Yellow were recently detected in saffron rice in Teheran (Iran) restaurants (in 44%, 9.1% and 8.4% of cases, respectively, in total 573 restaurants included) which suggests the new assessment of the population exposure and toxicity to be highly needed (Moradi-Khatoonabadi et al., 2015).

2.4. E 122 azorubinum, Azorubine

Description: red water-soluble anionic monoazo-dye, also known as carmoisine.

Toxicity of Azorubine was evaluated by JECFA in 1983 and the SCF in 1983 and 1984. Both panels concluded that the substance is safe at ADI of 0–4 mg/kg of body weight per day. An *in vitro* study which evaluated the genotoxicity of Azorubine detected alterations in the morphology of somatic chromosomes in evaluation of rye cells induced by this colorant (Zaharia and Pavel, 2003); however, the methodology was not compliant to the usual standards and no other study has confirmed this effect. Therefore, the EFSA Panel concluded in 2009 that the potential genotoxicity is negligible and further stated that there is no evidence which would enforce an ADI change. It may rarely cause skin and respiratory reactions in susceptible individuals, even at the approved dose (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009b).

Recent studies also report the ability of this dye to bind to human and bovine serum albumin (Basu and Kumar 2014a; Masone and Chanforan, 2015). However, so far, Azorubine does not seem to be of concern at common levels of exposure.

2.5. E124 rubor ponceau, Ponceau 4R

Description: red water-soluble anionic monoazo-dye, also known by more than 100 synonyms including Cochineal Red A, Brilliant Scarlet 4R or New Coccine.

Toxicity of Ponceau 4R was evaluated by JECFA in 1983 and SCF in 1984. It was concluded that the substance is safe at an ADI of 0–4 mg/kg of body weight per day. Recent preclinical experiments assessing reproductive and neurobehavioral toxicity of Ponceau 4R recorded cognitive deficits in the first generation of rat males at the NOAEL (in the amount of 0.12% of the diet, equivalent to approximately 205 mg/kg of body weight per day). A possible limitation of this study was inconsistency of findings in female rats, a small number of experimental animals in each group and a very high dose employed (Tanaka, 2006). Another study measuring DNA damage by different doses of Ponceau 4R (comet assay) in mice found a significant increase in migration of nuclear DNA in several

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organs. However, the authors conclude that this effect was not likely due to cytotoxicity (Tsuda et al., 2001). The EFSA Panel considered these results important and recommended further research in this area. Furthermore, based on all available data and the fact that the actual consumption of Ponceau 4R in the population is generally higher than the ADI, the Panel concluded that there are reasons for a re-definition of the ADI to 0.7 mg/kg of body weight per day (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009f). As a result, future studies may be useful in further safety evaluation.

2.6. E 127 erythrosinum natricum, Erythrosine

Description: red xanthen dye.

Toxicity of Erythrosine was evaluated in 1990 by JECFA and in 1984 by SFC. Both panels concluded that the substance is safe at an ADI of 0–0.1 mg/kg of body weight per day. Recent evaluation of the real consumption in the European population has reported that the average dose corresponds to 0.0031 mg/kg of body weight per day (0.01 mg/kg of body weight per day at the 95th percentile of the population), which is a much smaller dose than the ADI limit. Erythrosine is used only in a limited number of red foods in the EU, namely in dry candied cherries. Toxicokinetic data confirm that Erythrosine is poorly absorbed from the gastrointestinal tract (approximately 1%, see Table 1).

