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# CYP-450 isoenzymes catalyze the generation of hazardous aromatic amines after reaction with the azo dye Sudan III

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ABSTRACT

This work describes the mutagenic response of Sudan III, an adulterant food dye, using *Salmonella typhimurium* assay and the generation of hazardous aromatic amines after different oxidation methods of this azo dye. For that, we used metabolic activation by S9, catalytic oxidation by ironporphyrin and electrochemistry oxidation in order to simulate endogenous oxidation conditions. The oxidation reactions promoted discoloration from 65% to 95% of Sudan III at $1 \times 10^{-4}$ mol $L^{-1}$ and generation of $7.6 \times 10^{-7}$ mol $L^{-1}$ to $0.31 \times 10^{-4}$ mol $L^{-1}$ of aniline, o-anisidine, 2-methoxi-5-methylaniline, 4-aminobiphenyl, 4,4′-oxydianiline; 4,4′-diaminodiphenylmethane and 2,6-dimethylaniline. The results were confirmed by LC–MS–MS experiments. We also correlate the mutagenic effects of Sudan III using *S. typhimurium* with the strain TA1535 in the presence of exogenous metabolic activation (S9) with the metabolization products of this compound. Our findings clearly indicate that aromatic amines are formed due to oxidative reactions that can be promoted by hepatic cells, after the ingestion of Sudan III. Considering that, the use of azo compounds as food dyestuffs should be carefully controlled.



## 1. Introduction

Color is an essential characteristic for the success of industrialized food, considering that this organoleptic property is unconsciously related to the quality and taste of foodstuffs (Downham and Collins, 2000). In this context, various compounds are legally or illegally used to confer color or improve the appearance of foods.

However, there is a broad consensus in the scientific community that one of the biggest problems of the use of food dyes is their ability to be reductively metabolized in the gastric or intestinal tract leading to the formation of aromatic amines (Chen, 2006; Kleinow et al., 1987; Pielesz et al., 2002). Because of this, the International Agency for Research on Cancer (IARC) and the Ecological and Toxicological Association of the Dye and Organic Pigments Manufacturers (ETAD) have listed at least 22 amines that should not be formed during the degradation of a dye used for textiles, cosmetics, food and photography (ETAD, 2008).

After the ingestion of a xenobiotic, several biotransformation reactions may occur and the oxidation plays an important role because the products generated can be even more toxic than the original compound (Meunier, 1992; Montellano, 2004) and these bioactivation reactions are often catalyzed by the cytochrome P50 system (Guengerich, 1991; Miller, 1970). Thus, for the adequate risk assessment of a chemical used as food dye, it is essential to evaluate not only the toxicity of the compound itself but also the degradation products.

Sudan dyes are azo compounds commonly used to confer color to various materials as gasoline, solvents, oils, inks among others (Cheung et al., 2012; Oplatowska et al., 2011; Pan et al., 2012; Rebane et al., 2010). Due to their intense red color and low price, these compounds have been used illegally as food dye, mainly in chilli, curry and paprika powders, as well as palm oil, in order to intensify the color (Cheung et al., 2010; Mejia et al., 2007; Pan et al., 2012; Oplatowska et al., 2011; Qi et al., 2011; RASFF, 2004; Rebane et al., 2010; Xu et al., 2010).

Although the use of sudan compounds as food dye has been banned by the European Community (European Community, Commission Decision, 2002), these compounds are still a public health problem considering the large number of studies showing the presence of these dyes in food all over the world. In the review published in 2010, Rebane et al. showed that between 2003 and 2009, nearly 50 articles on this topic were published (Rebane et al., 2010).

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Almost all the studies involving mutagenicity of Sudan dyes are focused on Sudan I. An et al. (2007) report the induction of genotoxic effect of Sudan I in HepG2 cells as well as positive mutation response for *Salmonella typhimurium* assay in the presence of metabolic activation (S9) with TA1535 strain. This monoazo dye also forms DNA adducts after metabolic activation in the mammals liver cells (Stiborova et al., 2002, 2005, 2006). Some authors reported that the mutagenic responses of Sudan dyes are related to the reductive cleavage of the azo group by intestinal micro flora azoreductases or liver enzymes forming N-hydroxy derivatives. The dyes and/or their related by-products could also be acetylated by enzymes such as o-acetyltransferase, generating ions that are capable of reacting with DNA to form adducts (Hunger, 1994; Xu et al., 2007).

