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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 1×10^{-4} mol L^{-1} and generation of 7.6×10^{-7} mol L^{-1} to 0.31×10^{-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 (1.00×10^{-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 (1.0×10^{-4} mol L^{-1}) were treated with 10 μL of ironporphyrin (5.00×10^{-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.

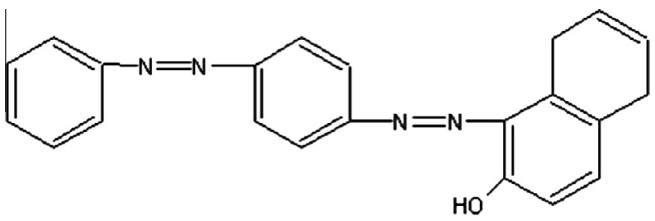


Fig. 1. Chemical structure of Sudan III dye (http://www.sigmaaldrich.com/catalog/product/sial/s4131?lang=pt&region=BR).

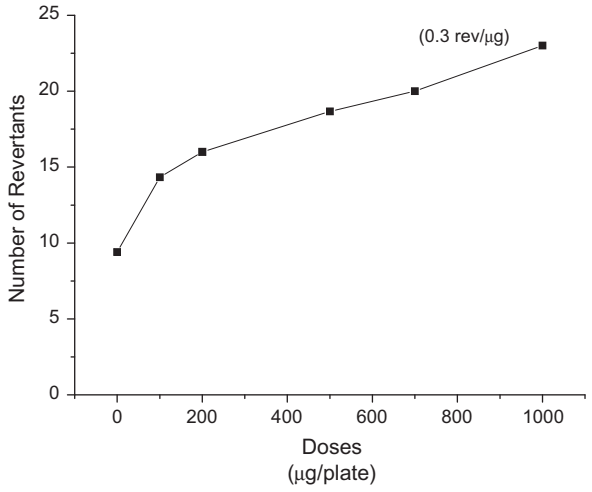


Fig. 2. Dose response curve for the dye Sudan III tested with *Salmonella* strains TA1535 in the presence of exogenous metabolic activation (S9), as described in Section 2. Number in parenthesis was generated as slope values by the model of Bernstein et al. (1982) and represent the potency of the compound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

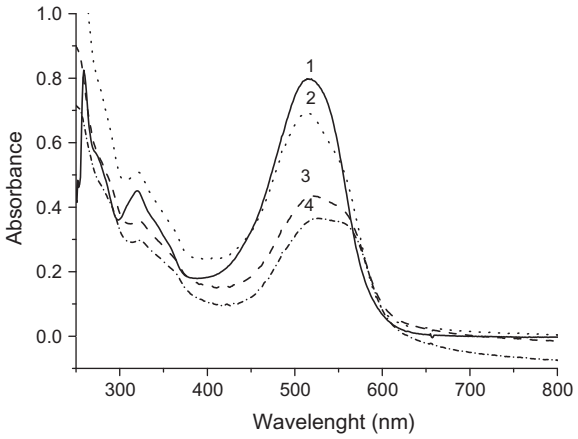


Fig. 3. UV–Vis spectra obtained for 5.0 mL of Sudan III dye solution (1×10^{-4} - mol L^{-1}) before (1) and after 90 min of incubation at 37 °C with 50 μL (2), 100 μL (3) and 200 μL (4) of S9 4% (v/v).

2.4. Electrochemical experiments

All electrochemical measurements were carried out on a Potentiostat EG&G model 283 (PAR). The measurements were performed in a conventional electrochemical cell of 10.0 mL, where three electrodes were inserted: a reference electrode of Ag/AgCl (KCl 3.0 mol L^{-1}), a platinum gaze as auxiliary electrode and a glassy carbon (area of 3.14 mm^2 for voltammetric measurements and 4.00 cm^2 for electrolysis experiments) as working electrode.

The voltammetric curves was obtained transferring 10.0 mL of DMSO/0.01 mol L^{-1} tetrabutylammonium tetrafluorborate solution into the voltammetric cell and the required volume of stock solution of Sudan III dye was added by micropipette. The solution was purged with nitrogen for 15 min and the voltammetric curves were recorded. For controlled potential electrolysis experiments, the dye degradation were conducted up to constant value of current recorded during current *vs* time curves. The generated products were analyzed by transference of electrolyzed samples at defined experimental conditions. The reaction was monitored using UV–Vis spectra and HPLC/DAD.

2.5. Chromatographic analysis

High Performance Liquid Chromatography coupled with a Diode Array Detector (HPLC-DAD) was used to monitor the dye as well as the products generated after the oxidation of Sudan III, using Shimadzu SCL-10AVP. For that, we used a reversed-phase column Shimadzu CLC-ODS (C18) (250 × 4.6 mm, 5 μm, 100 A) connected to a guard column Shimadzu CLC-ODS (C18) (1 cm × 4.6 mm, 5 μm, 100 A). The best experimental condition to analyze the dye was optimized by using isocratic mode: mobile-phase of methanol/acetonitrile 50:50 v/v, flow rate of 1.0 mL min^{-1} and column temperature of 40 °C. The analysis time was 10 min, and all the analyses were carried out in triplicate (Lizier et al., 2012).

