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1 **Mineralization of synthetic and industrial pharmaceutical effluent**  
2 **containing trimethoprim by combining electro-Fenton and activated sludge**  
3 **treatment**

4  
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26

## 1 **Abstract**

2 A combined process coupling an electro-Fenton and a biological degradation was investigated  
3 in order to mineralize synthetic and industrial pharmaceutical effluent containing  
4 trimethoprim, a bacteriostatic antibiotic. Electro-Fenton degradation of trimethoprim was  
5 optimized by means of a Doehlert experimental design, showing that 0.69 mM Fe<sup>2+</sup>, 466 mA  
6 and 30 min electrolysis time were optimal, leading to total trimethoprim removal, while  
7 mineralization remained limited, 12% for 30 min electrolysis times. The aromatic and  
8 aliphatic by-products were identified and a plausible degradation pathway was proposed.  
9 Biodegradability was improved, since the BOD<sub>5</sub>/COD ratio increased from 0.11 initially to  
10 0.32 and 0.52 after 30 and 60 min electrolysis times respectively, confirmed by activated  
11 sludge culture, 47 and 59% mineralization of the byproducts from electrolysis.

12 The relevance of the proposed combined process was then confirmed on an industrial  
13 pharmaceutical effluent. Its electrolysis under the above conditions showed an almost total  
14 removal of trimethoprim after 180 min of electrolysis, while TOC removal remained low, 14  
15 and 16% for 180 and 300 min reaction times, respectively. Overall removal yields of the  
16 industrial effluent during the combined process were therefore 80 and 89% for 180 and 300  
17 minutes of effluent pretreatment followed by 15 days activated sludge culture, respectively.

18

19 **Keywords:** Trimethoprim; Combined processes; Electro-Fenton; Activated sludge culture;  
20 Pharmaceutical effluents; Mineralization.

21

## 22 **1. Introduction**

23 In these last years, antibiotics were considered to be an emerging environmental problem due  
24 to their continuous entry and persistence in the aquatic ecosystem [1]. This pollution proceeds  
25 from human excretion after drug administration and passes through wastewater treatment

1 plants (WWTPs). Comparatively, veterinary antibiotics do not undergo WWTPs treatment  
 2 and may directly enter surface water. Animal manure, dispersed in the fields as fertilizer, can  
 3 contaminate soil and consequently surface and groundwater [2]. Moreover, waste effluents  
 4 from manufacture can also be considered as significant points of contamination [3].

5 Most conventional treatments applied in WWTPs were unsuccessful in the removal of  
 6 antibiotics [4–6]. Consequently, the accumulation and persistence of these compounds in the  
 7 environment can threat aquatic and terrestrial ecosystems [1]. Moreover, antibiotics residues  
 8 in water are also suspected to be responsible for the production of resistant microorganisms,  
 9 causing serious problems of public health, namely difficulties in treating pathologies and  
 10 imbalance of microbial ecosystems [7]. Therefore, efficient and economical methods must be  
 11 developed as an urgent need to remove these pollutants.

12 Advanced oxidation processes (AOPs) are potentially powerful methods; they are based on  
 13 the generation of hydroxyl radicals ( $\cdot\text{OH}$ ) which are very reactive and non-selective oxidizing  
 14 agents, leading to the degradation of organic pollutants by hydrogen atom abstraction reaction  
 15 (Eq. (1)), electron transfer (Eq. (2)), or electrophilic addition to  $\pi$  systems (Eq. (3)) [8–12].  
 16 These processes involve chemical, photochemical or electro-chemical techniques such as  
 17 Fenton, UV/ or  $\text{H}_2\text{O}_2$ /ozonation, photo-Fenton, heterogeneous photocatalysis and electro-  
 18 Fenton [11–15].

