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Electrochemical Reduction as a Powerful Tool to Highlight the Possible Formation of By-Products More Toxic Than Sudan III Dye

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The present work describes the electrochemical reduction of the azo dye Sudan III in methanol/0.01 mol l^{-1} Bu_4NBF_4 at applied potential of -1.2V, which promotes 98% discoloration of the commercial sample. The reduction products were analyzed by high performance liquid chromatography, after optimized conditions for 20 aromatic amines with carcinogenic potentiality. The harmful compounds such as: aniline, benzidine, o-toluidine, 2,6-dimethylaniline, 4,4'-oxydianiline, 4,4'-metileno-bis-2-methylaniline and 4-aminobiphenyl are formed after azo bond cleavage. The electrochemical reduction is compared with chemical reduction by using sodium thiosulfate. Our findings illustrates that commercial Sudan III under reductive condition can forms a number of products, which some are known active genotoxins. The technique could be used to mimic important redox reactions in human metabolism or environment, highlighting the possible formation of by-products more toxic than the original dyes.

Keywords: Aromatic amines, sub products, electrochemical reduction, HPLC, Sudan dyes

1. INTRODUCTION

The toxicity and carcinogenicity of certain azo dyes in mammalian systems may result either from their biotransformation via oxidation, hydrolysis, conjugation or reduction catalyzed by specific enzymatic reaction or from interactions of the original molecules with cytosolic receptors [1-4]. Although, many studies involving oxidation, including those catalyzed by cytochrome P-450 are

described in the literature [5-7] the studies focused on reduction and amine formation has attracted less attention. Nevertheless, it is widely known [4,8] that the main process involving azo reduction is the generation of amines that are either more toxic or less toxic than the parent molecule and therefore may either decrease or increase any toxic or carcinogenic effect of these dyes**.**

Pielesz [9] have studied the reduction of several disperse dyes by action of sodium hydrosulfite as a reducing agent in an alkaline medium and describes high concentrations of benzidine, p-phenylenediamine and aniline as main products**.** Garrigós [10] indicates that aromatic amines are formed after reduction of Disperse dyes Vermelho1 and 13, Disperse Orange 25, Solvent Black 3, Solvent Orange 7, Solvent Red 24, Solvent Yellow 2, Solvent Yellow 3 and Solvent Yellow 14 using sodium hydrosulfite as a reducing agent**.** The occurrence of carcinogenic amines derived from cleavage of azo bond in dye as pollutant is also described [11]. Among them, there is great concerning in the community about several amines condemned in a list of 22 aromatic amines classified as carcinogenic by International Agency for Research on Cancer (IARC) as possible product formed after biotransformation of azo dyes. The occurrence of benzidine, 3,3- dimethylbenzidine, o-toluidine and 3,3-dichlorobenzidine in the wastewater of a textile industry by HPLC coupled to electrochemical detector is also described in the literature [12].

In general, the most of dyes have functional groups capable of undergoing oxidation or direct electrochemical reduction of the substrate [13]. Thus, electrochemical techniques could be a simple way to be used as mimetic process to follow possible subproduct generated after reduction of azo dyes. The use of direct electrolytic processes of azo dye could be a valuable tool for diagnosis of system degradation of the molecule under controlled potential. The technique could be used to mimic important redox reactions in human metabolism or environment, highlighting the possible formation of by-products more toxic than the original dyes [11,14] or could serve as models for the biologically pathway [15].

Sudan dyes, 1-amino-2-naphthol-based azo dyes, are illegally used as food additives, particularly in chilly containing foods (such as chilly, curry and palm oil containing foodstuffs, frozen meat products and spice mix), because of their intense red/orange color and low price. However, great attention has been dispensed to this class of dyes, since there is some evidence that they have genotoxic effects [16-20]. Among them, Sudan III have been classified as category 3 carcinogens by International Agency for Research on Cancer and are therefore banned from the use in food in the EU (Official Journal of the European Union 2005)[21]. They generate metabolites that are converted to several active mutagens and carcinogens in humans. Despite of a few studies related to the metabolism of Sudan dye in rats, rabbits and human intestinal microflora [22], no reports regarding the probable aromatic amines generation during reduction of Sudan dyes have been published. In addition, attempts to identification of potential carcinogenic amines derived from azo dyes are limited [9,10]. Some authors have examined thirty-five prevalent species of human intestinal bacteria able to degrade Sudan dyes. Among these tested bacterial strains, only two strains were not able to reduce Sudan dyes [23].

Thus the aim of the present study was to investigate the reduction products obtained from the azo dye Sudan III, using electrochemical techniques. It also proposed to evaluate the carcinogenic amines generation as subproduct by using methodologies of HPLC/DAD and UV-Vis

spectrophotometry validated for 20 carcinogenic amines. Our particular interest was to focus on evidence of harmful byproducts generation for azo dyes under reduction reactions.

