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SCIENTIFIC OPINION

Statement on Allura Red AC and other sulphonated mono azo dyes authorised as food and feed additives[1]

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The ANS Panel has been asked by EFSA to assess the new scientific information that has become available since the adoption of the opinion on the re-evaluation of the food colouring agent Allura Red AC in 2009, in particular the positive findings from an *in vivo* comet assay in mice. The findings from this study have been interpreted in conjunction with all the available relevant data from genotoxicity testing, metabolism and carcinogenicity, and in consideration of possible species differences between mouse and rat. These new data have been considered in the context of the overall relevant data available not only for Allura Red AC but also for a number of other structurally related sulphonated mono azo dyes authorised as food additives, namely: Amaranth, Ponceau 4R, Sunset Yellow FCF, Tartrazine and Azorubine/Carmoisine. The Panel concluded that the new data by themselves were insufficient at this time to change the conclusions of the 2009 opinion on the safety of Allura Red AC and that there is currently no reason to revise the ADI. The read-across exercise has highlighted a shared pattern of effects for this class of substances that would warrant further investigation The Panel therefore recommended a repetition of the *in vivo* comet assay in mice, to be performed in compliance with the most recent and internationally validated experimental protocol, using whole cells and examining a wide range of tissues. These recommendations apply to all the sulphonated mono azo dyes included in this review.

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KEY WORDS

Allura Red AC, Amaranth, Ponceau 4R, Sunset Yellow, Tartrazine, Azorubine/Carmoisine, *in vivo* genotoxicity

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Additives and Nutrient Sources Added to Food (ANS) to deliver a scientific opinion addressing the new scientific information that has become available since the adoption of the opinion on the re-evaluation of Allura Red AC (E 129) when used as a food colouring agents in 2009 and assess whether this would alter its conclusions.

This request originates from an opinion adopted by the FEEDAP Panel in April 2012 on the re-evaluation of the substance for use in feedingstuffs for cats and dogs. In its opinion, the FEEDAP Panel had concluded that the genotoxic potential of Allura Red AC could not be excluded and that the data available were insufficient to demonstrate the safety of the substance for its proposed use.

In the context of the re-evaluation of Allura Red AC for use as a food additive, a first positive *in vivo* comet assay in mice by Tsuda et al. (2001) had already been considered by the ANS Panel. At the time, the Panel concluded that the positive findings observed in isolated nuclei in the mouse colon and in the stomach were not expected to result in carcinogenicity in the light of the negative results from the long-term toxicity studies in rats and mice.

Subsequent to the ANS Panel opinion in 2009, a new study was published by the same research group (Shimada et al., 2010) confirming observation of DNA damage in the mouse colon, three hours after administration of a single dose of 10 mg/kg bw by gavage. No such effects were observed in rats at doses up to 1 000 mg/kg bw.

The positive findings from the two *in vivo* comet assays in mice were considered by the FEEDAP Panel at the time of their re-evaluation of the safety of Allura Red AC for use in feedingstuffs. In its 2012 opinion the FEEDAP Panel concluded that, on the basis of these experimental results, it was not possible to exclude a genotoxic potential of Allura Red AC.

The ANS Panel critically reviewed the study by Shimada et al. (2010) and noted that these results were observed by a single group of researchers using an in-house developed protocol that uses isolated nuclei from homogenised tissues. Although this protocol was considered to meet the minimum criteria for acceptance of the *in vivo* comet assay (EFSA, 2012), the Panel noted that such protocol had not been included in the international validation exercise. Under the same experimental conditions, no effect could be observed in the rat. The Panel noted that the same findings observed for Allura Red AC were also reported by Shimada et al. (2010) for the two other authorised food dyes tested in this study, Amaranth and Ponceau 4R.

The Panel acknowledged the role of the comet assay as an indicator test for the detection of DNA lesions and/or DNA repair activity and concluded that the findings reported cannot be considered conclusive evidence of mutagenicity, i.e. of induced permanent genetic changes. The Panel also noted that the *in vivo* comet assay is typically performed as part of genotoxicity testing strategies for the follow-up of *in vitro* positives and, as such, its results should be interpreted in conjunction with the findings obtained from other genotoxicity tests.

In this specific case, however the Panel was faced with a limited dataset on the mutagenicity of Allura Red AC. A read-across exercise was therefore conducted in order to gather additional information from the relevant data available for a number of other structurally related sulphonated mono azo dyes, currently authorised as food and feed additives, namely Amaranth (E 123), Ponceau 4R (E 124), Sunset Yellow FCF (E 110), Tartrazine (E 102), and Azorubine/Carmoisine (E 122). The review performed has covered the data on genotoxicity and carcinogenicity previously included by the ANS Panel in the scientific opinions on the re-evaluations of these food colours and published studies which have become available in the literature since then. A similar exercise has been done for the data on metabolism, absorption, distribution and excretion, with a particular focus on the possible differences between metabolism in mouse and rat that could explain the different responses observed in the two species in the *in vivo* comet assays. Consideration has also been given to structure

relationship activities and possible modes of actions have been explored, such as the generation of reactive oxygen species, which could provide explanations of the different findings of the *in vivo* comet assay observed in mice and in rats.

When considering the structurally related sulphonated mono azo dyes as a chemically-related group, a number of genotoxicity studies have been retrieved, however their quality was inconsistent in terms of the reporting and conduct, some of the endpoints investigated were of only limited relevance to the investigation of the safety of the dyes under consideration and could not provide insight into any possible mode of action involved that could explain the species differences in the findings observed in the *in vivo* comet assay. On the other hand, from the data considered in this review, there appears to be a pattern of effects shared by the structurally related sulphonated mono azo dyes that would warrant further investigation.

The Panel concluded that the new data by themselves were insufficient at this time to change the overall weight of evidence in its 2009 opinion on the re-evaluation of Allura Red AC (E 129) as a food additive and that there is no reason to revise the ADI at this time.

After considering the available information on the metabolism of sulphonated and non-sulphonated mono azo dyes and species differences between rat and mouse, the Panel acknowledged that until the uncertainties regarding the *in vivo* comet assay were investigated it was not appropriate to undertake a full evaluation of the other points listed under the Terms of Reference at this stage.

The Panel therefore recommended a repetition of the *in vivo* comet assay in mice, to be performed in compliance with the more recent and internationally validated experimental protocol, using whole cells instead of isolated nuclei from the range of tissues included in the previous assays (glandular stomach, colon, urinary bladder, lung, liver, brain, kidney and bone marrow), for all the sulphonated mono azo dyes included in this review.

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BACKGROUND AS PROVIDED BY EFSA

The safety of Allura Red AC (E129) when used as a food colouring agent has been re-evaluated by the ANS Panel in 2009. On the basis of the evidence assessed, at that time the Panel concluded that there was no need to revise the ADI of 7 mg/kg bw/ day previously established by the JECFA and the SCF. In the context of this re-evaluation, the ANS Panel had noted the findings from a study (Tsuda et al, 2001) reporting effects on nuclear DNA migration in the mouse *in vivo* comet assay. The ANS Panel concluded that the effects on nuclear DNA migration observed in the mouse *in vivo* comet assay with Allura Red AC were not expected to result in carcinogenicity in the light of the negative results from the *in vitro* genotoxicity tests as well as from the long-term carcinogenicity studies in rats and mice.