Sudan III is a diazo dye also used as food adulterant and cosmetics (RASFF, 2004), with chemical structure (Fig. 1) more complex than Sudan I. There is a raised concern about Sudan III potential metabolic cleavage by intestinal bacteria and probable formation of 4-aminoazobenzene and aniline (Oplatowska et al., 2011; Pielesz et al., 2002; Xu et al., 2007). Our group has performed chemical and electrochemical reduction of Sudan III, showing that carcinogenic aromatic amines are formed after reductive reactions that could occur under endogenous conditions (Lizier et al., 2012).

Considering the high importance of oxidation reactions catalyzed by CYP-450 enzymes on xenobiotics toxicity, this work aimed to evaluate the generation of aromatic amines after the reaction of Sudan III with exogenous metabolization system (S9), widely used in mutagenic assays (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). Additionally, we also used a metalloporphyrin model that mimetize the oxidation activity of P-450 enzymes (Doro et al., 2000; Faria et al., 2008; MacLeod et al., 2007) and an electrochemical oxidation reaction to promote the oxidation of Sudan III. In order to evaluate the formation of hazardous by-products of the cited reaction, an HPLC/DAD method was developed to identify and quantify the amine content in the oxidized dye sample.

Furthermore in this work, the mutagenic response of Sudan III was evaluated by using *Salmonella* mutagenicity assay with different strains of *S. typhimurium*, which are able to detect base pair substitution mutations (TA100, TA1535 and YG1042) and frame shift mutations (TA98) in the presence and absence of exogenous metabolization (S9).

## 2. Materials and methods

### 2.1. Mutagenicity evaluation

The *Salmonella*/microsome mutagenicity assay or Ames test is widely used to detect chemical mutagens and potential carcinogens (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). In the present work, we used the strains TA100 (HisG461, rfa, Δbio, ΔuvrB, pKM101), TA98 (HisD30521, rfa, bio3, ΔuvrB, pKM101), TA1535 (HisG461, rfa, ΔuvrB) and YG1042 (HisD3052, rfa, Δbio, ΔuvrB, pKM101, nitroreductase and O-acetyltransferase overproducing enzyme activity). We used concentrations ranged from 0.0002 to 2 mg/plate of Sudan III (Sigma–Aldrich, 90% dye content) dissolved in alcohol:dimethylsulfoxide (DMSO) (3:7), based on preliminary assays (data not shown). We employed the standard pre incubation procedure with and without exogenous metabolic activation (S9) (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). Briefly, 100 μL overnight cultures of each strain of *S. typhimurium* (about $10^9$ cells/mL), 500 μL of 0.2 M sodium phosphate buffer or S9 mix and 100 μL of the dye solution were incubated at 37 °C for 30 min without shaking. After incubation, 2 mL of molten top agar was added, the mixture poured onto a minimal agar plate, and the plates incubated at 37 °C for 66 h. Colonies were counted by hand and the background was carefully evaluated.

DMSO (Merck, Darmstadt, Germany) was used as negative control. For TA98, TA100 and TA1535, the positive controls were 4-nitroquinoline-oxide – 4NQO (5 μg/plate, Sigma, St. Louis, USA) and 2-aminoanthracene – 2AA (25 μg/plate, Aldrich, Seelze, Germany). For YG1042 the positive controls were 100 μg/plate of 2-nitrofluorene – 2NF (Aldrich, Seelze, Germany) and 2-aminoanthracene – 2AA (25 μg/plate, Aldrich, Seelze, Germany), all dissolved in DMSO.

Metabolic activation was provided by Aroclor 1254 induced Sprague Dawley rat liver S9 mix (MolTox, Boone, USA), which was prepared at a concentration of 4% (v/v) according to Maron and Ames (1983).