The identification and quantification of the amines group: of 2-naphthylamine (Sigma, 98%), 2,4-diaminotoluidine (Fluka, 98%), benzidine (Fluka, 98%), 4,4′-methyleno-bis-(2-chloroaniline) (Aldrich, 85%), aniline (Sigma, 99%), o-toluidine (Aldrich, 98%), 4,4′-oxidianiline (Aldrich, 98%), o-dianisidine (Sigma, 98%), o-anisidine (Aldrich, 99%), 4,4′-diaminobiphenylmethane (Fluka, 97%), 3,3′-dimethylbenzidine (Sigma–Aldrich, 97%), 2-methoxy-5-methylaniline (Aldrich, 99%), 4-chloroaniline (Fluka, 99%), 2,6-dimethylaniline (Fluka, 98%), 2-chloro-4-nitroaniline (Fluka, 98%) were performed using a mobile-phase methanol/phosphate buffer 5.00×10^{-5} - mol L^{-1} (pH = 6.9) + 20.0 mM of triethylamine at composition of 50:50 v/v, a flow rate of 1.0 mL min^{-1} and a column temperature of 40 °C (condition 1). Other amines: 4,4′-metilene-bis-(2-methylaniline) (Fluka, 98.5%), 4-chloro-o-toluidine (Fluka, 98%), 4-aminobiphenyl (Sigma, 90%), 3,3′-dichlorobenzidine (Supelco, 99%) were better separated using similar experimental conditions but using methanol/phosphate buffer 5.00×10^{-5} mol L^{-1} (pH = 6.9) + 20.0 mM of triethylamine at composition 80:20 (v/v), a flow rate of 1.0 mL $min^{-1}$and a column temperature of 40 °C (condition 2). All these methodologies were carried out based on chromatographic parameters such as retention time (t_r), retention constant factor, selectivity and resolution between peaks and theoretical plate number (N) (Snyder et al., 1997). Standard curves and quantitative analysis of the target amines were carried out by linear regression plotting peak area *vs* concentration and further comparison with standard addition method by using spiking aliquots of the working standard in methanol. The procedure was carried out in triplicate for each sample. Characteristic UV–Vis spectra obtained by the diode array detector under the hydrodynamic conditions was recorded and used as a parameter to identify and to confirm the investigated species. Afterwards, these spectra were compared with that obtained from pure samples and treated dye sample (Lizier et al., 2012).

The degradation products generated during the oxidation of the Sudan III dye using all the procedures were also analyzed by mass spectrometry LC–ESI–MS–MS QTrap.

The developed method relied on a full scan (EMS – Enhanced Mass Spectrometry) and on the enhanced product ion (EPI) for structural elucidation. All the samples were diluted in MeOH/H2O 50:50, 0.1% formic acid before injection. The analytes were separated in an Agilent Zorbax C-18 (5 μm, 150 mm × 4.6 mm) column by using an Agilent 1200 automatic sampler and an Agilent 1200 HPLC (Agilent Technologies) pump. The elution was carried out in the isocratic mode by employing 0.1% formic acid as the phase modifier and the solvent H_2O (solvent A) and MeOH (solvent B) in the following conditions: 40% B and 60% A for 30 min. The flow rate was 500 μL min^{-1}.

ESI–MS/MS experiments were accomplished on a linear ion trap mass spectrometer (QTrap; Applied Biosystems) equipped with a TurboIonSpray connected to the liquid chromatography system. In order to obtain the spectral data, a temperature of 650 °C was applied to the vaporizer. The ESI source and the mass spectrometer were operated under positive ion using a voltage of 4.5 kV at TurboIonSpray. The desolvation potential was adjusted to 45 V, and ultrapure N_2 was utilized as the collision gas. The EPI analyses were performed at a scan speed of 4000 uma s^{-1}, 27 ± 2 V collision energy (CID), and 50 ms for ion capture. All the experiments were conducted in the m/z scan range of 50–600, with entrance potential of 8.0 V, which enabled trapping at Q^0. Direct infusion of 50 ppb of the individual standard solutions of each aromatic amine was utilized to compare