A re-evaluation of the substance for use in feedingstuffs for cats and dogs has also been completed by the FEEDAP Panel in April 2012. In the context of this re-evaluation the findings from another *in vivo* comet assay (Shimada et al, 2010) showed a similar genotoxic effect in the mouse colon whereas no genotoxic effect was observed in rats. On the basis of the evidence from these two studies, the FEEDAP Panel concluded that the genotoxic potential of Allura Red AC could not be excluded and that the data available were insufficient to demonstrate the safety of the substance for its proposed use.

In addition, in its opinion, the FEEDAP Panel questioned the suitability of the mouse as a good animal model for detecting colon carcinogenicity. During initial discussions between members of both Panels, a number of other matters was identified which would need to be considered; the specifics of the comet assay method used by the researchers, the species difference in the observed response, the potential species difference in the metabolism of Allura Red AC and related dyes, the recent SC opinion on genotoxicity testing (EFSA SC, 2011) and the context of the questions addressed by the two Panels. Moreover, the European Commission requested clarifications from EFSA on the impact that the FEEDAP Panel opinion would have on the former ANS opinion on Allura Red AC.

TERMS OF REFERENCE AS PROVIDED BY EFSA

In accordance with Article 29(1) of Regulation (EC) No 178/2002[4], the European Safety Authority asks its scientific Panel on Food Additives and Nutrient Sources added to food (ANS) to provide an updated Scientific Opinion on the re-evaluation of Allura Red AC (E 129) as a food additive.

In particular, the opinion should:

- Address the new scientific information that has become available since the adoption of the opinion of the Allura Red AC (E 129) by the ANS Panel in 2009 and assess whether this would alter its conclusions;

- Consider the differences in the metabolism of rat and mouse for Allura Red AC and, more generally, for the azo dyes;

- Consider the differences in the response of rat and mouse in the comet assay for Allura Red AC.

INTERPRETATION OF THE TERMS OF REFERENCE

The ANS Panel acknowledged that, in order to fulfil the questions contained in this self-task mandate, the scope of the assessment should not be limited solely to data on Allura Red AC (E 129) alone but should be broadened to allow consideration of available metabolism and genotoxicity data of structurally related sulphonated mono azo dyes, in particular those that are authorised as food

[4]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 laying down procedures in matters of food safety. OJ L. 31, 1.2.2002, p. 1-24.

additives. The Panel considered that read across of these data could be useful in addressing data gaps and interpreting the studies and their results.

EVALUATION

1. Introduction

The present statement deals with the apparent discrepancy between the conclusions reached by the EFSA Panel of Food Additive and Nutrient Sources added to food (ANS) Panel in 2009 and the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in April 2012, when re-assessing the colouring agent Allura Red AC (E 129) as food additive and feed additive for cats and dogs, respectively.

The different appraisal of the safety of this additive must be considered in the light of the specific terms of reference addressed in the two opinions, the different dates at which the two assessments have been conducted with the availability of new genotoxicity data published in 2010, and the evolution of guidance on genotoxicity testing strategies from the EFSA Scientific Committee.

The European Commission requested clarifications from EFSA on the impact of the FEEDAP Panel opinion on that of the ANS Panel.

The ANS Panel proposed a self task mandate to examine the new data and any implications for the existing opinions. This mandate was accepted by EFSA and a Working Group of experts from ANS, FEEDAP and other Panels and external experts was established.

In addressing the specific issue of Allura Red AC the Working Group noted that other sulphonated mono azo dyes are authorised food and feed colours and that there were indications that these could raise similar questions. It was considered that information on the genotoxicity and metabolism of other dyes should also be evaluated as it may assist in understanding and interpreting the results reported.

The following authorised colours were therefore considered in this opinion: Allura Red AC (E 129), Amaranth (E 123), Ponceau 4R (E 124), Sunset Yellow FCF (E 110), Tartrazine (E 102) and Azorubine/Carmoisine (E 122).

This opinion provides a critical analysis of the present knowledge on the genotoxicity of Allura Red AC and structurally related sulphonated mono azo dyes. This key element is placed in context with a summary of the metabolic fate (ADME) of these substances, including the role of gastrointestinal microbiota in their metabolism, the production of reactive oxygen species, and carcinogenicity studies. The implications are examined and conclusions drawn on the actions to be taken.

2. Summary of the findings from *in vivo* comet assays in isolated nuclei

The study that triggered this review (Shimada et al., 2010) was a follow-up from previous work by the same research group (Tsuda et al., 2001; Sasaki et al., 2002) in which a number of food additives, including some sulphonated mono azo dyes, were tested in a modified comet assay using isolated nuclei in mice from a wide range of tissues (liver, kidney, lung, brain and mucosa from urinary bladder, glandular stomach, colon). In addition to these tissues, in the study by Tsuda et al. (2001) the comet assay was also performed in embryos, whereas bone marrow was included in the study by Sasaki et al. (2002). Under the same experimental conditions the positive findings reported in mice were not observed in rats (Sasaki et al., 2002).

The studies of Tsuda et al. (2001) and Sasaki et al. (2002) have already been considered by the ANS Panel in the re-evaluations of Allura Red AC, Amaranth, Ponceau 4R, Sunset Yellow FCF and Tartrazine (EFSA ANS Panel, 2009a; 2009b; 2009c; 2009d; 2010) and are shortly summarised below.

In the study of Tsuda et al. (2001), Allura Red AC, Amaranth and Ponceau 4R were examined *in vivo* in the single cell gel electrophoresis (comet) assay using isolated nuclei separated from homogenized tissues (Tsuda et al., 2001). Groups of male CD-1 mice were dosed with 0, 1, 10, 100, and 2 000

mg/kg bw once by gavage. An additional dose of 1 000 mg/kg bw was also administered to animals treated with Allura Red AC and Amaranth.

In nuclei isolated from the colon, a significant increase in DNA migration was observed for all the three azo dyes tested at doses from 10 mg/kg bw onwards, three hours after administration,. DNA migration decreased with the time after the exposure, but remained significantly increased at doses of 2 000 mg/kg bw at six hours after administration for both Allura Red AC and Amaranth, and at 24 hours for Allura Red AC alone. In glandular stomach significant differences between treatment group and controls were observed at doses of 100 mg/kg bw onwards for Allura Red AC and Ponceau 4R and of 1 000 mg/kg bw for Amaranth, three hours after administration. In the highest dose group of 2 000 mg/kg bw, significant nuclear DNA migration was also determined six hours after administration of both Allura Red AC and Amaranth, whereas 24 hours after administration only Amaranth elicited a positive response. In the urinary bladder, Ponceau 4R was reported to produce a significant increase of DNA migration at doses of 100 mg/kg bw and above, from three hours up to 24 hours after administration whereas some positive findings were reported for Amaranth at 1 000 mg/kg bw, three hours after administration and at 2 000 mg/kg bw, 24 hours after administration. Isolated positive findings were reported for DNA migration from the other tissues examined. In the liver, Amaranth and Ponceau 4R produced a statistically significant increase of DNA migration at a dose of 1 000 mg/kg bw, at six and 24 hours after administration, respectively. In the lung, a significant difference between the control groups and the treated animals was reported for Allura Red AC at 1 000 mg/kg bw, three hours after administration and for Ponceau 4R at 2 000 mg/kg bw, 24 hours after administration. In the kidney, a statistically significant increase was observed for Ponceau 4R, at the highest tested dose, 24 hours after administration. Amaranth was reported to increase DNA migration in brain and bone marrow, at the highest dose tested, six hours after administration. A modest but significant effect was observed also in pregnant mice in the colon, three hours after treatment with a dose of 2 000 mg/kg bw of Allura Red AC and Amaranth. Positive findings in pregnant mice were also reported six hours after administration of Amaranth in the lung. No DNA migration was observed in nuclei from the other tissues examined. Necropsy and histopathological examination revealed no treatment-related effect on the colon and glandular stomach.