There is a potential risk of negative influences on the thyroid gland due to the fact that Erythrosine has four iodine atoms. In an experiment evaluating reproductive toxicity in rats, it was found that Erythrosine affects conversion of T4 to T3 and thus increases release of TRH from the pituitary. By this mechanism, Erythrosine-induced rodent thyroid tumours may arise (Jennings et al., 1990). A clinical study with thirty healthy male volunteers evaluated possible effects of sub-chronic administration of three doses of Erythrosine (20, 60 and 200 mg/kg for 14 days) on thyroid function. A dose dependent increase in total plasma iodide and an increase in the excretion of iodine was observed. A significant increase in thyroid-stimulating hormone (TSH) secretion was shown at the highest dose only. These results indicate that the raise in TSH levels correspond to an increase in serum iodide rather than a direct effect of Erythrosine on thyroid hormone secretion or an effect on peripheral metabolism (Gardner et al., 1987). Importantly, the doses employed in this study were a thousandfold higher than the consumption in food assessed in Europe. In regards to thyroid toxicity, the recently published Scientific Opinion of EFSA Panel concluded in that the effects recorded in animals have only limited validity due to the very low doses to which the European population is exposed. Furthermore, clinical trials so far do not provide evidence of small doses causing harmful effects. Altogether, the Panel concluded that the data on toxicity did not provide a reason to change the ADI (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2011b). However, the toxicity concern regarding Erythrosine might be different in the United States, where Erythrosine is used in a much wider range of foods.

Erythrosine has been demonstrated to have a toxic potential to human lymphocytes *in vitro* (cytotoxic effect) without evidence of genotoxicity, as assessed by Sister Chromatid Exchanges analysis at doses of 0.02–8 mM (Mpountoukas et al., 2010). However, due to low absorption (Table 1) and low exposure to the colorant in the EU, this evidence is unlikely to have any clinical implications.

Interestingly, Erythrosine was recently screened *in vitro* for its potential to inhibit hematopoietic prostaglandin D2 synthase, a member of the Sigma class glutathione transferases catalysing the isomerization of prostaglandin H2 to prostaglandin D2 and a mediator of allergy and inflammation responses. This mechanism could be of interest in future development of new drugs for treatment of symptoms related to allergy and asthma (Mazari et al., 2015) while Erythrosine use in the food industry does not seem to be of major concern in Europe.

2.7. E 129 rubor allura, Allura Red

Description: red water-soluble anionic monoazo-dye.

Toxicity of Allura Red was evaluated extensively by JECFA in 1980 and also by SCF in 1984 and 1989.

The colorant was claimed to be safe at ADI of 0–7 mg/kg of body weight per day. In 2009, the EFSA Panel considered all relevant results and recommended further research. DNA damage (comet assay) by different doses of Allura Red in mice has found a significant increase in migration of nuclear DNA in several organs. However, the authors conclude that the effect observed was not likely to be due to general cytotoxicity (Tsuda et al., 2001). Additionally, the Scientific Opinion of the EFSA Panel reported that Allura Red may cause allergic reactions (e.g. urticaria, asthma), especially when administered in mixes with other synthetic colour additives. However, the Panel concluded that there is no need to change the ADI due to the fact that current levels of use in the European population are far from reaching the official limit of 0.4–0.6 mg daily in the 95th percentile of the population (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2009a; Fallico et al., 2011). While future studies may reveal new concerns, the low exposure in the EU seems to warrant that the use of Allura Red is safe.

2.8. E 131 ceruleum protectum, Patent Blue

Description: blue water-soluble anionic triphenylmethan dye.

Toxicity of Patent Blue was evaluated by JECFA in 1970 and 1975 and the SCF in 1983; however, a final ADI dose was determined only by the SCF: 0–15 mg/kg of body weight per day. Review of all available studies has led to a reassessment of the ADI dose by EFSA in 2013 to 5 mg/kg of body weight per day. Patent Blue did not show any mutagenic activity, DNA damaging capabilities or reproductive toxicity. However, reduced values for haemoglobin, haematocrit and red blood cell counts were detected after chronic exposure to a high dose of Patent Blue. These findings contributed to the new ADI dose (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2013).