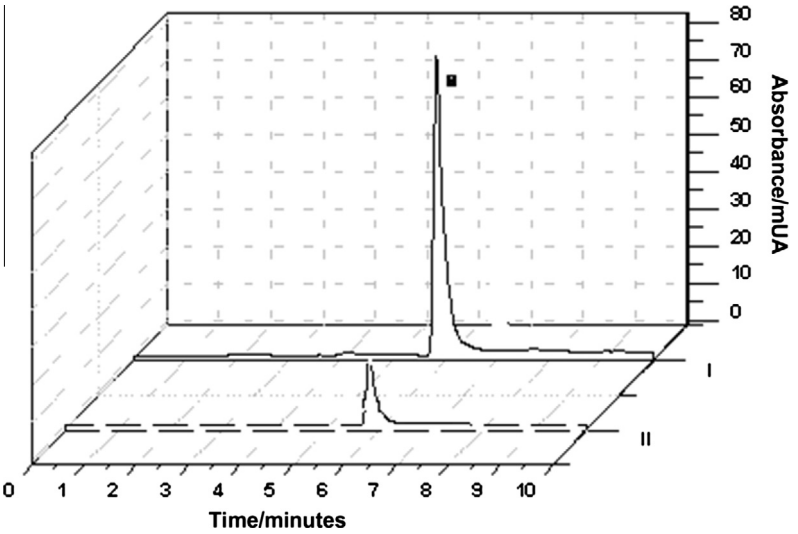
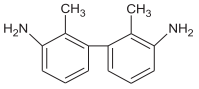
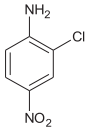
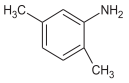
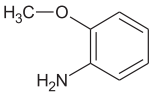
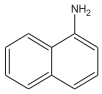
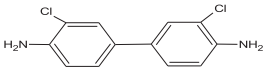
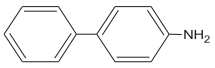
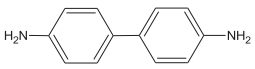
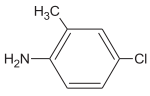
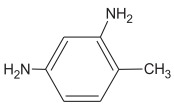
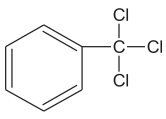


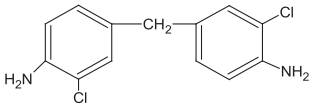
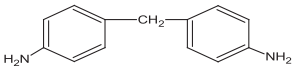
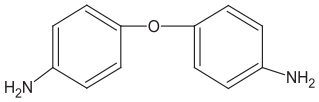
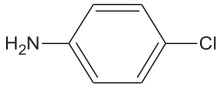
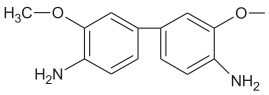
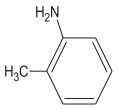
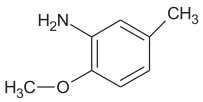
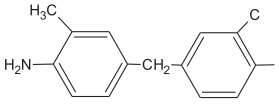
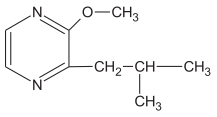
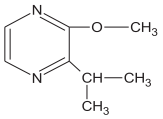
Fig. 4. Chromatogram obtained for 20 μL of 1.0×10^{-4} mol L^{-1} Sudan III dye before (I) and after 4 h (II) of S9 oxidation. Mobile phase: ACN/MeOH 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, column = C18, λ = 500 nm.

Table 1
Chemical structures of studied aromatic amines, IARC classification and retention time corresponding to HPLC/DAD obtained at (a) condition 1: $T = 40\ ^{\circ}C$. Flow rate = 1.00 mL min^{-1}. Detection = 230 nm. Mobile phase: MeOH/phosphate buffer 5.00×10^{-5} mol L^{-1} (pH = 6.9) 50:50 (v/v) and 20 mM of triethylamine and (b) condition 2: $T = 40\ ^{\circ}C$. Flow rate = 1.00 mL min^{-1}. Detection = 230 nm. Mobile phase: MeOH/phosphate buffer 5.00×10^{-5} mol L^{-1} (pH = 6.9) 80:20 (v/v) and 20 mM of triethylamine.

Substances	Structure	Classification IARC[A]	Retention time (min)
3,3′-Dimethylbenzidine		2B	9.0[a]
Aniline		3	5.2[a]
2-Chloro-4-nitroaniline		–	11.5[a]
2,6-Dimethylaniline		2B	11.2[a]
o-Anisidine		2B	8.0[a]
2-Naphthylamine		1	3.0[a]
3,3′-Dichlorobenzidine		2B	4.7[b]
4-Aminobiphenyl		1	4.4[b]
Benzidine		1	4.9[a]
4-Chloro-2-methylaniline		2A	4.1[b]
2,4-Diaminotoluidine		2B	3.7[a]
α,α,α-Trichlorotoluene		2A	8.4[b]

with the analyzed sample. The optimized separation conditions were as follows: solvent (A) methanol with 0.1% of formic acid, solvent (B) water with 0.1% formic acid at a flow rate of 0.5 mL/min. The gradient elution was: initial conditions 30% A; 0–30.0 min, 30–100% A; 30.0–40.0 min, 100% A; from 40.1 return to initial conditions; 40.2–45.0, equilibration of the column. The sample volume injected was 10 μL.