The results from Tsuda et al. (2001) are also reported in a further publication from the same research group (Sasaki et al., 2002) providing additional information on the testing of a number of other food additives, including Tartrazine and Sunset Yellow FCF, using the same modified protocol for the *in vivo* comet assay.

In the study by Sasaki et al. (2002), groups of male CD-1 mice were administered once by gavage at doses of 0, 1, 10, 100 and 2 000 mg/kg bw. Isolated nuclei from glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow, were analysed 3 and 24 hours after treatment In the nuclei isolated from colon cells, Tartrazine was reported to induce a significant increase in DNA migration at doses from 10 mg/kg bw onwards, three hours after administration. In the highest dose group (2 000 mg/kg bw) the migration remained statistically significant increased 24 hours after administration. In the glandular stomach, Tartrazine was reported to produce an increase in DNA migration at doses from 100 mg/kg bw onwards, however the effect observed with the highest dose of 2 000 mg/kg bw was no longer statistically significant at 24 hours after administration. No DNA migration was observed in nuclei from the other tissues examined. Necropsy and histopathological examination revealed no treatment related effect on the colon and glandular stomach. Under the same experimental conditions, Sunset Yellow FCF tested at doses of 2 000 mg/kg bw did not yield a statistically significant increase in DNA migration in any of the organs examined.

The more recent study by Shimada et al. (2010) was aimed at examining potential differences in the response between two species (rat and mouse). The study was conducted in male ICR (CD-1) mice and male Fischer (F344) rats. The substances tested in the study were Allura Red AC, Amaranth and Ponceau 4R from certified lots approved for food use. In mice, single doses of 0, 1 and 10 mg/kg BW were administered by gavage (4 animals per group, 6 controls), three hours before sacrifice. In rats single doses of 0, 10, 100 and 1 000 mg/kg bw were administered by gavage to the animals (4 animals

per group, 5 controls), three hours before sacrifice (10 mg/kg bw) and 6, 12 and 24 hours before sacrifice (100 and 1 000 mg/kg bw). Comet assay was performed using the same protocol previously described by Tsuda et al. (2001), using nuclei isolated from homogenised mucosa of glandular stomach, colon and urinary bladder and from lung, kidney, liver, brain and bone marrow. Migration was calculated as the difference between the length of the whole comet and the diameter of the head for each of 50 nuclei from slide. Mean migration of 50 nuclei from each organ was calculated for each individual animal. An increase in migration length was considered as an increase in DNA damage. As previously reported, in the colon all the three dyes tested induced an increase in migration length in mice at a dose of 10 mg/kg bw, three hours after their administration. In mice, no other statistically significant increase in DNA migration was observed in any of the tissues from the organs examined In rats, none of the tested dyes produced a significant effect at any sampling time and for any of the doses tested, with the only exception of Amaranth that produced one isolate finding in the stomach at a dose of 10 mg/kg bw, three hours after administration. No DNA migration was observed in nuclei from the other tissues examined. The comet assay was accompanied by histological examination of the glandular stomach and colon tissues, after removal of the mucosa. No treatment-related effect was observed in any of the organs examined.

As part of this experiment, the species difference in transit time through the gastrointestinal tract was also examined but did not provide any possible explanation to the difference observed in the response to the comet assay.

The Panel noted that according to the recent EFSA Scientific Report on Minimum Criteria for the acceptance, interpretation and reporting of the *in vivo* alkaline comet assay (EFSA, 2012), examination of the whole cells is the preferred option, however examination of isolated nuclei accompanied by histological examination for the evaluation of cytotoxicity is also considered acceptable. The Panel was also aware of a large validation programme (JaCVAM, online) undertaken in support of the development of an OECD Test Guideline on the *in vivo* rodent alkaline comet assay (OECD, 2013, online), and noted that only whole cells were included in the experimental protocol adopted and refined during this international validation exercise.

In interpreting these findings, the Panel also noted that the comet assay is never used for hazard identification, but rather as an indicator test, capable of detecting DNA lesions or DNA repair activity, not necessarily leading to permanent DNA damage. For this reason, in genotoxicity testing strategies, this assay is recommended to follow-up *in vivo* those substances that have shown *in vitro* the ability to induce irreversible mutations at gene or chromosomal level. In this specific case, the *in vitro* dataset available for Allura Red AC, although generally negative, is extremely limited and does not include any mammalian cell genotoxicity assay nor were the bacterial reverse mutation assays performed in the full range of recommended strains (EFSA ANS Panel, 2009a). In its evaluation from 2009, the Panel considered also a negative result reported for Allura Red AC in a mouse heritable translocation test (unpublished, Jorgenson et al., 1978 as referred to in EFSA ANS Panel, 2009a) which, however was not available for re-evaluation (neither in 2009 nor for the current evaluation).

3. Read-across from data from other structurally related sulphonated mono azo dyes

In order to fulfil this mandate a read-across exercise was conducted in order to put the new findings in the context of the overall data available for a number of other structurally related sulphonated mono azo dyes, currently authorised as food and feed additives, namely Allura Red AC (E 129), Amaranth (E 123), Ponceau 4R (E 124), Sunset Yellow FCF (E 110), Tartrazine (E 102) and Azorubine/ Carmoisine (E 122). The data on ADME, genotoxicity and carcinogenicity for the sulphonated mono azo dyes 2009b; 2009c; 2009d; 2009e; 2010) have been reviewed together with any additional relevant literature that has become available since the adoption of these opinions (the literature search was conducted in the SciFinder authorised as food additives previously considered by the ANS Panel (EFSA ANS Panel, 2009a; database, encompassing MEDLINE and CAPLUS databases, with a cut-off date 15 October 2012) and are presented in Appendix A. Data on metabolism, including the possible

species difference between mice and rats, and on structure relationship with genotoxicity and carcinogenicity are also presented in Appendix A.

4. Discussion

The Panel noted the study by Shimada et al. (2010) which had become available after the finalisation of the 2009 ANS opinion on Allura Red AC confirming positive findings previously reported by Tsuda et al. (2001) in an *in vivo* comet assay in mice using isolated nuclei. It was noted that Shimada et al. (2010) also reported positive findings for the two other sulphonated mono azo dyes authorised as food additives tested in this study, namely Amaranth and Ponceau 4R.

The *in vivo* mouse comet assays (Tsuda, *et al*., 2001; Sasaki et al., 2002; Shimada, et al., 2010) were performed using a single non-standard methodology that nevertheless conformed with EFSA Scientific Report on Minimum Criteria for the acceptance, interpretation and reporting of the *in vivo* alkaline comet assay (EFSA, 2012). The results in mice for the four sulphonated mono azo dyes Allura Red AC, Amaranth, Ponceau 4R and Tartrazine were all positive in the glandular stomach and the colon (and, in the case of New Coccine/Ponceau 4R, also in the urinary bladder), with negative results for all other organs tested, including brain, lung, liver, kidney, bone marrow and embryo. In a study performed by the same research group, using the same methodology, Sunset Yellow FCF was negative (Sasaki et al., 2002). In contrast, in rats, all of these dyes gave negative results for all organs investigated in this assay. The reasons for the organ specificity and the species difference in results are not known.

Only a few genotoxicity studies of Allura Red AC were available for assessment, but more studies covering all of the relevant endpoints have been retrieved for the sulphonated mono azo dyes when considered as a chemically-related group. The quality of the genotoxicity studies available was inconsistent in terms of the reporting and conduct of the studies and some of the endpoints investigated were of only limited relevance to the question under consideration.