2. EXPERIMENTAL

2.1. 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 were obtained transferring 10 mL of Methanol/0.01 mol l^{-1} tetrabuthylammonium 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 minutes 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. Color of the solution was measured using UV-VIS spectra. HPLC/DAD analysis was carried in a Shimadzu SCL-10AVP apparatus coupled to a diode array detector using a pre step of filtration of the sample in a system MILLEX Millipore (0.45 μm) using the best chromatographic conditions previously optimized.

2.4. Chemical reduction of Sudan III.

Solution pattern of 0.10 mol l^{-1} of sodium thiosulfate (Sigma-Aldrich) used as reducing agent was prepared in deionized water 2.00 mL of this standard solution was added to 50.0 mL of Sudan III (Sigma-Aldrich, 95%) and submitted to 2h of stirring and temperature of 40ºC in a covered system. After cooling, the solution was filtered in filter MILLEX Millipore (0.45 μm) and submitted immediately to analysis by HPLC/DAD system.

2.5. Chromatographic analysis.

The HPLC analysis of Sudan dyes and aromatic amines were performed in a reversed-phase column Shimadzu CLC-ODS (C18) (25.0 cm x 4.60 mm x 5.00 μm, 100 A) connected to a guard column Shimadzu CLC-ODS (C18) (1.00 cm x 4.60 mm x 5.00 μm, 100 A). The best experimental conditions for Sudan dyes under optimized isocratic mode were: a mobile-phase Methanol/Acetonitrile 50:50 v/v, a flow rate of 1.00 mL min^{-1} and a column temperature of 40°C. The analysis time was 10 minutes, and all the analyses were carried out in triplicate.

The optimized conditions for HPLC/DAD identification and quantification 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'-diamino-bis-phenylmetane (Fluka, 97%), 3,3'-dimethylbenzidine (Sigma-Aldrich, 97%), 2-metoxi-5-metilaniline (Aldrich, 99%), 4-chloroaniline (Fluka, 99%), 2,6-dimethylaniline (Fluka, 98%), 2-chloro-4-nitroaniline (Fluka, 98%) were a mobile-phase methanol/phosphate buffer $5.00x10^{-5}$ mol l^{-1} (pH = 6.9) + 20.0 mM of triethylamine at composition of 50:50 v/v, a flow rate of 1.0mL min^{-1} and a column temperature of 40°C (condition 1). For other amines: 4,4'-metilene-bis-(2-methylaniline) (Fluka, 98,5%), 4-chloro-o-toluidine (Fluka, 98%), 4-aminobyphenile (Sigma, 90%), 3,3'-dichlorobenzidine (Supelco, 99%) were better separated using similar experimental conditions but using methanol/phosphate buffer $5.00x10^{-5}$ 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 the aromatics amines are Sigma-Aldrich, analytical grade. All these methodologies were carried out based on chromatographic parameters such as retention time (t_r), retention constant factor (k), selectivity (α) and resolution between peaks (r) and theoretical plate number (N) [24]. 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 detection under the hydrodynamic conditions was recorded and used as a parameter to identify and confirm the investigated species; and then afterwards to compare this with the one recorded for the pure samples of each component of the CVS sample.

3. RESULTS AND DISCUSSION

3.1. Electrochemical reduction of Sudan III.

The electrochemical reduction of 50 ppm Sudan III was investigated in 0.10 mol l^{-1} Bu_4NBF_4/Methanol at Pt electrode. The typical cyclic voltammogram is shown in Fig. 1. The azo group in the dye molecule is reduced in only one step at E_{pc}= -0.75V and present no peak on the reverse scan. The effect of scan rate (ν) on the cyclic voltammetric behavior was investigated from 10 mV s^{-1} to 500 mV s^{-1}. The cathodic peak current increased linearly with $\nu^{1/2}$ ($(I_{pc}$ (mA) = 1.45+ 6.25 ν $^{1/2}$ (mV s^{-1}) ^{½}, R= 0.999, N=9), indicating that the process is diffusion controlled. The peak potential shifts more negative with an increase in the scan rate, and the current function ($I_{pc}/\nu^{1/2}$) decreases with scan rate increases suggesting that the electrodic process is complicated by subsequent chemical reaction [25].

In order to check the possible cleavage of azo group in the dyes, controlled potential electrolysis was carried out using 50 ppm Sudan III in 0.10 mol L^{-1} Bu_4NBF_4/Methanol. The electrolysis was performed at -1.20 V using Pt gauze as cathode. The current *vs* time was followed

during 2 hours of electrolysis and the number of electrons (n) consumed in the total oxidation was around 3.87 electrons (media of 3 measurements). At the end of electrolysis the products at the cathode were examined by UV-Vis spectrophotometry and HPLC/DAD.