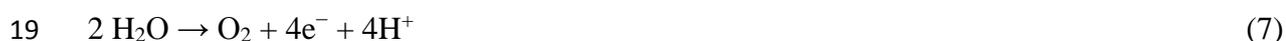
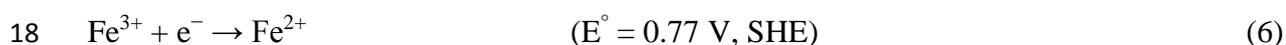
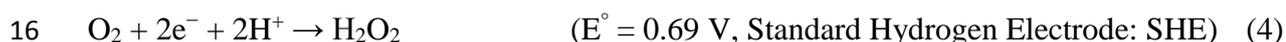


22 Nevertheless, AOP used for complete mineralization can be expensive, and hence its  
 23 combination with a biological treatment can significantly reduce operating costs [16]. This  
 24 combination is especially suitable when microorganisms present in activated sludge are not  
 25 able to metabolize the parent compound; the AOP, applied as a pre-treatment step, may

1 enhance the overall biodegradability by transforming this parent compound into easily  
2 biodegradable and less toxic intermediates, preventing cellular lysis [1,17,18].

3 The present work intends to perform three objectives. The first one is the use of Doehlert  
4 methodology design to optimize the electro-Fenton operating conditions for the removal of  
5 trimethoprim (TMP) i.e. its degradation into organic by-products; the second aim is to  
6 combine electro-Fenton process and biological treatment for the mineralization of TMP i.e. its  
7 transformation into water, carbon dioxide and inorganic ions; and the last one is the validation  
8 of the combined process through the remediation of an industrial pharmaceutical effluent.

9 Electro-Fenton is an electrochemical advanced oxidation process based on the continuous  
10 generation of H<sub>2</sub>O<sub>2</sub> in an acidic medium through the electrochemical reduction of O<sub>2</sub> at the  
11 cathode (Eq. (4)). The generated H<sub>2</sub>O<sub>2</sub> reacts with the added Fe<sup>2+</sup> ions to produce hydroxyl  
12 radicals (<sup>•</sup>OH) and Fe<sup>3+</sup> ions via the Fenton's reaction (Eq. (5)), which is favored by the  
13 catalytic action of the Fe<sup>3+</sup>/Fe<sup>2+</sup> system, mainly from the regeneration of Fe<sup>2+</sup> by the cathodic  
14 reduction of Fe<sup>3+</sup> (Eq. (6)) [8,11,13,19]. At the anode, oxygen is formed by the oxidation of  
15 water (Eq. (7)). Moreover, the method and the involved reactor are easy to handle and to use.



20 As mentioned above, the chosen target compound is trimethoprim (C<sub>14</sub>H<sub>18</sub>N<sub>4</sub>O<sub>3</sub>,  
21 290.32 g mol<sup>-1</sup>, CAS 738-70-5), a bacteriostatic antibiotic commonly prescribed in  
22 combination with sulfamethoxazole for the treatment of infectious diseases in humans. It is  
23 also widely used in veterinary medicine, for prevention and treatment of infections and as a  
24 growth promoter. The removal of TMP in wastewater treatment plants has been reported to be  
25 less than 10% [20]. Therefore, the TMP (water solubility of 400 mg L<sup>-1</sup> at 25°C) has been

1 detected in many environmental monitoring studies in the concentration range of  $\mu\text{g L}^{-1}$   
2 (0.1 to  $5 \mu\text{g L}^{-1}$ ) in WWTP effluents [21].

3

## 4 **2. Materials and Methods**

### 5 *2.1. Chemicals*

6 Trimethoprim (98%) was purchased from Sigma Aldrich (Saint Quentin Fallavier, France).  
7  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (purity 99%) and  $\text{Na}_2\text{SO}_4$  (purity 99%), used as a catalyst source and inert  
8 supporting electrolyte respectively, were provided from Acros Organics (Thermo Fisher  
9 Scientific, Illkirch, France). Acetonitrile (purity 99.9%) (HPLC grade) was also obtained from  
10 Sigma Aldrich. The initial pH of the solutions was adjusted using analytical grade sulfuric  
11 acid from Acros. All solutions were prepared in ultra-pure water and all the other chemicals  
12 used for analysis were purchased from Acros Organics and Sigma Aldrich.