The survey of available genotoxicity data shows some similarities in the genotoxic profile of the six sulphonated mono azo dyes considered in this statement. All of these dyes generally tested negative in the bacterial reverse mutation assays, with and without metabolic activation and under experimental conditions optimized for the metabolic reduction of the azo bond. Negative results were also obtained in tests with the chemically reduced component amines of three of the dyes (Allura Red AC, Amaranth and Tartrazine), both in presence and absence of exogenous metabolic activation. Some positive findings have been observed in some *in vitro* tests with extracts from or rat metabolites of Allura Red AC, Amaranth and Sunset Yellow FCF (Henschler et al., 1985; Münzner and Wever, 1987; Prival et al., 1988; Wever et al., 1989).

The results obtained in bacterial reverse mutation assays of the sulphonated mono azo dyes under consideration did not match the results anticipated if they caused mutagenicity by some suggested mechanisms. Overall, the available negative results from bacterial reverse mutation assays do not point to the reduction of the azo linkage and metabolic oxidation of the resulting amine group as a plausible mechanism explaining the *in vivo* positive comet findings. Among alternative mechanisms, the extrahepatic activation of aromatic amines to phenoxy radicals and quinine imines (Eyer, 1994) has been proposed (Tsuda et al., 2001), but it should be noted that the required o- or p-hydroxylation does not occur with all sulphonated mono azo-dyes that tested positive in comet assays (e.g. in Tartrazine). Another suggested alternative mechanism relies on the pro-oxidant activity of azo-reduction products (Sweeney et al., 1994; Ben Mansour, 2007), with production of reactive oxygen species. In this respect it should be noted that bacterial reverse mutation tests with azo dyes were performed with set of tester strains which, although generally sensitive to carcinogenic aromatic amines (McCann et al., 1975), did not include a strain specifically sensitive to oxidative mutagens such as *Salmonella* Typhimurium TA102, and thus oxidative damage may have not been efficiently detected. Indeed, a positive response in this strain has been reported after reduction of some dyes (Amaranth and Sunset Yellow FCF) by intestinal bacteria flora (Sweeney et al., 1994). In the absence

of information about the nature of the DNA lesions detected in comet assays (Tsuda et al., 2001; Sasaki et al., 2002; Shimada et al.; 2010) with sulphonated mono azo dyes, it is not possible at present to draw any firm conclusions on the mechanism by which the dyes cause positive results in these *in vivo* comet assays.

Overall *in vitro* test systems in mammalian cells gave negative or inconclusive results. No *in vitro* mammalian cell clastogenicity data for Allura Red AC and Azorubine/Carmoisine are available.

Recently published *in vitro* studies investigating the DNA binding capacity of these dyes (Mpountoukas et al., 2010; Kashanian and Zeidali, 2011; Williams et al., 2012) have shown that Amaranth, Ponceau 4R, Sunset Yellow FCF and Tartrazine can bind to DNA in a non-covalent fashion.

Other *in vivo* mammalian tests gave an inconsistent pattern of results. Mouse bone marrow micronucleus tests gave negative results for Ponceau 4R (Hayashi et al., 1988) as did a Chinese hamster bone marrow micronucleus test of Sunset Yellow FCF (Wever et al., 1989). A micronucleus test of Amaranth, Tartrazine and Sunset Yellow FCF in mouse gut was also negative (Poul et al., 2009). Positive results were reported in tests for chromosomal aberrations in mouse bone marrow with Sunset Yellow FCF (Sayed et al., 2012), Tartrazine (Giri et al., 1990; Farag et al., 2001) and Ponceau 4R (Agarwal et al., 1993); for Sunset Yellow FCF, increased incidences of SCEs in mouse bone marrow and SCEs and chromosomal aberrations in spermatocytes were also reported (Sayed et al., 2012). The Panel however identified a number of shortcomings in these studies and considered the relevance of these findings questionable. Negative results were reported in other studies for Sunset Yellow FCF (Wever et al., 1989), Tartrazine (Durnev et al., 1995 and Das and Moukherikijee, 2004 as referred to in EFSA ANS Panel, 2009d), Amaranth (Das and Moukherikijee, 2004) and Azorubine/Carmoisine (Durnev et al., 1995 as referred to in EFSA ANS Panel, 2009e). In Chinese hamster bone marrow, Sunset Yellow FCF gave a positive result for chromosomal aberrations at an intermediate dose but not at the highest dose level (Wever et al., 1989). In *in vivo* tests for possible effects on germ cells, a rat dominant lethal assay of Amaranth (BIBRA, 1982 as referred to in EFSA ANS Panel, 2010) and mouse heritable translocation test of Allura Red AC (Jorgenson et al., 1978, as referred to in EFSA ANS Panel, 2009a) gave negative results.

In the light of the ADME data reviewed some conclusions can be drawn on the structure activity relationships within these compounds. There appear to be a correlation between the presence of a sulphonated group in the azo dye structure and a decrease in the reactivity of the dye and/or its metabolites, whereas generally inclusion of an aromatic amine or nitro group into the azo dye structure increases the genotoxicity and carcinogenicity of the dye itself and/or its metabolites. The metabolic fate of the dyes under assessment, including the prevalent reduction of the azo function by the gut microbiota, is common to most species, including the human.

The gut microflora of mice and rat comprises a great number of anaerobic bacteria; it is highly variable along the development of the animals but also day-to-day, depending on anatomical, physiological and nutritional factors. Some bacterial species are common to both mice and rat, whereas a large number of them are species-specific. However, many of these bacterial species possess azoreductases. Consequently, it is unlikely that the genotoxic end-points (comet assay) revealed as positive in mice and negative in the rat may be directly related to a difference in the gut microflora azo-reduction capability of these animals.

Evaluation of the data from *in vivo* comet assays (Tsuda et al., 2001; Sasaki et al., 2002; Shimada et al., 2010) shows a target organ profile that does not fit with the suggested mechanism that genotoxicity only occurs subsequent to azoreduction. In particular, the stomach and colon, appear to be important targets for Allura Red AC, Amaranth, Tartrazine and Ponceau 4R, showing extensive migration of nuclear DNA. This experimental finding is difficult to explain given that the azo linkage is expected to be stable under the acidic stomach conditions. The Panel noted that these results were obtained by a single research group using isolated nuclei obtained from homogenised tissues, and that this procedure

was not included in the international validation exercise coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) in support of the development of an OECD Technical Guideline for the in vivo comet assay (JaCVAM, online).

In risk assessment, with respect to the carcinogenic potential of a compound, it is generally accepted that negative carcinogenicity studies overrule positive *in vivo* genotoxicity. The Panel agreed with this conclusion but noted that clear evidence of genotoxicity in somatic cells *in vivo* has to be considered an adverse effect per se, even if the results of cancer bioassays are negative, since genotoxicity is also implicated in somatic diseases other than cancer (EFSA SC, 2011). Moreover, it has been shown by Gold et al (1991) that the mouse is in general not a good model for detecting cancer of the colon and has a very low background incidence of colon cancer, one of the main sites of genotoxicity identified in mice in the *in vivo* comet assays of Allura Red AC, Amaranth, Tartrazine and Ponceau 4R. The Panel however, noted that histopathological examination of the intestines did not demonstrate any abnormality, neither tumours nor degenerative hyperplasic lesions (EFSA ANS Panel, 2009a, 2009b, 2009d, 2010) which would have been expected after repeated daily exposure to a DNA damaging substance. Moreover the Panel also noted the negative results obtained in the comet assays and in the carcinogenicity studies in rats (EFSA ANS Panel, 2009a, 2009b, 2009d, 2010).