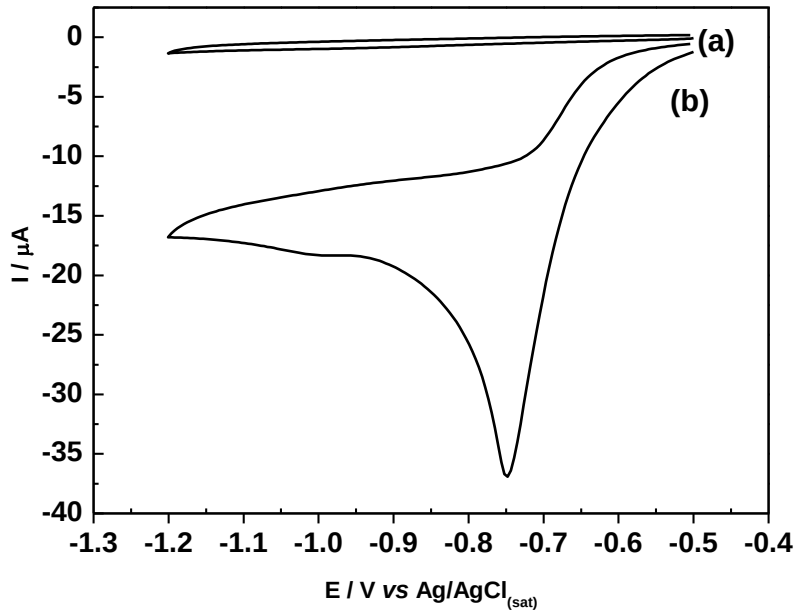

Figure 1. Cyclic voltammograms for electrochemical reduction of 50 ppm Sudan III in 0.1 mol l^{-1} Bu_4NBF_4/Methanol (b) at Pt electrode. Scan rate = 100 mV s^{-1}. (a) Supporting electrolyte.

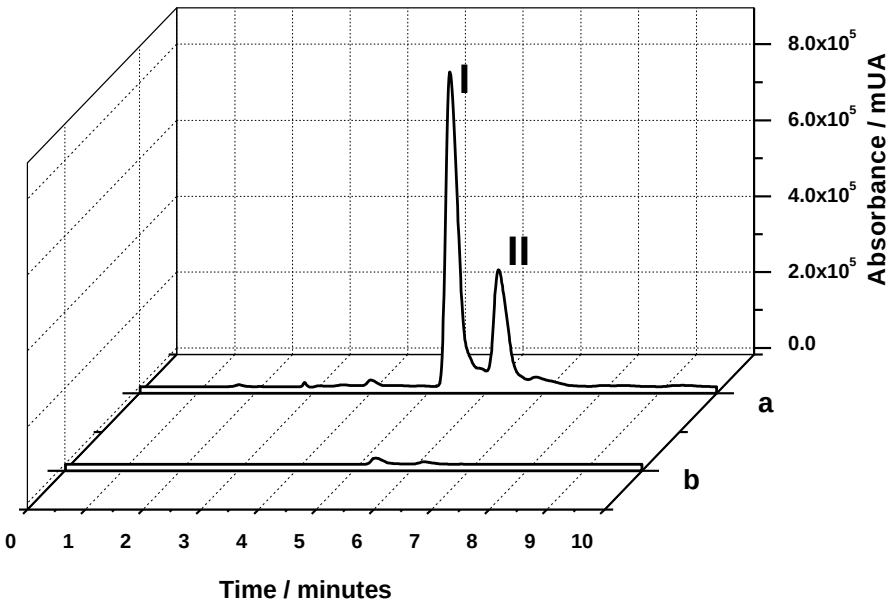

Figure 2. Chromatograms obtained for commercial sample of 50 ppm Sudan III in 0.1 mol l^{-1} Bu_4NBF_4/Methanol, before (Curve a) and after 60 min of electrolysis performed at -1.2 V using Pt gauze as cathode (Curve b). Column C18, isocratic mode, mobile-phase Methanol/Acetonitrile 50:50 v/v, flow rate of 1.00 mL min^{-1} and T= 40°C.

The analysis of products generated after electrolysis were also examined by HPLC equipped with a diode array detector. The chromatograms obtained before and after electrochemical reduction are shown in Fig. 2. The respective chromatogram obtained for commercial sample of Sudan III is shown in Curve a, Fig.2. The chromatogram is characterized by two intense peaks at a retention time of t_r = 5.70 min (A=7.0x10^5) (peak I) and t_r= 6.50min (A=2.0x10^5) (peak II). The first peak was attributed to Sudan III and the second one to Sudan II, presents as contaminant in the sample. Both peaks were identified by injection of individual standard sample of Sudan II and Sudan III in previous purified sample.