The samples were considered positive when a significant ANOVA and dose response was obtained using the Bernstein model (Bersntein et al., 1982). The results were expressed as the mean of number of revertants per plate ± standard deviation.

All the experiments were performed in triplicate.

### 2.2. Oxidation of Sudan III by exogenous metabolic activation (S9)

We promoted the metabolic activation of Sudan III using the S9 mixture at 4% v/v, prepared following the Ames test protocol (Maron and Ames, 1983). Briefly: the lyophilized S9 fraction (MolTox, Boone, USA) was resuspended using ultra pure water. Then, NADP (0.1 M), phosphate buffer (0.2 M), glucose-6-phosphate (1.0 M), KCl (1.65 M) and $MgCl_2$ (0.40 M) were added.

Five millilitre of Sudan III dye solution ($1.00 \times 10^{-4}$ mol $L^{-1}$) were incubated with different volumes of S9 at 4% (v/v), varying between 50 and 200 μL, at 37 °C for 90 min. These conditions were chosen based on a preliminary study (data not shown). After the incubation period, the sample was extracted by a liquid–liquid technique, using dichloromethane (1:1). This procedure was repeated three times. After solvent evaporation, the residue was dissolved in methanol.

The absorbance of the extracted sample was monitored using a Hewlett Packard 8453 UV–Vis spectrophotometer. Concomitantly, another aliquot of the sample was analyzed by HPLC/DAD. All analysis was compared to a blank, using PBS instead of the dye.

### 2.3. Oxidation of Sudan III by tert-butylhydroperoxide catalized by an ironporphyrin (FeTMPyP)

Aliquots of 1.00 mL of Sudan III ($1.0 \times 10^{-4}$ mol $L^{-1}$) were treated with 10 μL of ironporphyrin ($5.00 \times 10^{-8}$ mol $L^{-1}$, MidCentury) and 50 μL of the oxidant agent tert-butylhydroperoxide-concentrated (Faria et al., 2008). After constant agitation for 4 and 15 h, the samples were submitted to liquid–liquid extraction and analyzed by HPLC/DAD and UV–Vis spectrophotometry.

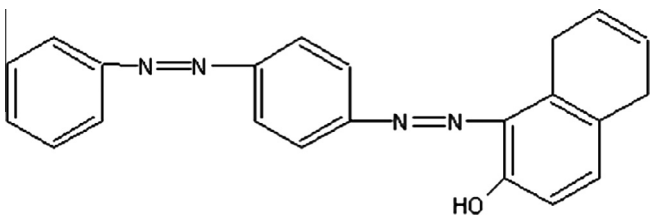


**Fig. 1.** Chemical structure of Sudan III dye (http://www.sigmaaldrich.com/catalog/product/sial/s4131?lang=pt&region=BR).

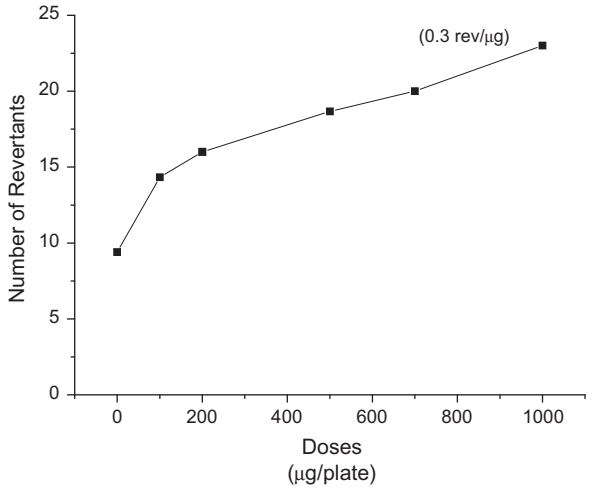


**Fig. 2.** Dose response curve for the dye Sudan III tested with *Salmonella* strains TA1535 in the presence of exogenous metabolic activation (S9), as described in Section 2. Number in parenthesis was generated as slope values by the model of Bernstein et al. (1982) and represent the potency of the compound expressed in revertants per μg.