The Panel considered that the new data by themselves were insufficient at this time to change the overall weight of evidence in its 2009 opinion on Allura Red AC and there is no reason to modify the ADI at this time. The Panel noted that the new results from this *in vivo* comet assay by Shimada et al. (2010) added uncertainty to its earlier evaluation and therefore it would be desirable to conduct a repetition of the *in vivo* comet assay in mice using whole cells in line with most recent and validated protocol. The Panel in reading across data on other structurally related sulphonated mono azo dyes to evaluate the results with Allura Red AC has recognised that the need for additional test described above should also apply to these dyes.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The Panel has addressed the new scientific information that has become available since the adoption of the opinion on the re-evaluation of Allura Red AC (E 129) for use as a food additive in 2009 and whether the new study by Shimada et al. published in 2010 would alter the conclusions reached by the Panel at that time. The Panel noted its previous assessment included earlier studies from these researchers using the same method.

Positive findings from the *in vivo* comet assays in mice are reported for Allura Red AC, Amaranth, Ponceau 4R. Under the same experimental conditions, no effect could be observed in rats. In a previous study by the same research group, positive findings were also reported for Tartrazine, in mice. The Panel noted that these results were observed by a single group of researchers, using a protocol evaluated as acceptable albeit not validated and not aligned to the latest standards.

The comet assay is typically performed as part of genotoxicity testing strategies and, as such, its results should be interpreted in conjunction with the findings coming from other genotoxicity tests. In this specific case, however, the Panel was faced with a limited dataset on the mutagenicity of Allura Red AC and for the other five structurally related sulphonated mono azo dyes considered for read across, namely Amaranth, Ponceau 4R, Tartrazine, Sunset Yellow FCF and Azorubine/Carmoisine. The Panel noted that the available studies, many of which were not conducted as part of a testing strategy for regulatory approval, had important flaws that would not allow the drawing of firm conclusions and could not provide insights into the mode of action involved. Nevertheless, in the absence of well-conducted studies, the positive findings in some of these imperfect genotoxicity tests raise uncertainties about possible mutagenicity that should be addressed.

Despite the limitations of the databases, and concerns about the experimental procedures applied in the *in vivo* comet assays, the Panel considered that the results of comet assays in mice added uncertainties over a possible *in vivo* genotoxic potential of sulphonated mono azo dyes. Therefore a further investigation on the *in vivo* genotoxicity of the sulphonated mono azo dyes, using an internationally validated experimental protocol for comet assay is recommended.

The Panel concluded that the new data by themselves were insufficient at this time to change the overall weight of evidence in its 2009 opinion on the re-evaluation of Allura Red AC (E 129) as a food additive and that there is no reason to revise the ADI at this time.

RECOMMENDATIONS

In the light of all the data considered within the limited time available, the Panel considered that Allura Red AC and the other structurally related sulphonated mono azo dyes covered by this review share a pattern of effects in some studies that would warrant further investigation. In particular, for the evaluation of the genotoxic potential, it is recommended that additional testing in accordance to the latest guidelines is undertaken.

In particular the Panel recommended an *in vivo* comet assay in mice using whole cells from the range of tissues included in the Shimada study (glandular stomach, colon, urinary bladder, lung, kidney, liver, brain and bone marrow), in a validated protocol, for all the substances included in this review. The Panel considered that it would be sufficient to repeat the comet assay in the mouse alone, given that this species has shown to be the more sensitive. It was considered that repeating the study in the rat would not be of sufficient added value to decrease the existing uncertainties arising from the findings in mice.

As these uncertainties appear to apply to this whole class of sulphonated azo dyes, further testing should not be limited to Allura Red AC and the other substances which resulted positive to the *in vivo* comet assay reported by Shimada et al. (2010), i.e. Amaranth and Ponceau 4R, but also to untested dyes that were included in the current review.

No effects observed in this additional testing, will allow the Panel to conclude that the substance can be regarded as of no genotoxic potential *in vivo*.

In the case of positive results obtained from this additional testing, and in line with the EFSA Scientific Committee "Opinion on genotoxicity testing strategies applicable to food and feed safety assessment" (EFSA SC, 2011) the sulphonated mono azo dyes would have to be considered as *in vivo* genotoxic agents. In the absence of any further mechanistic explanation the Panel would not normally establish an ADI for an *in vivo* genotoxin.

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APPENDIX

Appendix A. Read-across from data from other structurally related sulphonated mono azo dyes

GENOTOXICITY DATA

In vitro studies in bacteria

Allura Red AC was not mutagenic or genotoxic in tests in bacteria with and without metabolic activation using several procedures, including the protocol optimized for the testing of azo dyes. Negative results were also obtained in tests with the chemically reduced component amines, both in presence and absence of exogenous metabolic activation (EFSA ANS Panel, 2009a).

Positive findings were observed for some batches of Allura Red AC, following chemical reduction and solvent (ether) extraction. Ether extracts (without chemical reduction) of some batches also showed mutagenic activities, which were attributable to possible impurities of the dye tested. (Prival et al., 1988).

In mutagenicity tests in bacteria with and without metabolic activation, Amaranth was generally negative when tested using several procedures, including the protocol optimized for the testing of azo dyes. Negative results were also obtained in bacterial tests with products of the chemical reduction of the azo group (EFSA ANS Panel, 2010).

Sunset Yellow FCF was not mutagenic in standard plate-incorporation and preincubation bacterial reverse mutation assays as well as in some non-guideline studies conducted in microorganisms: rec assay with *Escherichia coli*, fluctuation assays with *E. coli* and *Salmonella* Typhimurium, and mitotic gene conversion assay in *Saccharomyces cerevisiae* (EFSA ANS Panel, 2009c).

Bile, urine and faecal extracts were analysed for mutagenicity in the bacterial reverse mutation assay after oral administration (gavage) to rats (3 x 1.5 mg/kg bw in 48 hours) of Sunset Yellow FCF (certified for food additive purposes, dye content 85 %). Aqueous faecal extracts from rats orally treated with Sunset Yellow FCF produced a volume-dependent increase in the number of revertants in TA100 strain, only in the presence of metabolic activation. The experiments were negative in all the other experimental conditions (Wever et al., 1989).

Sunset Yellow FCF and Amaranth, after reduction by the intestinal bacteria *Enterococcus faecalis* and *Bacteroides thetaiotaomicron*, induced differential cytotoxicity in a DNA repair deficient/proficient *E. coli* strains and mutagenesis in the *S.* Typhimurium strain TA102, particularly sensitive to oxidative mutagens, but not in TA98 and TA100 (Sweeney *et al.* 1994).

Ponceau 4R was negative in bacterial reverse mutation assays, also when tested with the modified protocol for the reduction of azo compounds (EFSA ANS Panel, 2009b).

In bacterial reverse mutation assays, with and without metabolic activation, Tartrazine was generally negative when tested using several procedures, including the protocol optimized for the testing of azo dyes (EFSA ANS Panel, 2009d). The compound was negative also when tested in *E. coli* polA recA DNA repair test and in fluctuation test in *S.* Typhimurium TA 1538 and *E. coli* WP2uvrA (Haveland-Smith and Combes, 1980). Negative results were also obtained in bacterial tests with products of the chemical reduction of the azo group (Chung, 1981).

Tests on body fluids and excreta of rats administered orally with Tartrazine indicated the presence of an unidentified urinary metabolite, positive in the *S.* Typhimurium strain TA98 in presence of rat liver S9 (Henschler and Wild, 1985). Weak mutagenic activity was also observed in faecal extracts with strain TA100 in presence of metabolic activation; bile extracts were negative (Münzner and Wever, 1987).