Table 1 (*continued*)

Substances	Structure	Classification IARC[A]	Retention time (min)
4,4′-Methylenebis(2-chloroaniline)		1	4.9[b]
4,4′-Diaminodiphenylmethane		2B	8.5[a]
4,4′-Oxydianiline		2B	6.7[a]
4-Chloroaniline		2B	10.8[a]
o-Dianisidine		2B	7.4[a]
o-Toluidine		1	6.5[a]
2-Methoxy-5-methylaniline		2B	12.2[a]
4,4′-Methylene-bis(2-methylaniline)		2B	3.8[b]
2-Isobutyl-3-methoxypyrazine		–	5.9[b]
2-Isopropyl-3-methoxypyrazine		2B	6.2[b]

[A] According to IARC the group 1: carcinogenic to humans; group 2A: probably carcinogenic to humans; group 2B: possibly carcinogenic to humans; and group 3: not classifiable as to its carcinogenicity to humans. – Not listed (IARC, 2012).
[a] Chromatographic condition 1.
[b] Chromatographic condition 2.

3. Results and discussion

3.1. Evaluation of mutagenic activity

Sudan III tested negative in the *Salmonella* assay for all strains, except for TA1535 in the presence of S9 that exhibited mutagenic potency of 0.3 rev/plate (Fig. 2). So, after the activation of Sudan III by the isoenzymes of cytochrome P-450 present in S9 mixture, this compound induces to base pair substitution, considering that this is the mutagenic effect detected by the strain 1535. Therefore, we hypostatize that the oxidative metabolism by cytochrome P-450 plays an important role on the toxicity of Sudan III. Consider-

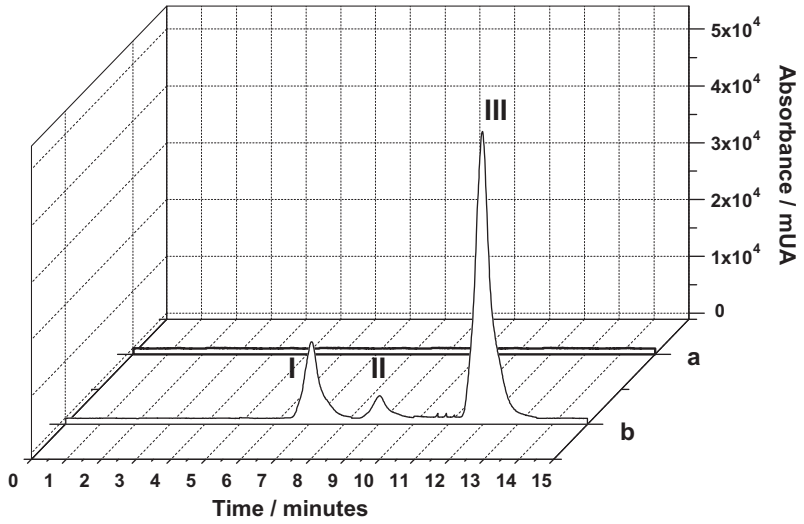


Fig. 5. Chromatogram of the Sudan III (a) and of the aromatic amines obtained after 4 h of reaction with S9 (b). Mobile phase: MeOH/buffer phosphate 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, e column C18, λ = 280 nm; (I) 4,4′-oxydianiline; (II) 4,4′-diaminodiphenylmethame; and (III) 2,6-dimethylaniline.

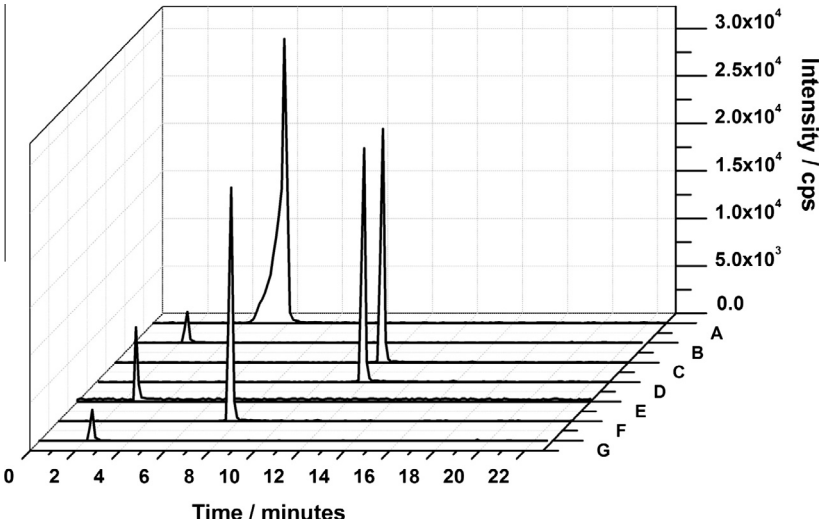


Fig. 6. LC–MS pattern obtained for 10 μL of 50 ppb of aromatic amines: (A) 2-methoxy-5-methylaniline, (B) 4,4-diaminodiphenylmethane, (C) 4,4′-oxydianiline, (D) 4-aminobiphenyl, (E) o-anisidine, (F) 2,5-dimethylaniline, and (G) aniline.