Moreover, several reports of serious allergic or anaphylactic reactions to Patent Blue from different countries (Langner-Viviani et al., 2014; Maranhao et al., 2014; Viegas et al., 2015; Wu et al., 2015b) have been published. However, this effect is usually experienced after intravenous injection of the dye, with the aim to visualize and excise a sentinel lymph node in cancer patients (Bezu et al., 2011) and is not of concern for Patent Blue when it is used as food additive. Nevertheless, other types of exposure (cosmetics, foods, etc.) could cause sensitization to the dye. More specifically, Patent Blue has been shown to be absorbed by shaved skin as well as oral mucosa to the bloodstream (Lucova et al., 2013) and this evidence may require a re-assessment of exposure and also of the ADI value in the future.

2.9. E 132 indigocarminum, Indigo Carmine

Description: blue water-soluble anionic pyrrole-based dye.

Toxicity of Indigo Carmine was evaluated first by JECFA, which established a temporary ADI of 0–2.5 mg/kg of body weight per day in 1969. This value was increased to a final ADI of 0–5 mg/kg of body weight per day in 1975. In the same year, SCF endorsed this ADI value and it was also retained in another evaluation done in 1984. EFSA has recently collected new data on the Indigo Carmine

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toxicity and issued an updated Scientific Opinion. Indigo Carmine did not show any genotoxicity, developmental toxicity or modifications of haematological parameters in chronic toxicity studies. The only report of an adverse effect was in testis with a NOAEL of 17 mg/kg of body weight per day (Dixit and Goyal, 2013). However, the results remained inconclusive due to the limitations of the study, such as impossibility to exclude effects of impurities in the tested solution (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2014). Interestingly, there has been a case report of possible atrioventricular blocking capacity of Indigo Carmine (Takeyama et al., 2014) and one case of acute severe hypotension (Lee et al., 2015). However, these phenomena are likely to occur due to individual patients' idiosyncrasies rather than the general toxicity of the dye, which otherwise can be considered as safe at exposure levels below the ADI.

2.10. E 133 ceruleum nitens, Brilliant Blue FCF

Description: blue water-soluble anionic triphenylmethan dye, also known as Blue 1.

Toxicity of Brilliant Blue FCF was evaluated by JECFA in 1970 and also the SCF in 1975. Both panels defined the ADI as 0–12.5 mg/kg of body weight per day. In 1984, the available findings from long-term studies were revised and the ADI value was adjusted to 10 mg/kg of body weight per day. Toxicokinetic data confirmed that Brilliant Blue is poorly absorbed from the gastrointestinal tract and mainly excreted unchanged in faeces (see Table 1). The NOAEL was determined in a study of chronic and reproductive toxicity in rats and corresponds to 631 mg/kg of body weight per day (Borzelleca et al., 1990). On this basis, SFC set the new ADI level to 6 mg/kg of body weight per day. This value corresponds approximately to the actual intake recorded in the European population. The most recent evaluation by EFSA Scientific Opinion warns that even these reduced doses may cause hypersensitive reactions in susceptible individuals (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010c).

Recent *in vitro* studies have yielded results demonstrating certain cytotoxic and genotoxic potential of this dye on human blood lymphocyte cell cultures. In these studies, a dose-dependent decrease was seen in the values of mitotic index frequencies, while micronucleus frequency was increased in the same manner (Kus and Eroglu, 2015). As has been shown with Patent Blue, Brilliant Blue has also been proven to be absorbed by shaved skin as well as oral mucosa to the bloodstream (Lucova et al., 2013). However, the International Association of Colour Manufacturers claims that the total amount absorbed by this route is at least 3600 times below the ADI, as established by EFSA, and hence can be considered insignificant (Codrea, 2013).

Another type of possible toxic reaction to Brilliant Blue was identified in ophthalmology, where it is used for staining the internal limiting membrane. In several patients, foveal thinning and perifoveal hyperpigmentation was observed (Jindal et al., 2014). Interestingly, Brilliant Blue may have certain anti-inflammatory and anti-depressant effects as recorded in a preclinical study (Ma et al., 2014). However, this finding does not concern food industry regulations. Given that the ADI reflects approximate exposure levels of the European population, new data on chronic exposure effects may lead to a re-definition of ADI in future evaluations.