No evidence of mutagenicity in five *in vitro* studies were reported in the JECFA evaluation of Azorubine/Carmoisine and considered by the ANS Panel in its 2009 opinion (EFSA ANS Panel, 2009e).

In vitro studies in mammalian cells

No *in vitro* studies with mammalian cells are available for Allura Red AC.

Positive results were referred to in cytogenetic tests without metabolic activation, where Amaranth, Ponceau 4R and Tartrazine (0.25 mg/ml) increased the incidence of structural chromosomal aberrations in Chinese hamster lung fibroblasts (CHL) (Ishidate et al., 1984) The Panel noted that no conclusions can be drawn from the the cytogenetic study of Ishidate and co-workers, because of the limited study protocol applied and because chromatid and chromosome gaps were included in the computation of chromosomal aberration incidences.

In an *in vitro* sister chromatid exchanges (SCE) study conducted in human peripheral lymphocytes, Amaranth (0.02-8 mM) and Tartrazine (0.02-2mM) were tested. Amaranth showed an increase of SCE (1.7-fold over control), whereas no effect on this endpoint was observed for Tartrazine (Mpountoukas et al., 2010). The Panel also noted that the *in vitro* SCE assay is an indicator test, with questionable relevance for genotoxic hazard identification.

Ponceau 4R was negative in the mouse lymphoma $TK^{+/-}$ assay (Cameron *et al*., 1987).

NIH3T3 cells were exposed for 4 hours to different concentrations of Ponceau 4R and the DNA damage was analysed by comet assay. Tail length, DNA contents in tail, and tail moment had positive correlations with the concentrations of the test item (Zeng et al. 2008, article in Chinese, only abstract available in English).

Sunset Yellow FCF was tested for its mutagenic potential in the L5178Y $tk^{+/-}$ mouse lymphoma cell forward mutation assay (McGregor et al. 1988) using a non-standard procedure (according to Clive and Spector, 1975; Clive et al., 1979). Cultures were exposed to the chemicals for 4 hours, then cultured for 2 days before plating in soft agar with or without the selective agent trifluorothymidine (TFT). The chemicals were tested at least twice. Sunset Yellow FCF induced a weak increase in mutation frequency, only in the presence of rat liver S9 mix. Since the increased mutation frequency did not exceed the "Global Evaluation Factor", the Panel considered the results as being negative.

No induction of micronuclei or sister chromatid exchange was reported in the bone marrow of Chinese hamster treated with Sunset Yellow FCF (certified for food additive purposes, dye content 85 %) while a slight increase in chromosome aberrations was observed at an intermediate dose, but was not confirmed at the upper one (Wever *et al.*, 1989). In the same study, the test item was negative for clastogenic effects in three different strains of mice. (Wever *et al.*, 1989).

In vitro studies in other non-mammalian cells

Results in other non-mammalian systems considered by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1980 are referred to in the 2009 ANS Opinion on Allura Red AC, even though they were not available for evaluation (EFSA ANS Panel, 2009a). In these studies, Allura Red AC was negative in genotoxicity assays in three yeast strains (not specified) and in *Drosophila melanogaster* in tests for loss of X or Y chromosome, visible mutations at specific loci, sex-linked recessive lethals and chromosomal translocations after oral feeding for 24 days at the LD_{50} dose (presumably the oral LD_{50} for *Drosophila*).

Amaranth and Tartrazine were not mutagenic in somatic (wing spot test) and germ cells (sex linked recessive lethals test) of *Drosophila* (Tripathy et al., 1995 as referred to in EFSA ANS Panel, 2009d; 2010).

In another *in vitro* study, Amaranth and Tartrazine were reported to interact non-covalently with calf thymus (CT) DNA, as measured by spectroscopic titration (Mpountoukas et al., 2010).

In a thermodynamic study in native (CT) DNA, Tartrazine was reported to interact with DNA (Kashanian and Zeidali, 2011).

In a fluorescence quenching assay, Amaranth, Ponceau 4R, Sunset Yellow FCF and Tartrazine were shown to interact non-covalently with naked DNA (Williams et al., 2012).

***In vivo* studies**

In vivo, negative results were reported for Allura Red AC in a test for heritable translocations in mice after dietary exposure at 4 000 – 20 000 ppm for 8 weeks (unpublished, Jorgenson et al., 1978 as referred to in EFSA ANS Panel, 2009a).

In rodent assays *in vivo* Amaranth was negative in a cytogenetic assay in mouse bone marrow after intraperitoneal (i.p.) administration at 50, 100 and 200 mg/kg bw 18 h before sacrifice (Das and Mukherijee, 2004), and in a rat dominant lethal test, not available for evaluation (BIBRA, 1982 as referred to in EFSA ANS Panel, 2010).

The genotoxic and toxic activity of Amaranth, Sunset Yellow FCF and Tartrazine in the lower gastrointestinal tract was evaluated in a gut micronucleus assay in male Swiss mice (Poul et al., 2009). Animals were administered with the dye by gavage at 200 or 1 000 mg/kg bw twice at 24-hour intervals, and sacrificed 24 hours later. Micronucleated, apoptotic, and mitotic cells were assessed as a measure of induced genotoxicity, cytotoxicity and cell proliferation. The concentrations of parent compound and its main metabolite (naphthionic acid) were measured in faeces during a 24-hour period after single oral administrations of the food dye. Administration of Amaranth, Sunset Yellow FCF and Tartrazine did not increase the incidence of micronucleated cells in mouse gut, but significantly raised the incidence of mitotic cells, suggesting a local toxic effect. Parent dye compound and its main aromatic amine metabolites were detected in significant amounts in the environment of colonic cells.

Ponceau 4R was negative in the *in vivo* mouse micronucleus assay when administered by i.p. injection up to 2 400 mg/kg bw (Hayashi et al., 1988) The sampling time was 24 hours. At the highest dosage 5 out of 6 animals died and in the survivor the percentage of polychromatic erythrocytes (PCE) was reduced by 30 %.

Clastogenicity in bone marrow cells was reported in mice after i.p. injection with Ponceau 4R (Agarwal et al. 1993). The substance was administered in a dose range from 4 to 20 mg/kg bw and the animals were sacrificed 24 hours after treatment. Significant induction of chromosome aberrations was observed from 4 mg/kg bw, with a dose response trend. The Panel noted that local toxicity in the bone marrow was reported in this study at a very low dosage if compared to the Hayashi study described above. This casts doubts on the identity/purity of the sample analysed.

Negative results were obtained in a cytogenetic tests in mice, with *per os* administration of Tartrazine and Azorubine/Carmoisine for 5 days at daily doses of 0.5 and 5 mg/kg bw (Durnev et al., 1995 as referred to in EFSA ANS Panel, 2009d; 2009e), and after i.p. injection of 50, 100 and 200 mg/kg bw of Tartrazine (Das and Moukherikijee, 2004 as referred to in EFSA ANS Panel, 2009d).

Increases in SCE and chromosomal aberrations in rodent bone marrow were reported in a limited study following acute or dietary administration of Tartrazine (Giri et al., 1990). In this study SCE in Swiss mice bone marrow cells were increased up to 2-fold 24 hours after i.p. injection of 100 and 200 mg/kg tartrazine; a 2-fold increase of chromosomal aberrations (“breaks”) was also reported in bone marrow cells of mice and rats after 3, 6, and 9 months feeding at 1 000 ppm. The Panel noted the limited study protocol and the inappropriate presentation and evaluation of experimental results as aggregated means, considering cells instead of animals as experimental units. Overall, the Panel considered this study inconclusive.