Table 2
MS/MS transition ion scan obtained by LC–MS–MS for aromatic amines generated after chemical, electrochemical and biological oxidation.

Compounds	t_r (min)	m/z	Fragments	CE (V)
4,4′-Diaminodiphenylmethane	2.72	198.3	199.3 → 124.1 (199.3 → 80.1)	19 37
2,5-Dimethylaniline	7.77	121.2	122.1 → 77.0 (122.1 → 105.1)	28 14
4,4′-Oxydianiline	13.2	200.2	201.1 → 80.1 (201.1 → 108.1)	33 19
4-Aminobiphenyl	14	169.2	170.1 → 152.1 (170.1 → 153.1)	30 19
o-Anisidine	3.67	123.2	124.0 → 109.0 (124.05 → 80.0)	14 29
2-Methoxy-5-methylaniline	5.33	137.2	138.1 → 123.1 (138.1 → 122.1)	15 25
Aniline	2.73	93.5	94.01 → 77.1 (94.01 → 50.9)	19 34

t_r = retention time at LC–MS–MS; m/z = $[M+H]^+$ of the MS/MS transition; CE = collision energy.

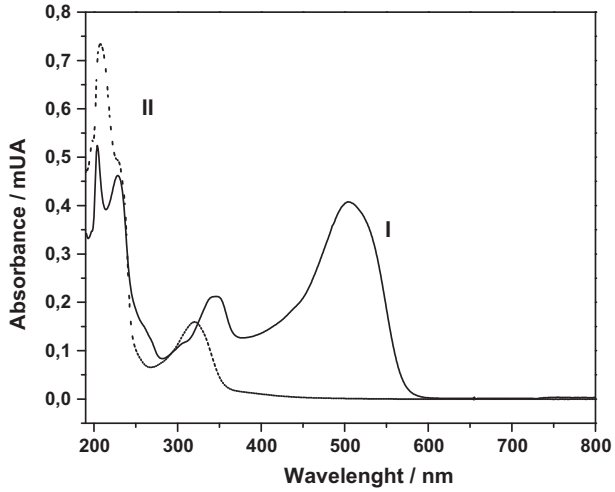


Fig. 7. UV–Vis hydrodynamic spectra obtained for 20 μL of 1×10^{-4} mol L^{-1} of Sudan III before (I) and after 4 h (II) of ironporphyrin oxidation. Mobile phase: ACN/MeOH 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, column = C18, $\lambda_{initial}$ = 190 - nm; λ_{final} = 800 nm.

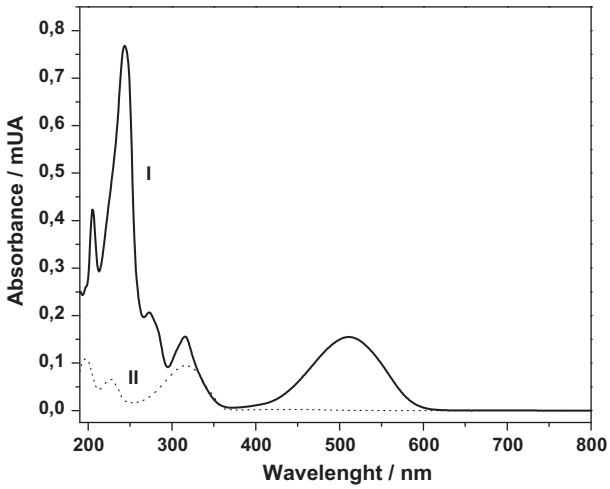


Fig. 9. UV–Vis spectra obtained for 1.0×10^{-4} mol L^{-1} Sudan III dye before (I) and after (II) electrooxidation for 2 h, with E = + 1.5 V. Mobile phase: ACN/MeOH 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, column = C18, $\lambda_{initial}$ = 190 nm; λ_{final} = 800 nm.

ing that, we monitored the generation of toxic products, such as aromatic amines, after different types of oxidation reactions, i.e. S9, ironporphyrin and electrochemical oxidation in order to mimic the *in vivo* biotransformation.