2.11. E 142 viride S, Green S

Description: green water-soluble anionic triarylmethane dye.

Toxicity of Green S was evaluated by JECFA in 1970 and 1975, and by the SCF in 1984. JECFA concluded that the substance is safe at an ADI of 0–25 mg/kg of body weight per day but this decision was withdrawn in 1975 and to date, has not been re-established. The SCF in 1984 set the ADI value to 5 mg/kg of body weight per day which so far seems safe. The most recent evaluation in 2010 by the EFSA has also deemed this ADI as safe.

Toxicokinetic data confirms that Green S is poorly absorbed from the gastrointestinal tract and excreted mainly unchanged in faeces (Table 1). There is no evidence of allergic or hypersensitivity reactions, carcinogenicity or reproductive toxicity. Currently, there are no adequate genotoxicity data available. The EFSA Panel noted that the lack of genotoxicity data could be balanced by studies of carcinogenicity and concluded that there is no scientific evidence which would require a change of ADI. However, the Panel warns that the permitted dose may cause hypersensitivity reactions in susceptible individuals, although they have not been yet recorded (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010d). Overall, Green S seems to be safe at the actual levels of exposure; however, the research assessing its potential toxicity is very limited and requires further investigation.

2.12. E 155 fuscum HT, brown HT

Description: brown water-soluble anionic diazo-dye.

Toxicity of Brilliant Black was repeatedly evaluated by JECFA in 1977 and in 1984, and also the SCF in 1975 and in 1984. The panels defined two different values of ADI: 1.5 (JEFCA) and 3 mg/kg (SCF) of body weight per day. The EFSA panel has reassessed the evidence of toxicity and set a new ADI value of 1.5 mg/kg. The Panel warns that even the permitted doses may cause hypersensitive reactions in susceptible individuals; however there is so far no evidence of such idiosyncrasies (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010a). Brown HT can be considered safe with the new ADI value; however, no studies are currently being conducted to assess its potential toxicity.

2.13. E 151 nigrum nitens, Brilliant Black

Description: black water-soluble anionic diazo-dye.

Toxicity of Brilliant Black was evaluated by JECFA in 1975, 1978 and 1981, and by SCF in 1984. JECFA established an ADI of 1 mg/kg of body weight per day, whereas the SCF concluded that the substance is safe at an ADI of 5 mg/kg of body weight per day. EFSA re-evaluated the colorants' toxicity in the 2010.

Brilliant Black has shown mild genotoxic properties in two cell models: human lymphocytes *in vitro* and Vicia faba root tip meristems *in vivo*, as assessed by the micronucleus and comet assays (Macioszek and Kononowicz, 2004). The EFSA Panel concluded that there is no scientific evidence which would change the dose of the ADI due to, among other things, the current levels of use in the European population, which is far from reaching the legal limit (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010b).

Interestingly, Brilliant Black has been shown to inhibit affinity of a non-selective antagonist on the A1 and A3 adenosine receptors by an allosteric modulation. This could be a promising direction for new drug development (Jacobson et al., 2011; May et al., 2010), hence suggesting that Brilliant Black has certain pharmacological activity at higher doses.

3. Specific issues of food colorants' toxicity

In order to exert a systemic pharmacological effect, every xenobiotic has to be absorbed in the bloodstream. Furthermore, the effect can be prolonged by production of active metabolites, in the case of azo-dyes this concerns mainly azoreduction by intestinal bacteria (Chung et al., 1992; Puvaneswari et al., 2006). Generally all

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azo-dyes are poorly absorbed and many of them are not metabolized, therefore their toxicity is generally low. However, in some cases, toxicity studies have been performed in the past however, current data is lacking for re-evaluations to be conducted. Furthermore, only few clinical studies evaluating effects of chronic exposure were published and they have many limitations. The complete overview of toxicokinetic properties is presented in Table 1.