In another study (Farag et al. 2001), pregnant Swiss mice were treated orally with Tartrazine (1.7 mg/25 g bw corresponding to 68 mg/kg bw) for the first seven days of gestation; embryos were collected at day 11, and cultured for further 48 h *in vitro*, or collected at day 13 and directly processed for cytogenetic analysis. Bone marrow cells of mothers were also collected and analysed for chromosomal aberrations. Increased frequencies of structural chromosomal aberrations and decreased mitotic index were reported in the group of animals treated with Tartrazine compared to untreated controls and mice treated with β-carotene (1.7 mg/25 g bw). The Panel noted some inconsistencies in the effects reported, such as the presence of unstable chromosomal aberrations and decreased mitotic index both in mothers and in embryos several days after the termination of treatment, and the persistence of such effects in embryos also after 48 hours of *in vitro* culture. The Panel also noted that the presentation of aggregated data did not allow to assess the consistency of the effects reported within the experimental group. The Panel concluded that the reliability of these finding was questionable and did not take into account this study in the assessment of weight of evidence of genotoxicity.

In a recently published study aimed to assess the protective effect of selenium and vitamins A, C, and E, Sayed and coworkers tested a commercial product containing 96 % Sunset Yellow FCF (Sayed et al., 2012). Male mice were given orally single doses of Sunset Yellow FCF up to 1.3 mg/kg bw or 0.325 mg/kg bw/day daily for 1, 2 or 3 weeks. Sunset Yellow FCF was reported to elicit genotoxic effects as indicated by increased frequency of SCE's, chromosomal aberrations in both somatic (bone-marrow) and germ cells (spermatocytes), and by increased morphological sperm abnormalities and DNA fragmentation. The oral administration of selenium and vitamins A, C, and E significantly reduced the genotoxic effects of Sunset Yellow FCF. The Panel noted several inconsistencies in the results reported which cast doubts on the reliability of this study: in particular the Panel noted that accumulation of unstable chromosomal aberrations in bone marrow cells with prolonged treatment, as well as the reported increase of chromosomal aberrations and reciprocal translocations in spermatocytes 24 hours after chemical treatment lack of biological plausibility, given the cycling of bone marrow cells and the timing of mouse germ cell maturation. The Panel also noted that such results were reported after treatments with exceedingly low dosages of the dye, lower than the powerful positive control substance. Based on these concerns, the Panel did not consider the study by Sayed and coworkers in the genotoxicity weight of evidence evaluation.

METABOLISM

Absorption, distribution, metabolism and excretion (ADME)

The overall conclusions on the ADME data available for the sulphonated mono azo dyes already assessed by the ANS Panel (EFSA, 2005, 2007; EFSA ANS Panel, 2009a, 2009b, 2009c,2009d, 2009e, 2010) can be summarised as follows:

i. absorption of the intact dye from the gastrointestinal tract is very limited. However, faecal elimination of the parent compound is also low, up to 95% of the administered dose being found as metabolites.

ii. the initial metabolic step is the reduction of the azo group which occurs in the gastrointestinal lumen. Azoreductases in the anaerobic microbiota play a major role, as observed in the rat, mouse, rabbit, guinea-pig and humans.

iii. After intraperitoneal administration, most of the dye (60 to 95%) is excreted unchanged in the urine, indicating that the hepatic azoreductase(s) has a limited capacity.

iv. the amino compounds resulting from the reduction process are absorbed to different extents, depending on the degree of sulphonation of these molecules. The absorbed metabolites are then oxidized and/or conjugated in the liver and excreted in the bile and urine.

v. A common reduction metabolite of the dyes under assessment is 1-amino-2-naphtol, free or substituted (mono-, di- or tri-sulphonated)

vi. Differences in the chemical reactivity of aromatic/cyclic amines produced following azoreduction have been reported: generally, the presence of an amine or nitro group substituent increases the chemical reactivity (electrophilicity) of these metabolite(s) and/or the dye itself, whereas the sulphonation of the aromatic ring decreases it.

Metabolism and intestinal microbiota

The gastrointestinal tract contains a diverse microbiota comprised of at least several hundred species at concentrations of up to 10^{14} colony forming units (CFU) per ml (or g) of contents. The distribution, types and concentration of bacteria varies along the gastrointestinal tract. There is an increase in concentration and alteration in composition along the small intestine. In general there is an increase in strict (obligate) anaerobes at the expense of aerobes and facultative anaerobes further down the gastrointestinal tract. There is also a difference in distribution within the gastrointestinal lumen with a more consistent bacterial population associated with the gastrointestinal wall. The prevalence of bacteria in different parts of the gastrointestinal tract appears to be dependent on several factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial cooperation, mucin secretion, nutrient availability, diet, and bacterial antagonism. 99.9 % of colonic microflora are obligate anaerobes.

The phylogenetic analysis (randomly cloned16S rDNA comparative sequence) of the intestinal microflora of mice revealed 40 unique 16S rDNA sequences, of which 25 % corresponded to already described intestinal organisms (including *Lactobacillus* spp., *Helicobacter* spp. and segmented filamentous bacteria) and 75 % represented novel sequences with a large number (11/40) corresponding to an operational taxonomic unit (OTU) belonging to the *Cytophaga–Flavobacter–Bacteroides* phylum (Salzman, 2002). A similar analysis of the faecal flora of the rat revealed a total of 109 near full-length 16S rDNA clones representing 69 unique OTUs. Estimates of species richness indicated that approximately 338 species were present in the faeces, suggesting that only 20 % of species were identified. Only two of 39 Gram-negative clones aligned with previously cultured species, the remainder fell into a separate lineage within the *Bacteroides-Cytophaga* phylum. Several clones within this new group were related to 16S rDNA sequences previously identified from mouse faeces. *Lactobacilli* spp. were the most abundant Gram-positive species, representing 23 % of the total clones but only 7 % of OTUs. The remaining Gram-positive clones were distributed among the *Clostridium coccoides* group (9 %), the *Clostridium leptum* subgroup (18 %), and throughout the low GC Gram-positive bacteria (13 %) (Brooks et al., 2003).

The anaerobe *Bacteroides* thetaiotaomicron predominates (6% of all bacteria) in the human intestinal microbiota, accounting for 6% of all bacteria in the human intestine (Zocco et al., 2007). B. thetaiotaomicron has been shown to reduce Amaranth, Sunset Yellow FCF (Chung et al. 1978, Sweeney et al. 1994), Tartrazine, Ponceau SX, Methyl Orange, Orange II, Allura Red AC (Chung et al. 1978).

Azoreduction by the intestinal microflora

Azoreductase activity is commonly found in intestinal bacteria and mediated by three types of enzymes. Flavin dependent NADH preferred azoreductase and flavin dependent NADPH preferred azoreductase have broad substrate specificity, whereas flavin free NADPH preferred azoreductase, which involves a sophisticated binding mode, shows narrower substrate specificity. In some cases, the azo dyes can be degraded via oxidative reactions catalysed by oxidases and peroxidases.

The location of the azoreductase in the bacterial cell determines the class of azo dyes that can be metabolised. Almost 100 % of azoreductase activity of *E. coli* was found in the cytoplasm and proved to be unable to significantly reduce water-insoluble azo dyes, whereas 55 % of the azoreductase activity of *Enterococcus faecalis* was membrane bound and capable of reducing the azo bond of some water-insoluble dyes (Xu et al., 2010).