3.2. Identification of the oxidation products generated after the reaction of Sudan III with S9

To evaluate the role of cytochrome P-450 on activating of Sudan III dye we tested the effect of different concentrations of S9 (exogenous metabolization) monitoring the UV–Vis spectra of the dye. According to Fig. 3, Sudan III (1×10^{-4} mol L^{-1}) presents a peak of maximum absorbance at 520 nm attributed to azo chromophore group. Analyzing Fig. 3, we clearly observe the effect of S9 on the UV–Vis spectra of the dye, with the reduction of around 50% of this main peak with the highest concentration of S9, suggesting the cleavage of the azo bond in a dose dependent manner. In order to confirm this find, the solution resultant of the reaction of S9 with Sudan III was extracted using liquid/liquid procedure and submitted to HPLC/DAD analysis, following the procedure described in the experimental section. The chromatograms indicates almost 60% of peak suppression at retention time t_r = 5.7 min attributed to the Sudan III dye (Fig. 4).

In this context, we analyzed several aromatic amines using HPLC/DAD, in order to identify the products formed after the biotransformation of Sudan II by S9. The list of aromatic amines, their IARC classification and respective retention time obtained from chromatograms are shown in Table 1. Fig. 5 exhibits the chromatogram obtained after the reaction with S9 (curve b), where we can observe three well-defined peaks at retention time of t_r = 6.7 min (I), t_r = 8.5 min (II) and t_r = 11.2 min (III) identified as 4,4′-oxydianiline; 4,4′-diaminodiphenylmethane and 2,6-dimethylaniline, respectively. The respective chromatogram obtained before S9 incubation shows no peaks (Fig. 5, curve a).

Analytical curves were constructed for each aromatic amine under best experimental conditions by HPLC/DAD detection based on peak area (Y) *vs* concentration (X). A linear relationship was ob-

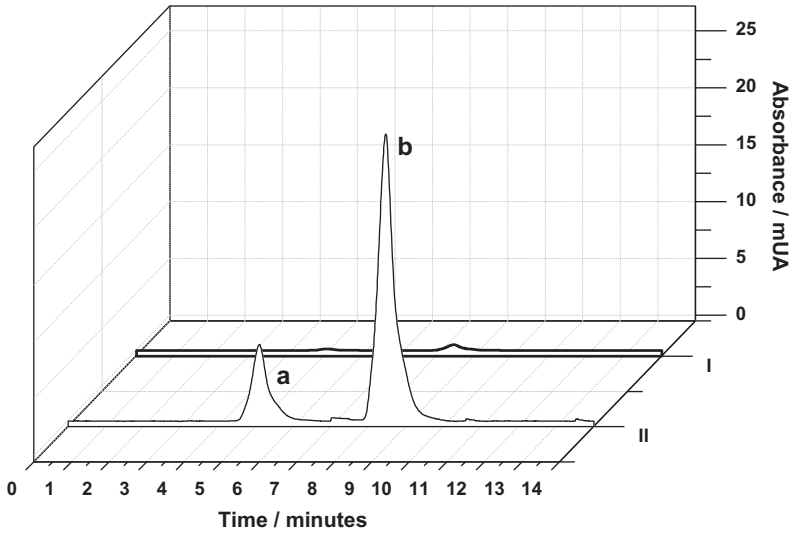


Fig. 8. Chromatogram of the Sudan III (I) and of the aromatic amines (II) obtained after ironporphyrin oxidation. Mobile phase: MeOH/buffer phosphate 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, e column C18, λ = 280 nm; (a) 4-aminobiphenyl, and (b) o-anisidine.

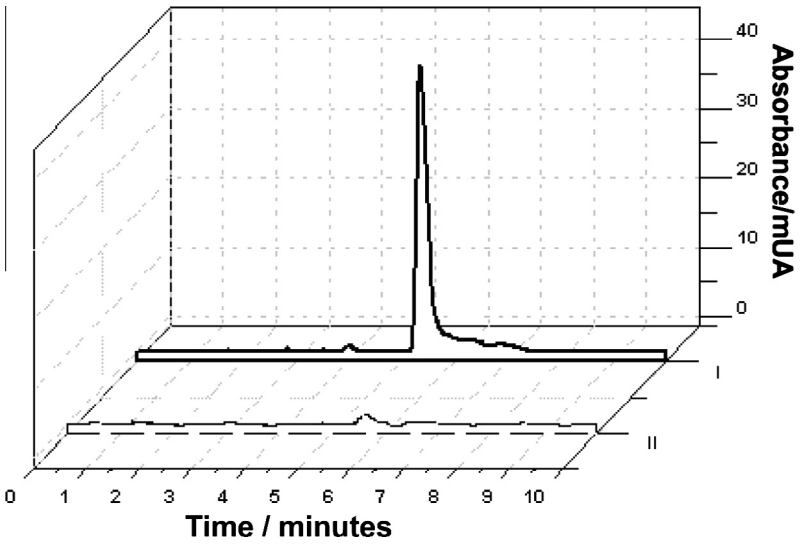


Fig. 10. Chromatogram obtained for 20 μL of 1.0×10^{-4} mol L^{-1} Sudan III dye before (I) and after (II) of electrooxidation for 2 h, applied E = +1.5 V. Mobile phase: ACN/MeOH 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, column = C18, λ = 500 nm.