13

### 14 *2.2. Characterization of the pharmaceutical effluent*

15 The effluent used in this study was supplied by a pharmaceutical industry located in north  
16 Tunisia, collected in a container, closed and stored in obscurity at  $4^\circ\text{C}$ . This effluent  
17 contained a high TMP concentration of  $3.56 \text{ g L}^{-1}$  and was characterized by a conductivity of  
18  $4.36 \text{ mS cm}^{-1}$ , a COD of  $438.50 \text{ g L}^{-1}$  and a TOC of  $125.40 \text{ g L}^{-1}$ . Before treatment, the  
19 effluent was diluted to obtain an initial TMP concentration of 0.2 mM, in order to conduct the  
20 treatment in the same conditions as the synthetic solution.

21

### 22 *2.3. Analytical determinations*

#### 23 *2.3.1. High Performance Liquid Chromatography (HPLC)*

24 The evolution of trimethoprim concentrations was monitored by HPLC using a Waters 996  
25 system equipped with Waters 996 PDA (Photodiode Array Detector) and Waters 600LCD

1 Pump. The separation was achieved on a Waters C<sub>18</sub>, (5 μm; 4.6 × 250 mm) reversed-phase  
2 column. The eluent consisted of a mixture of acetonitrile/ultra-pure water (20/80 v/v) buffered  
3 at pH 3 with phosphoric acid and 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, delivered at a flow rate of 1 mL min<sup>-1</sup>.  
4 Detection of trimethoprim was carried out at 204 nm.

5

### 6 2.3.2. Chemical Oxygen Demand (COD) measurements

7 Chemical Oxygen Demand (COD) was measured by means of Nanocolor<sup>®</sup> tests CSB 160 and  
8 1500 from Macherey-Nagel (Düren, Germany). The amount of oxygen required for the  
9 oxidation of the organic and mineral matter at 148°C for 2 h was quantified after oxidation  
10 with K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> at acidic pH [22].

11

### 12 2.3.3. Total Organic Carbon (TOC) measurements

13 The solutions were filtered on Sartorius Stedim Minisart 0.40 μm GF prefilters (Goettingen,  
14 Germany). TOC was measured by means of a TOC-V<sub>CPH/CPN</sub> Total Organic Analyzer  
15 Shimadzu. Organic carbon compounds were combusted and converted to CO<sub>2</sub>, which was  
16 detected and measured by a non-dispersive infrared detector (NDIR). Reproducible TOC  
17 values were always obtained using the standard NPOC (Non Purgeable Organic Carbon)  
18 method. For each sample, each measurement was triplicated [16].

19

### 20 2.3.4. Activated Sludge Preparation

21 Activated Sludge used in this study was collected from a local wastewater treatment plant  
22 (Rennes Beaurade, Bretagne, France). It was washed several times by successions of  
23 centrifugation, supernatant withdrawing and resuspension of pellet in water, in order to  
24 remove any residual carbon or mineral nutrients.

25

### 1 2.3.5. Biological Oxygen Demand (BOD<sub>5</sub>) measurements

2 BOD<sub>5</sub> measurements were carried out in Oxitop IS6 (WTW, Alès, France). The following  
3 mineral basis was used for all experiments (g L<sup>-1</sup>): MgSO<sub>4</sub>·7H<sub>2</sub>O, 22.50; CaCl<sub>2</sub>, 27.50; FeCl<sub>3</sub>,  
4 0.15; NH<sub>4</sub>Cl, 2.00; Na<sub>2</sub>HPO<sub>4</sub>, 6.80; KH<sub>2</sub>PO<sub>4</sub>, 2.80. Washed activated sludge was added in  
5 order to have 0.05 g L<sup>-1</sup> initial concentration of dry matter. The BOD<sub>5</sub> value was initially  
6 estimated based on the COD value experimentally measured, BOD<sub>5</sub> = COD/1.46. The range  
7 of expected BOD<sub>5</sub> measurement was then deduced and hence led to the volumes of sample, of  
8 activated sludge solution and nitrification inhibitor (10 mg L<sup>-1</sup> solution of N-allylthiourea)  
9 which have to be added to the shake flask of the Oxitop apparatus. Similar protocol was  
10 applied for the control sample except that it was replaced by a solution of easily  
11 biodegradable compounds, namely glutamic acid (150 mg L<sup>-1</sup>) and glucose (150 mg L<sup>-1</sup>).  
12 Before use, KOH was added to achieve neutral pH (7.0 ± 0.2). Similar protocol was also  
13 considered for the blank solution, for which the sample was replaced by water to have a  
14 negligible BOD<sub>5</sub> value.