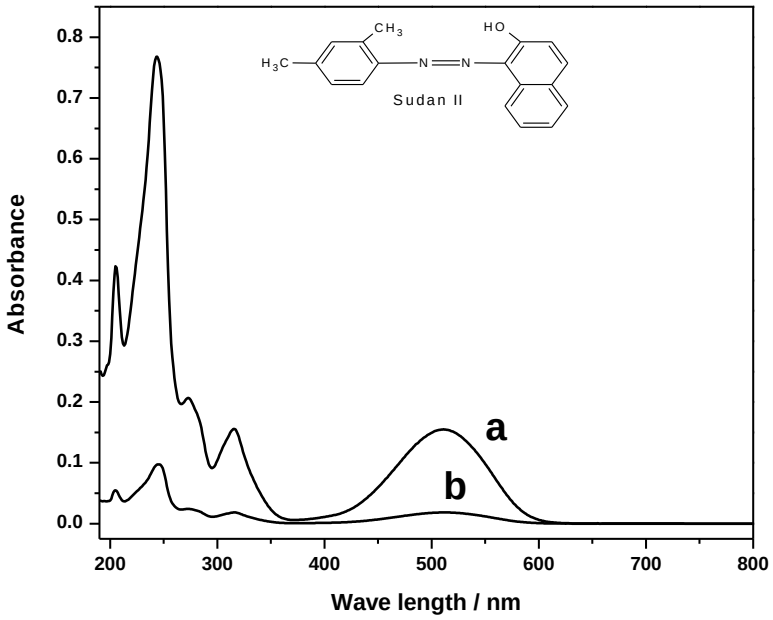

Figure 3. UV-Vis spectra obtained from hydrodynamic conditions of HPLC coupled to a diode array detector, using the retention time of each dye component shown in Figure 2.

The respective spectra of each dye even in the commercial sample was monitored by recording of the UV-Vis spectra in the hydrodynamic conditions of HPLC coupled to a diode array detector, using the retention time of each dye component and are the curves are shown in Fig. 3. Each spectrum exhibited a set of bands arising from a substituted azobenzene system with maximum absorption at 510 (intense peak), 340 and 240 nm for Sudan III dye; and at 510 (intense peak), 320, 270 and 250 nm for Sudan II dye [23]. Quantitative analysis of each component in the commercial sample was carried out using HPLC chromatographic separations, and the results indicate that the mass contribution of the dyes is 77.4% (m/m) for Sudan III dye and 22.6% (m/m) for Sudan II dye in the commercial sample. An analytical curve was obtained for Sudan II and III, using the best experimental conditions by HPLC with DAD detection based on peak area *vs* concentration, which showed good linearity. The following equations were obtained: Sudan III: area = 18754+ 1.120 x 10^{12} [dyes] (ppm), n = 4, R = 0.999 and Sudan II: area = 4778 + 2.730x10^{12} [dyes] (ppm), n = 5, R= 0.999. A linear relationship was obtained

in all the concentration investigated from 0.024 to 38 ppm. The limit of detection (LD) for each compound was of 0.0059 and 0.0049 ppm, respectively from Sudan III and Sudan II.0.00509 ppm.

After two hours of electrolysis (Curve b, Fig. 2) both peak heights decreases 96 % due electrochemical reduction of Sudan III and Sudan II. The UV-Vis spectra obtained from chromatograms are also shown in Curve b of Fig. 3. The absorption spectra recorded for Sudan III presents a reduction 96 % on bands attributed to azo group (around 510 nm), revealing the most part of coloration was removed from the solution. However, small bands around 330 and 240 nm are still remained in the absorption spectra, indicating that intermediate products are being formed. The relative decreasing in the peak attributed to Sudan II (peak II) was also verified. It is possible to see that after reduction of 95 % of chromophore peak at 510 nm. These results clearly indicate that the reduction of azo group leads to the discoloration of the dye solution, as verified previously in other azo dyes [26], but other products are being formed.

3.1. Otimization of chromatographic method for selected aromatic amines

In order to identify the occurrence of aromatic amines as byproducts generated during electrochemical reduction of commercial Sudan III, an HPLC-DAD methodology was optimized using separation and identification of 20 selected aromatic amines considered harmful to the human being and environment by IARC.