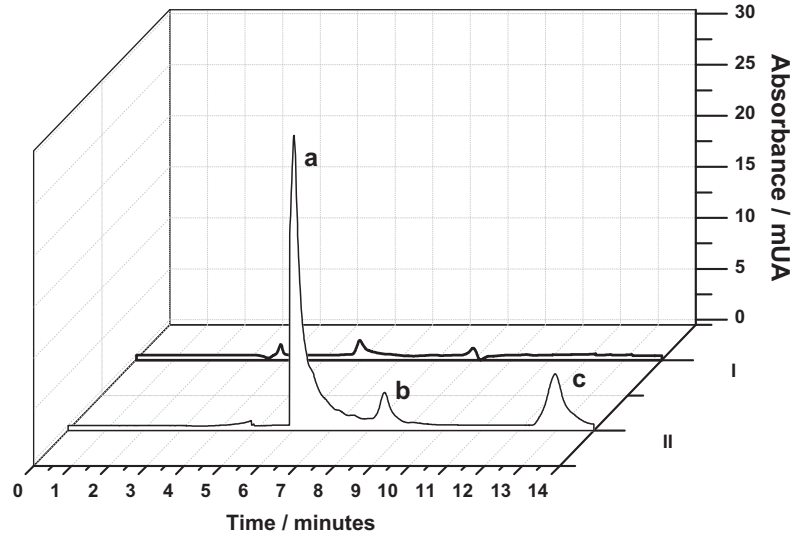


Fig. 11. Chromatogram of the Sudan III (I) and of the aromatic amines (II) obtained after electrooxidation. Mobile phase: MeOH/buffer phosphate 50:50 (v/v), T = 40 °C, flow rate = 1.00 mL min^{-1}, e column C18, λ = 280 nm; (I) before (II) after electrooxidation. (a) aniline; (b) o-anisidine and (c) 2-methoxy-5-methylaniline.

served from 5.0×10^{-7} mol L^{-1} to 2.5×10^{-4} mol L^{-1}, following the equation: 4,4′-oxydianiline ($Y = -2550.23 + 1.94 \times 10^{10}$ X, N = 8, r = 0.997, limit detection of 1.18×10^{-7} mol L^{-1}); 4,4-diaminodiphenylmethane ($Y = 813.6 + 1.60 \times 10^{10}$ X, N = 6, r = 0.999, limit detection of 7.54×10^{-8} mol L^{-1}) and 2,6-dimethylaniline ($Y = 3574.6 + 1.31 \times 10^{10}$ X, N = 6, r = 0.999, limit detection of 5.02×10^{-8} mol L^{-1}). So, the oxidation of 1×10^{-4} mol L^{-1} of Sudan III leads to the formation of: 1.07×10^{-5} mol L^{-1} of 4,4′-oxydianiline, 9.7×10^{-6} mol L^{-1} of 4,4′-diaminodiphenylmethane and 4.65×10^{-5} mol L^{-1} of 2,6-dimethylaniline. The detection of 4,4′-oxydianiline, 4,4′-diaminodiphenylmethane and 2,6-dimethylaniline was confirmed by LC–ESI–MS/MS analysis, which chromatogram is shown in Fig. 6. It the peaks are similar to the standard that present a well-defined peak at retention times (t_r) at 2.23 min (m/z = 201.1), 2.72 min (m/z = 217.2) and 7.77 min (m/z = 122.1), respectively. The transitions in LC–MS–MS are shown in Table 2.

These three aromatic amines are classified as group 2B by IARC as shown in Table 1, which means that they are possibly carcinogenic to humans, based on studies with experimental animals (IARC, 2012). However, they are known not to be mutagenic in *Salmonella* assay, corroborating with the weak positive response that we observed using strain TA1535 in the presence of exogenous metabolic activation.

This behavior indicates that Sudan III could be oxidized by typical enzymes of CYP-450, promoting the cleavage of the azo bonds leading to the formation of toxic aromatic amines. Thus, the generating of noxious aromatic amines may be hazardous to humans considering that this *in vitro* model simulates a reaction that could occur *in vivo* after the ingestion of food or water containing this dye.

3.3. Sudan III oxidation by ironporphyrin

We chosen the porphyrin FeTMPyP (5, 10, 15, 20-tetrakis-(4-N-methylpyridyl)porphyrin iron III chloride) to catalyze the Sudan III dye oxidation in the tert-butylhydroperoxide assay because it mimics the reaction of cytochrome P-450 present in mammalian li-

ver cell (Barros et al., 2008; Liu et al., 2002). Fig. 7 shows the degradation of the Sudan III (1.0×10^{-4} mol L^{-1}) (curve I) after 4 h of reaction with FeTMPyP/tert-butylhydroperoxide (curve II). We observed total suppression of the characteristic absorbance bands at 500 nm and, additionally, we also observed an extra peak at around 280 nm, clearly indicating the generation of other sub products. The HPLC/DAD data confirms these results, when there is 100% of peaks disappearance after 4 h of reaction.