15

### 16 2.3.6. Ultra-pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

17 Aromatic by-products of trimethoprim were separated using a Waters (Acquity UPLC) liquid  
18 chromatographic system (Milford, USA). 5 µL of samples were injected into an Acquity BEH  
19 C<sub>18</sub> column (2.1 mm × 100 mm, 1.7 µm) thermostated at 45°C. Isocratic elution was  
20 performed with a mixture of acetonitrile/ultra-pure water (30/70 v/v) (with the addition of  
21 0.1% formic acid when needed), delivered at a flow rate of 400 µL min<sup>-1</sup>. A Quattro Premier  
22 triple-quadrupole mass spectrometer (Manchester, UK) equipped with an electrospray  
23 ionization source (ESI) was used for by-products detection. MS/MS detection was performed  
24 in negative or positive mode with a capillary voltage of ±3 KV. The desolvation gas flow  
25 (N<sub>2</sub>) was set to 750 L h<sup>-1</sup>. The source temperature and desolvation gas temperature were

1 120°C and 350°C, respectively. The analytical device was controlled by Micromass Masslynx  
2 4.1 software.

3

#### 4 2.3.7. Ion chromatography (IC)

5 Generated carboxylic acids were identified by ion chromatography (Dionex DX120), coupled  
6 to a conductivity detector, using an anion-exchange column Dionex AS19 (4 × 250 mm)  
7 equipped with a Dionex AG19 guard column (4 × 50 mm) coupled to a ASRS suppressor.  
8 The eluent gradient was generated using the Dionex ECG-KOH Elu GenII cartridge; it was as  
9 follows: 0-10 min: 10 mM isocratic; 10-25 min: gradient from 10 to 45 mM; 25-35 min:  
10 45 mM isocratic with a flow rate of 1 mL min<sup>-1</sup>.

11

### 12 2.4. Experimental Procedure

#### 13 2.4.1. Electro-Fenton process

14 The degradation of the organic matter by the electro-Fenton process was carried out in a 1 L  
15 undivided cylindrical glass cell equipped with two electrodes. The dimensions of the carbon  
16 felt piece placed on the inner wall of the cell (Le Carbone Lorraine RVG 4000 – Mersen,  
17 Paris La Défense, France), which was used as the cathode, were 260 mm × 80 mm. Its  
18 specific area, measured by the BET method was 0.7 m<sup>2</sup> g<sup>-1</sup>, its thickness was 12 mm, its  
19 density was 0.088 g cm<sup>-3</sup> and its carbon yield was 99.9%. The anode was a cylindrical  
20 platinum electrode (50 mm × 20 mm) located in the center of the electrochemical reactor to  
21 have a good potential distribution. Prior to electrolysis, compressed air was bubbled for 10  
22 min through the solution at a flow rate of 450 cm<sup>3</sup> min<sup>-1</sup> to saturate the aqueous solution with  
23 oxygen.

24 The pH of the solutions was adjusted to 3 by sulfuric acid (H<sub>2</sub>SO<sub>4</sub>). A catalytic quantity of  
25 FeSO<sub>4</sub>·7H<sub>2</sub>O was introduced into the cell just before the beginning of the electrolysis. The

1 electrodes were connected to a DC power supply (Metrix, model AX 322, Chauvin Arnoux  
2 Group, Paris, France) operating in galvanostatic mode to control the current intensity. The  
3 ionic strength was maintained constant by the addition of 0.05 M Na<sub>2</sub>SO<sub>4</sub>. The electrolytic  
4 solution was in circulation with the help of a peristaltic pump (flow rate of 2 L min<sup>-1</sup>). The  
5 temperature was maintained at 18°C and the initial synthetic solution or the industrial  
6 pharmaceutical effluent was diluted to achieve an initial trimethoprim concentration of  
7 0.2 mM.