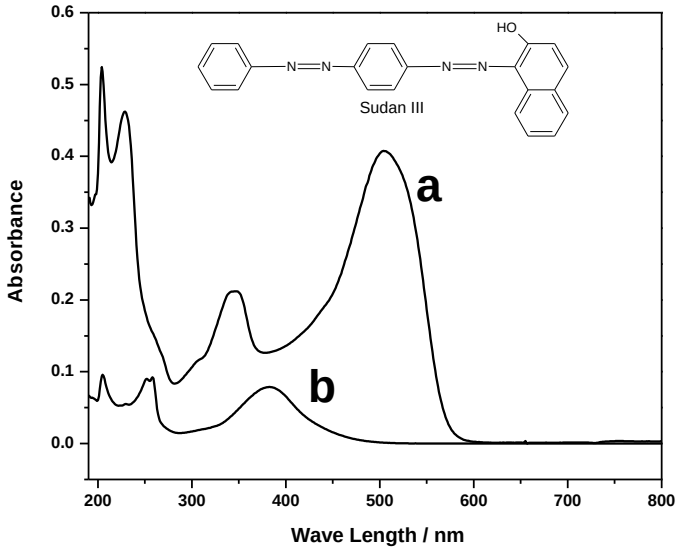

Figure 4. Chromatograms obtained for 20.0 μL of mixture of 20 ppm of the selected aromatic amines: 1) 2-naphtilamine (t_r= 3.0 min), 2) 2,4-diaminotoluidine (t_r= 3.7 min), 3) benzidine (t_r= 4.7 min), 4) 4,4'-methilenebis(2-chloroaniline) (t_r= 4.9 min), 5) aniline (t_r= 5.2 min), 6) o-toluidine (t_r= 6.5 min), 7) 4,4'-oxidianiline (t_r= 6.7 min), 8) o-dianisidine (t_r= 7.4 min), 9) o-anisidine (t_r= 8.0 min), 10) 4,4'-diaminodiphenilmetane (t_r= 8.5 min), 11) 3,3'-dimethylbenzidine(t_r= 9.0 min), 12) 2-methoxi-5-methilaniline (t_r= 9.3 min), 13) 4-chloroaniline (t_r= 10.8 min), 14) 2,6-dimethylaniline(t_r= 11.2 min), 15) 2-chloro-4-nitroaniline (t_r= 11.5 min). Column C18, isocratic mode, mobile-phase methanol/phosphate buffer $5.00x10^{-5}$ mol l^{-1} (pH = 6.9) + 20 mM of triethylamine at composition of 50:50 v/v, flow rate of 1.00 mL min^{-1} and T= 40°C.

The performance of the DAD detector to separate the 20 aromatic amines was conducted using standard solution containing mixture of all compounds using modified method of literature (10). Best chromatographic separation was obtained using two different experimental conditions assigned condition 1 and 2, and described in the experimental part.

Table 1. Analytical parameters obtained from analytical curves recorded for individual aromatic amines analyzed by HPLC/DAD using mobile phase: MeOH/Phosphate buffer $5.00x10^{-5}$ mol l^{-1} (pH=6.9) 50:50 (v/v) and 20mM of triethylamine, T= 40°C. flow rate = 1.00 mL min^{-1}. Detection at λ=230 nm.

Amines	Tr	A[#]	B[# #]	Linear range**	N	R	SD	L.D.*	L.Q.*
2-naphthylamine	3.0	- 12290.42	$5.03x10^5$	0.230 to 237	8	0.998	1.98	0.003	0.009
2,4-diaminotoluidine	3.7	3898.75	$8.18x10^5$	0.23 to 927	7	0.998	3.12	0.041	0.12
Benzidine	4.9	- 42.00	$4.20x10^5$	0.12 to 1900	8	0.991	8.08	0.003	0.010
4,4'-metilenebis (2-chloroaniline)	5.0	5990.31	$1.42x10^5$	0.24 to 144	6	0.999	4.99	0.006	0.017
Aniline	5.2	82195.13	$1.37x10^5$	0.27 to 69.2	8	0.999	1.37	0.036	0.108
o-toluidine	6.5	3406.41	$1.58x10^5$	0.46 to 1900	7	0.990	2.70	0.032	0.099
4,4'-oxidianiline	6.7	- 2550.23	$1.94x10^5$	0.23 to 950	8	0.997	7.32	0.045	0.14
o-dianisidine	7.4	- 574.28	$1.54x10^5$	0.23 to 1900	7	0.999	7.50	0.004	0.012
o-anisidine	8.0	7913.16	$2.24x10^5$	0.93 to 1900	7	0.999	7.50	0.054	0.16
4,4'-diaminobiphenilmetame	8.5	- 813.57	$1.60x10^5$	0.23 to 950	6	0.999	3.95	0.027	0.083
3,3'-dimethylbenzidine	9.0	- 8173.30	$9.98x10^4$	3.8 to 1700	6	0.998	10.0	0.088	0.27
2-metoxi-5-methylaniline	9.3	- 65686.21	$7.70x10^5$	3.73 to 1700	6	0.998	12.5	0.34	1.02
4-chloroaniline	10.8	- 80955.37	$1.05x10^5$	15.1 to 1806	5	0.998	12.1	0.038	0.12
2,6-dimethylaniline	11.2	3574.61	$1.31x10^5$	0.92 to 1900	6	0.999	9.67	0.018	0.055
2-chloro-4-nitroaniline	11.5	76292.25	$3.86x10^5$	1.08 to 691.6	8	0.999	0.65	0.25	0.77

Tr: retention time (minutes); [#] linear coefficient; [# #] angular coefficient; ** linearity (ppm); N number of measurements; R correlation coefficient; SD: standard deviation; * LD: detection limit (ppm); ** LQ: quantification limit (ppm).