Recently, a number of studies have been published on the issue of synthetic colorants binding to human serum albumin (HSA). Specifically, binding to HSA was reported for Tartrazine (Pan et al., 2011), Azorubine (Basu and Kumar 2014a; Datta et al., 2013; Masone and Chanforan, 2015), Allura Red (Masone and Chanforan, 2015; Wang et al., 2014; Wu et al., 2015a) Sunset Yellow, Quinoline Yellow (Masone and Chanforan, 2015), and Patent Blue (Tellier et al., 2012). This indicates a potential for drug–dye interactions. However, a unique study evaluating the interactions between different food colorants did not demonstrate evidence of such interactions. Toxicity increase of any target organ at expected population exposure levels were also not observed, hence the study concluded that such interactions are rather theoretical (Groten et al., 2000). However, this does not rule out potential interactions with drugs.

Furthermore, systematic screening of the potential toxicity exerted by different mixtures of colorants in preclinical and clinical studies is lacking. Available studies focus mostly on the connection of colorants' exposure with behavioural disorders in children and respiratory infections or allergies, as described in detail below.

3.1. Food colorants and their effect on children's behaviour

Recently, there have been growing concerns that food colorants may contribute to the development of attention deficit hyperactivity disorder (ADHD) in children (Vojdani and Vojdani, 2015). In 2004, a meta-analysis study looked at the relationship between ADHD and food colorants; however, no clear evidence of ADHD was provided and the study concluded that only certain groups of responders may be affected, hence not providing a clear clinical recommendation for food colorant use (Schab and Trinh, 2004). A few years later, in 2007, a large clinical trial evaluating the effects of two mixtures of synthetic dyes on the development of ADHD in children, aged three or eight to nine year old, was published (McCann et al., 2007). This study evaluated the effects of two drinks stabilized by sodium benzoate containing: (A) Sunset Yellow, Azorubine, Tartrazine, Ponceau 4R and (B) Sunset Yellow, Azorubine, Quinoline Yellow, Allura Red in two age groups of children: three-year and eight to nine-year old and their age-matched normal controls. The doses of colorants were selected to be similar to the amount contained in one 56 g bag of sweets for the younger group and two bags for the older group. The main challenge, but also a limitation of this study, was the use of dye mixtures, thereby avoiding their individual evaluations. The results demonstrated a small clinical difference between the groups. A statistically significant association was found between the intake of colorants and the development of ADHD (McCann et al., 2007). EFSA processed an extensive analysis of this study and concluded that no revision of the ADI is necessary. The main limitations of the study, as identified by the EFSA Panel were: the study investigated mixtures, not separate colorants; unverified validity the innovative behavioural scoring; small sample size; absence of information about the relationship between dose and effect and absence of suggestion of possible biological mechanism of induction of behavioural changes due to consumption of colorants (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2008). Furthermore, current literature deals with methodological problems and attempts to fix the standard testing of the ADHD (Kleinman et al., 2012). A recent meta-analysis lead to analogous conclusions as a study from 2004: available studies do not provide a sufficiently powerful record due to the large variability of the results of an insufficient number of subjects. However, the study noted that approximately 8% of children with ADHD improved clinical outcome while limiting the amount of colorants in the diet (Nigg et al., 2012). EFSA Panel and FDA consider that on the basis of this data, elaboration of relevant studies and detailed analysis of this phenomenon is required (Cheeseman, 2012).

Another concern is a possible neurodevelopmental effect of food additives and their mixtures. These properties, due to ethical reasons, can only be assessed in experimental animals, therefore a study using a mixture of food colorants consisting of Erythrosine, Ponceau 4R, Allura Red, Sunset Yellow, Tartrazine, Amaranth, Brilliant Blue, Azorubine and Indigotine at NOAEL was conducted in female rats and their offspring. Prenatal exposure to the mixture has shown a detrimental effect on the spatial working memory in female offspring. However, it also showed an increase in anxiolytic like and antidepressive like behaviours with sex-dependent differences (Doguc et al., 2015) and gender specific alterations in expressions of glutamate and acetylcholine receptor densities (Doguc et al., 2013). Furthermore, a similar mixture of colorants was reported to affect the glutamatergic signalling in the brain of prenatally exposed rats (Ceyhan et al., 2013). Clearly, these findings cannot be directly translated to human medicine, however, all the previously mentioned studies suggest an evidence of a possible synergism in the toxic effects that mixtures of colorants may exert.