In order to investigate the products generated from oxidation of Sudan III dye by tert-butylhydroperoxide catalyzed by FeTMPyP, the reaction supernatants were analyzed by HPLC/DAD using the experimental conditions optimized for aromatic amines detection, as stated before. The chromatograms (Fig. 8, curve II) reveal the occurrence of two aromatic amines with retention times at 4.4 and 8.0 min, which corresponds to 4-aminobiphenyl (a) and 0-anisidine (b). This identification was confirmed by comparing both the retention time and UV–Vis spectral data with standard solutions. The quantity formed was evaluated by calibration curve extrapolation, which shows that the oxidation of Sudan III at 1.0×10^{-4} mol L^{-1} forms 3.8×10^{-6} mol L^{-1} of 4-aminophenyl and 3.2×10^{-5} mol L^{-1} of o-anisidine.

LC–ESI–MS/MS analyze were also conducted aiming to confirm the diagnostic of the main products generated for Sudan III solution exposure to ironporphyrin. Analysis are based on retention times (t_r), molecular mass (MM) and fragments obtained (Table 2). Fig. 6 shows the chromatograms obtained by ESI (+) MS analysis. It can be seen the occurrence of o-anisidine, peak at $t_r = 3.67$ min identified by $m/z = 124.0$ and 4-aminophenyl at 14.0 min ($m/z = 170.1$). The spectrum presents fragments for both aromatic amines, which are transitions are shown in Table 2, confirming the chemical structure of the aromatic amines detected previously.

Our findings are of great concern because 4-aminobiphenyl is carcinogenic to humans (IARC, 2012) while o-anisidine is possible human carcinogen. Considering that FeTMPyP has high cytochrome P-450-like activity (Liu et al., 2002), the generation of these toxic compounds are expected after oral exposure to Sudan III.

3.4. Sudan III oxidation by electrochemistry

The UV–Vis spectra shown in Fig. 9 indicate that Sudan III at 1×10^{-4} mol L^{-1} (curve I) was completely discoloration after 2 h of electrochemical oxidation (curve II) at potential of +1.5 V on glassy carbon electrode. In addition, we observe the generation of sub-products detected at 310 nm. The chromatograms obtained for the same solution show a peak at retention time of 5.7 min (Fig. 10, line I) attributed to Sudan III that exhibited 95% of suppression after 2 h of electrolysis at +1.5 V (Fig. 10, line II). The chromatograms obtained for the products generated after electrochemical oxidation is shown in Fig. 11. We detected three aromatic amines that were quantified as followed: (a) aniline ($t_r = 5.7$ min, 0.31×10^{-4} mol L^{-1}); (b) o-anisidine ($t_r = 8.0$ min, 7.6×10^{-7} mol L^{-1}) and (c) 2-methoxy-5-methylaniline ($t_r = 12.2$ - min, 5.9×10^{-5} mol L^{-1}). Between then, only aniline has no evidence to be a carcinogen. The formation of these aromatic amines was also confirmed by LC–ESI–MS/MS, comparing with the standard chromatogram shown in Table 2. It was seen in the chromatograms the occurrence of aniline at $t_r = 3.67$ min identified by $m/z = 124.0$, o-anidisine, peak at $t_r = 3.67$ min identified by $m/z = 124.0$, and 4-aminophenyl at 14.0 min ($m/z = 170.1$).

Both levels and the activity of cytochrome P450 isoforms vary greatly due to environmental and genetic factors, such as genetic polymorphism and presence of enzyme inducers (Parkinson and Ogilvie, 2008). In this paper, we catalyzes the oxidation of Sudan, by using different reactions in order to predict the generation of different by-products under endogenous conditions. Therefore, Sudan III is a drug metabolizing enzyme inducer (Fujita et al., 1984), for this reason, it is important to simulate more drastic oxidation conditions such as electrochemical and reaction with system S9, obtained from rat liver previously exposed to inducer Aroclor (Maron and Ames, 1983).

Although, one of the main hazard criteria for a dye is the ability to reductively cleave the azo bond, our findings clearly indicate that hazardous aromatic amines, including a recognized human carcinogen, can be formed after oxidation reactions that can be catalyzed by cytochrome P-450. Therefore, our results corroborate with the recommendation of The European Union Countries that prohibits the importation and marketing of products that could produce carcinogenic amines at levels of ppm (European Community, Commission Decision, 2002).

4. Conclusion

We concluded that the oxidative metabolism of azo dyes plays an important role on the toxicity of these compounds, mainly considering the generation of aromatic amines possibly carcinogenic for humans. Therefore, the use of Sudan III as food dye should be carefully controlled, since hepatic cells can promote the same oxidative reactions.

Conflict of Interest

The authors report no declarations of interest.

Acknowledgments

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