Figure 4 exhibits the chromatogram obtained for separation of 20 μL of mixture of 50 ppm of the selected aromatic amines: 2-naphthylamine (t_r= 3.0min), 2,4-diaminotoluidine (t_r= 3.7min), benzidine (t_r= 4.7min), 4,4'-methylenebis(2-chloroaniline) (t_r= 4.9min), aniline (t_r= 5.2min), o-toluidine (t_r= 6.5min), 4,4'-oxidianiline (t_r= 6.7min), o-dianisidine (t_r= 7.4min) , o-anisidine (t_r= 8.0min), 4,4'-diaminodiphenilmetane (t_r= 8.5min), 3,3'-dimethylbenzidine (t_r= 9.0min), 2-methoxi-5-methilaniline (t_r= 9.3min), 4-chloroaniline (t_r= 10.8min), 2,6-dimethylaniline (t_r= 11.2min) and 2-chloro-4-nitroaniline (t_r= 11.5min). Peak identification was based on the retention time, which was confirmed by spiking authentic standard solutions of each amine sample as assigned in the experimental section. Characteristic UV-Vis spectra obtained by the diode array detection under the hydrodynamic conditions was recorded and used as a parameter to identify and confirm the investigated species.

The parameters corresponding to the analytical curve constructed for all the evaluated amines are shown in Table 1. They were obtained using the best experimental conditions by HPLC with DAD

detection based on peak area *vs* concentration in mol L^{-1} of amines (Table 1). A linear relationship was obtained from 0.038 to 380 ppm amines concentrations. The limit of detection (LD) and others parameters of the analytical curve for each compounds are also shown in Table 2.

Table 2. Analytical parameters obtained from analytical curves recorded for individual aromatic amines analyzed by HPLC-DAD using mobile phase: MeOH/Phosphate buffer $5.00x10^{-5}$ mol l^{-1} (pH=6.9) 80:20 (v/v) and 20.0 mM of trietilamine, T= 40°C. Flow rate = 1.00 mL min^{-1}. Detection at λ=230nm.

Amines	Tr	A#	B##	Linear range**	N	R	SD	LD*	LQ**
4,4'-metileno-bis-(2-metilanilina)	3.8	-373.55	$2.66 x10^5$	0.23 to 953	5	0.999	9.40	0.003	0.009
4-chloro-o-toluidine	4.1	70751.41	$4.14x10^5$	3.71 to 1900	6	0.997	9.21	0.11	0.34
4-aminobiphenila	4.4	30152.18	$5.71x10^5$	3.71 to 1900	6	0.999	1.35	0.47	1.43
3,3'-dichlorobenzidine	4.7	174317.37	$1.06x10^5$	19.2 to 308	5	0.998	8.20	0.69	2.10

Tr: retention time (minutes); # linear coefficient; ## angular coefficient; ** linearity (ppm); N number of measurements; R correlation coefficient; SD: standard deviation; * LD: detection limit (ppm); ** LQ: quantification limit (ppm).