3.2. Food colorants and their effect on the respiratory system or allergies

In the last decades, an increase in the incidence of allergies and asthma has been observed. This phenomenon has not yet been satisfactorily explained. Besides the well-known hygiene theory (which suggests that sterile environments may lead to reduced immunity of the organism), other factors, such as administration of antioxidant supplements, food preserving agents and colorants, have also been suggested to be correlated with the increase in the incidence of allergies and asthma (Vojdani and Vojdani, 2015). A review analysing contemporary knowledge concludes that the situation is still unclear and epidemiological studies are insufficient to evaluate the actual issue (Zaknun et al., 2011).

Studies have shown contradictory results on this topic, as Erythrosine was shown to inhibit hematopoietic prostaglandin D2 synthase, which is a member of the glutathione transferases, catalysing the isomerization of prostaglandin H2 to prostaglandin D2. This is a mediator of allergy and inflammation responses and hence could be of therapeutic importance in the treatment of allergy and asthma (Mazari et al., 2015). Indeed, this particular mechanism is unlikely to be responsible for any major health effect. Furthermore, it was found in other studies that even small doses of azo-dyes absorbed from tattoos were recently suggested to trigger immune responses of the body (Baumler, 2015). Nevertheless, this finding will require further investigation to assess the potential risks. These studies thus highlight that it is still possible to unravel new and surprising pharmacological effects that food colorants may have, hence this may be worth further investigation.

4. Conclusion

European Food Safety Authority (EFSA) has re-evaluated safety concerns of all synthetic dyes since 2008 and has revised ADI values. As a result, the EFSA has concluded that according to the available literature and clinical studies, it is unlikely that these

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agents have a significant detrimental effect on human health in normal consumption. However, it should be noted that most of the well interpretable and valid studies evaluate an intake of single colorants and not mixtures thereof, what is more commonly encountered in the general population. Importantly, there are new studies demonstrating that children may actually consume more coloured foods than expected by the regulatory authorities, e.g. in the USA the amount has risen from 12 mg/capita/day in 1950 to 62 mg/capita/day in 2010 (Stevens et al., 2014). Furthermore, new toxicity concerns have arisen in several colorants due to their ability to bind to human serum albumin. This was reported in Sunset Yellow (Kus and Eroglu, 2015), Tartrazine (Pan et al., 2011), Azorubine (Basu and Kumar 2014a; Datta et al., 2013), Allura Red (Wang et al., 2014; Wu et al., 2015a) and Patent Blue (Tellier et al., 2012). Hypersensitivity reactions have also been reported to become more frequent with new indications of the dyes, e.g. blue dyes used to visualize and excise a sentinel lymph node in cancer patients (Bezu et al., 2011).

There are some clinical studies aimed to evaluate effects of different colorant mixtures, as they may be ingested in normal life (McCann et al., 2007; Schab and Trinh, 2004). Unfortunately, these studies have severe limitations; hence it is impossible draw a clear conclusion on the matter.

Therefore, future research on unknown pharmacological mechanisms of dyes should be promoted and toxicity studies should be carried out with modern methodological approaches, despite the apparent abundance of knowledge. Also the issue of pharmacological properties and toxicity of food colorants has not yet been exploited, as new and possibly useful pharmacological mechanisms of the routinely used colorants have been identified, e.g. Erythrosine inhibiting hematopoietic prostaglandin D2 synthase (Mazari et al., 2015) and Brilliant Blue having anti-inflammatory and antidepressant effects (Ma et al., 2014). Therefore, systematic studies should be performed in order to elucidate pharmacological, neurodevelopmental and other effects that various colorants or their mixtures may have.

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