Metabolism and genesis of reactive oxygen species

The production of reactive oxygen species (ROS) from o-hydroxy aromatic amine products has been suggested (Kimura et al., 1979; Nakayama et al., 1983). The findings suggest that various azo dye-derived amines are genotoxic, not through N-hydroxylation and esterification, which is characteristic of many aromatic amines (Farr and Kogoma, 1991), but rather through a mechanism involving oxygen radicals (Nakayama et al., 1983; Basaga, 1989).

In a study of Sweeney et al. (1994), the production of $O_2\cdot^-$ radical from bacterial reduction of azo dyes was detected and tested against repair-deficient *E. coli* and *S.* Typhimurium TA102 sensitive to oxidative mutagens. The compounds shown to generate reactive oxygen species (ROS) after azo-reduction (Amaranth, Sunset Yellow FCF and, to a much lesser extent, Azorubine/Carmoisine) had a hydroxyl substituent ortho to the amine function, whereas no production was detected with Azorubine/ Carmoisine which exhibits a different chemical structure. The mechanism suggested relates to reactions postulated by Nakayama et al. (1983) involving a requirement for iron, as in Fenton chemistry, where O^0 is converted via H_2O_2 to the highly reactive ·OH species that is known to damage DNA (Basaga, 1989).

One of the points to address within the current mandate was the different response in the comet assay in mice and in rats and whether there could be some species differences that could provide an explanation to the observed effects. For what concerns the generation of ROS, some examples have been found in the literature that can illustrate species differences. In a study of dieldrin using mouse, rat and human hepatocytes a dose dependent increase in lipid peroxidation and oxidised DNA bases was observed only in mouse hepatocytes. Vitamin E content of hepatocytes was shown to decrease concomitantly in the three species. However, as the vitamin E stores in the human and rat hepatocytes were much greater that that measured in mouse hepatocytes, the dieldrin-induced oxidative stress only damaged mouse cells (Klaunig et al., 1998). In mouse and rat, after oral paracetamol, although overall paracetamol metabolism was similar in both species, the oxidative stress, mitochondrial protein adducts and nuclear DNA fragmentation were significantly lower in rats compared to mice (Mc Gill et al., 2012). These examples however do not allow the drawing of any firm conclusions for this specific assessment.

STRUCTURE RELATIONSHIP WITH GENOTOXICITY AND CARCINOGENICITY

Azo dyes can be separated into two groups, those containing an aromatic amine group (R_1-N=N-R_2-NH_2), such as 4-aminoazobenzene, and a second group that can only produce amine compounds after reduction of the azo bond (R_1-N=N-R_2 → R_1-NH_2 + R_2-NH_2). Most of the azo dyes of the first group are carcinogenic, which is not true for those of the second group that have been tested for carcinogenicity. In addition, some azo dyes or their metabolites were selectively toxic or carcinogenic, for example, 4-aminoazobenzene is hepatocarcinogenic in male but not female mice. This result is not consistent with the hypothesis that azoreduction is the only cause of carcinogenicity, and it suggests there may be another mode of action.

4-aminobenzenesulphonic acid (4-ABS), a metabolite of the food dyes Sunset Yellow FCF and Tartrazine, was shown not to possess a mutagenic effect with or without metabolic activation in bacterial reverse mutation assays (Ben Mansour et al., 2007; 2009a, 2009b).

The 1-amino-2-naphthol based azo dyes produce 1-amino-2-naphthol, which has been reported to be a carcinogen. Although a study reported that it was not mutagenic in the *Salmonella*/microsome test system (Chung et al., 1981; Chung, 1983), later studies showed that this compound was mutagenic (Dillon et al., 1994). In standard protocols with metabolic activation, 1-amino-2-naphthol was mutagenic to *S.* Typhimurium strain TA100 but not to strain TA98 (Dillon et al., 1994).

In another study, the metabolite of Sunset Yellow FCF 1-amino-2-naphthol-6-sulphonate was shown to be not mutagenic in the *E. coli* differential lethality test with DNA repair proficient/deficient strains,

which the authors suggested could be due to the decrease in membrane permeability of this compound (Gottlieb et al., 2003).

Another study on the mutagenicity of 1-amino-2-naphthol-based azo dyes, indicated inhibition of the mutagenicity when 1-amino-2-naphthol was sulphonated at strategic sites (Rosenkranz and Klopman, 1990). Acid Orange 8, Acid Red 88, Orange II, Para Red, Ponceau BS, 1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, and 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol can be reduced to produce 1-amino-2-naphthol; which is genotoxic and carcinogenic. The food dyes Allura Red AC and Sunset Yellow FCF produce 1-amino-2-naphthol-6-sulphonate which is not genotoxic and carcinogenic (Feng et al., 2012).

Benzidine is known as a carcinogen for the human urinary bladder (Haley, 1975). In the testing systems with mouse liver enzyme preparations, benzidine was mutagenic to S. Typhimurium strain TA98 and TA100 (Lazear and Louie, 1978). In the testing systems with flavin-mononucleotide (FMN) and hamster liver S9, benzidine was also mutagenic (Prival et al., 1984). The nitro derivative of benzidine was more mutagenic than that of parent compound in TA98 without metabolic activation (You et al., 1994). The addition of a sulphonic acid group to benzidine reduced the mutagenicity (Ashby et al., 1982). In addition, when both sides of the azo linkage of the azo dyes were sulphonated, the compounds were not carcinogenic in any of the animal species tested (Brown and De Vito, 1993). The similar situation was also found with the mutagenicity of aniline and 4-aminobenzenesulphonic acid, 1-amino-2-naphthol, and 1-amino-2-naphthol-6-sulphonate.

Thus, generally inclusion of an aromatic amine or nitro group into the azo dye structure increases the genotoxicity and carcinogenicity of the dye itself and/or its metabolites. In contrast the sulphonation of the aromatic ring decreases the genotoxicity and carcinogenicity of the dye itself and/or its metabolites.

CARCINOGENICITY DATA

The data from carcinogenicity studies conducted with the sulphonated mono azo dyes authorised as food additives and previously considered by the ANS Panel were reviewed (EFSA ANS Panel, 2009a; 2009b;2009c; 2009d; 2009e; 2010). A literature search did not reveal any new publications (the literature search was conducted in the SciFinder database, encompassing MEDLINE and CAPLUS, with a cut-off date 15 October 2012).

Based on the results of the carcinogenicity studies the Panel concluded that there is no evidence of a carcinogenic potential of Allura Red AC (E 129), Amaranth (E 123), Ponceau 4R (E 124), Sunset Yellow FCF (E 110), Tartrazine (E 102) and Azorubine/Carmoisine (E 122).

It was noted that at the time of the re-evaluation of these food dyes, Red 2G was considered as being of safety concern since it is extensively metabolised to aniline, which should be considered as a carcinogen for which a genotoxic mechanism cannot be excluded (EFSA, 2007). Bearing in mind the chemical structure of the sulphonated mono azo dyes included in this opinion, it should be noted that none of these would yield aniline upon reduction of the azo bound.

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ABBREVIATIONS

4-ABS:	4-aminobenzenesulphonic acid
ADME:	Absorption, distribution, metabolism and excretion
CFU:	Colony forming units
CHL:	Chinese hamster lung fibroblasts
CT:	Calf thymus
i.p.:	intraperitoneal
JaCVAM:	Japanese Center for the Validation of Alternative Methods
OTU:	Operational Taxonomic Unit
PCE:	polychromatic erythrocytes
ROS:	Reactive Oxygen Species
SCE:	Sister chromatid exchanges
TFT:	Trifluorothymidine