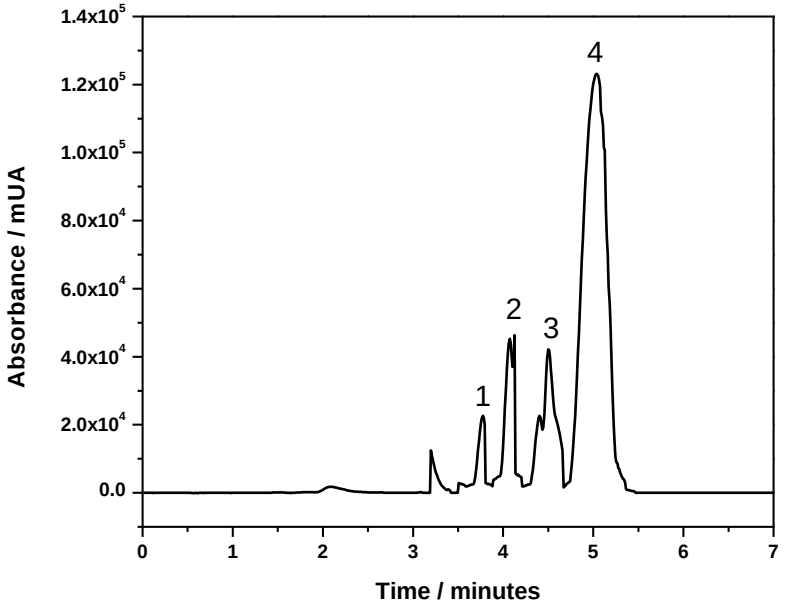

Figure 5. Chromatogramas obtained for 20.0 μL of mixture of 50 ppm of the selected aromatic amines: 1) 4,4'-methylene-bis(2-methylaniline) (t_r= 3.8 min), 2) 4-chloro-2-methylaniline (t_r= 4.1 min), 3) 4- aminobiphenile (t_r= 4.4 min), 4) 3,3'-dichlorobenzidine (t_r= 4.7 min). Column C18, isocratic mode, methanol + phosphate buffer pH 6.9 ($5x10^{-5}$ mol l^{-1}) at ratio of 80:20 v/v, containing 20 mM of triethylamine, flow rate of 1.0 mL min^{-1} and T= 40°C.

Figure 5 illustrate the separation of eight other aromatic amines that required another chromatographic separation methodology assigned as condition 2: 4,4'-methylene-bis(2-methylaniline (t_r= 3.8min), 4-chloro-2-methylaniline (t_r= 4.1min), 4- aminobyphenil (t_r= 4.4min), 3,3'-

dichlorobenzidine (t_r= 4.7min). The respective parameters obtained for the analytical curves for each amine are shown in Table 2.

In order to identify aromatic amines as potential byproduct, a water sample was spiked to 0.23 ppm of the 20 selected substances, with the aim of simulating the sub-product concentration levels that could be detected by the treated sample using the proposed method. Recoveries from 94 % and 110 % was obtained from the water samples (n = 3) using the proposed method. This is evidence of the accuracy of the proposed procedure. The statistical calculations for the assay results showed suitable precision of the HPLC/DAD method. According to the t-test, there were no significant differences between the calculated and added concentrations at the 95% confidence level, being within an acceptable range of error, indicating that the proposed method could be used for detection and determinations aromatic amine as side product during electrolyzed samples of the dye.

3.2. Byproducts generated from electrochemical reduction of Sudan III.

The fading of coloring 38 ppm of Sudan III after 2 hours of treatment was observed by HPLC/DAD, which was discussed previously. The main electrochemical processes take place during the electrolysis of azo dyes, when direct reduction of azo group occurs on the surface of electrodes, but is also accepted that indirect reactions play important role, which occur in the bulk solution if there is a sufficient amount of intermediates produced by electrolysis [27]. As diagnosed previously the commercial Sudan III dye used in the present work shows the occurrence of Sudan II as impurities with a higher retention time and the electrolysis product did not separate the effect of this impurity in the final sample.

Table 3. Aromatic amines generated after 2 h of electrochemical reduction of 38 ppm of commercial Sudan III dye at applied potential of -1.2 V analyzed by HPLC-DAD using previous optimized condition (Table 1 and 2).

Byproducts	(ppm) T= 0 min	(ppm) T= 120 min
Benzidine	0	0.11
o-toluidine	0	0.92
4.4'-oxydianiline	0	0.22
2.6-dimetilaniline	0	0.83
4.4'-methyleneo-bis-2-methylaniline	0	0.44
4-biphenilamine	0	0.21

Table 3 shows the byproducts identified by HPLC/DAD after 2 h of electrochemical reduction of 38 ppm Sudan III in methanol/0.01 mol l^{-1} Bu_4NBF_4 at -1.2V *vs* Ag/AgCl. As shown Figure 2 and 3 there is almost total suppression of the chromatographic peaks and visible bands after 120 min, respectively. The electrolyzed sample indicates the occurrence 6 aromatic amines identified as: benzidine (tr= 4.7 min, 11 ppm), o-toluidine (tr= 6.5 min, 9.2 ppm), 4,4'-oxidianiline (tr= 7.0 min, 2.2

ppm), 2,6 dimethylaniniline (tr=11.2 min, 7.4 ppm), 4,4'-methylene-bis-2-methylaniline (tr=3.8min, 0.44 ppm), 4-aminobiphenil (tr= 4.4 min, 0.21 ppm), o-toluidine (tr=6.5 min, 0.92 ppm) and aniline (tr= 5.2 min, 0.56 ppm). The peaks were identified by characteristic profile of the amines using the optimized condition, retention time and UV-Vis spectra relative to the standard substances. The quantity of generated products indicates values of harmful amines variation from 0.21 to 11 ppm in the reduced sample. Taking into consideration that limit values in water for this aromatic amines is less than 30 ppb or the dangerous power of these aromatic amines in the metabolism, some of the generated product are superior to the legislation, even for the initial concentration of the dye as small as 38 ppm.

In agreement with the results obtained it is possible to conclude that the electrochemical reduction of Sudan III, which commercial sample is contaminated with Sudan II dye in methanolic solution involving the cleavage of azo group. The reduction of the azobenzene group follows a four electron reaction diagnosed by coulometry, in agreement with literature [27]. Afterwards, there is substantial change in the chromophore group and therefore color removal. The main characteristic of the reduction mechanism is the cleavage of the nitrogen-nitrogen bond, which product is identified as aromatic amines as aniline, benzidine, o-toluidine, 4,4-oxydianiline, 2,6-dimethylaniline, 4-aminobiphenila and 4,4-methylene-bis-2-methylaniline and others by HPLC-DAD studies.

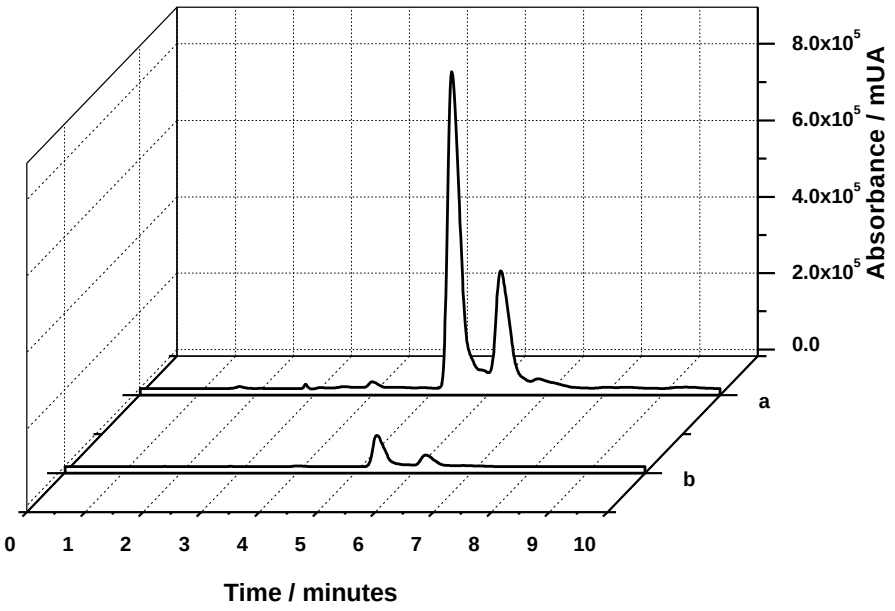

Figure 6. Chromatogramas obtained for 38 ppm Sudan III dye before (a) and after reduction by sodium thiosulfate, during 2 hours under stirring. Column C18, isocratic mode, mobile-phase Methanol/acetonitrile 50:50 v/v, flow rate of 1.00 mL min^{-1} and T= 40°C.

The chemical reduction of dye Sudan III with dithionite of sodium was also carried out with the aim to compare the byproducts. For this solutions 38 ppm of the Sudan III dye were treated with dithionite of sodium, during 2 hours of chemical reaction under stirring, as described in experimental part. The chromatograms obtained before and after chemical reaction are presented in the Figure 6.

There is a 98 % suppression of the peaks 1 and 2 attributed to Sudan III (tr= 5.7min) and Sudan II (tr= 6.5min), respectively. The respective spectra of UV-Vis obtained before and after chemical reduction agree with the reduction of the chromophore group. In addition, it is seen significant decreasing of bands around 250 nm, and the formation of extra peak at 400nm, evidencing the presence of byproducts in a similar way verified under electrochemistry reduction. But, the results obtained indicated the occurrence of only two main products: aniline (tr= 5.2min, 11.6 ppm) and o-toluidine (tr= 6.5min, 14.4 ppm). This behavior suggests that electrochemical reduction can forms radical species as intermediate, which can produce other chemicals than those originated from a punctual rupture of the azo reduction. But all situations indicated that harmful compounds are generated during reduction of Sudan III and could be a simple tool to diagnostic the harmful use of this kind of dye.

4. CONCLUSION

The azo group of Sudan III dye and Sudan II dye present as contaminant in a commercial sample can be reduced by both chemical and electrochemical methods, leading to a high discoloration. In both methods there is evidence of formation of aniline, benzidine and o-toluidine, 2,6-dimethylaniline, 4,4'-oxydianiline, 4,4'-metileno-bis-2-methylaniline and 4-aminobiphenila whose are considered hazardous due of its toxicity and carcinogenity. The results are similar to that obtained for reduction of Sudan III by *E. faecalis* intestinal bacteria (15). This initial investigation provides useful information as a model to determine risk assessment involving the consumption of this azo dye.

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