#### 8 2.4.2. Biological treatment

9 Culture media were prepared in duplicate in 500 mL serum bottles containing 200 mL of  
10 synthetic trimethoprim solution or pharmaceutical effluent, beforehand electrolyzed. Minerals  
11 were spiked in the medium as highly concentrated solutions to reach the following initial  
12 composition (mg L<sup>-1</sup>): Na<sub>2</sub>HPO<sub>4</sub>, 334; K<sub>2</sub>HPO<sub>4</sub>, 208; KH<sub>2</sub>PO<sub>4</sub>, 85; CaCl<sub>2</sub>, 27.4; MgSO<sub>4</sub>.7H<sub>2</sub>O,  
13 22.6; NH<sub>4</sub>Cl, 2; FeCl<sub>3</sub>.6H<sub>2</sub>O, 0.26; and the initial pH was adjusted to 7. Activated sludge was  
14 added in order to have 1 g L<sup>-1</sup> initial concentration of dry matter. Cultures were agitated at  
15 350 rpm and kept at room temperature (25°C). Samples (5 mL) were taken regularly, filtered  
16 through 0.45 µm-syringe filters and injected for TOC measurements.

17

#### 18 2.5. Doehlert experimental design

19 Doehlert experimental design [23] was used to determine the optimal operating conditions for  
20 the degradation of trimethoprim. The influence of three factors: current intensity (U<sub>1</sub>), initial  
21 Fe<sup>2+</sup> concentration (U<sub>2</sub>) and electrolysis time (U<sub>3</sub>) were studied. The analyzed response (Y)  
22 was the removal rate of trimethoprim. The Doehlert matrix consists of N experiments with  
23  $N = K^2 + K + 1$ , where K is the number of variables. For K = 3, the matrix comprised 13  
24 experiments which were uniformly distributed within the space of the coded variables (X<sub>i</sub>).  
25 The number of replicates in the central point of the design was fixed at 3 (experiments 13-15)

1 in order to obtain an estimation of the experimental error. The transformation of natural  
 2 variables ( $U_i$ ) into coded variables ( $X_i$ ) was made according to the following equation [24]:

$$3 \quad X_i = \left[ \frac{U_i - U_{i(0)}}{\Delta U_i} \right] \alpha_i \quad (8)$$

4 Where  $U_{i(0)}$  is the value of  $U_i$  at the center of the study domain,  $\Delta U_i$  is the variation step and  
 5  $\alpha_i$  is the maximum coded value of  $X_i$ :  $\alpha_1 = 1$ ;  $\alpha_2 = \sqrt{3} / 2$ ;  $\alpha_3 = \sqrt{6} / 3$ .

$$6 \quad U_{i(0)} = \frac{\text{upper limit of } U_i + \text{lower limit of } U_i}{2} \quad (9)$$

$$7 \quad \Delta U_i = \frac{\text{upper limit of } U_i - \text{lower limit of } U_i}{2} \quad (10)$$

8 For the Doehlert experimental design construction, the retained domain for each variable (300  
 9  $< U_1/\text{mA} < 500$ ;  $0.050 < U_2/\text{mM} < 1.000$  and  $6 < U_3/\text{min} < 30$ ) was determined after  
 10 preliminary tests (data not shown).

11 The experimental response associated to the Doehlert matrix is represented by a quadratic  
 12 polynomial model:

$$13 \quad Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 \quad (11)$$

14 Where  $Y$  is the experimental response,  $b_0$  is a constant of the model,  $b_i$  is the estimation of the  
 15 main effects of the factor  $i$ ,  $b_{ii}$  is the estimation of the second order effects and  $b_{ij}$  is the  
 16 estimation of the interactions between factor  $i$  and factor  $j$ .

17 The calculation of coefficients is carried out through the least squares method by means of

$$18 \quad B = (X^T X)^{-1} X^T Y \quad (12)$$

19 Where  $B$  is the vector of estimates of the coefficients,  $X$  is the model matrix,  $X^T$  is the  
 20 transposed model matrix and  $Y$  is the vector of the measured response.

21 The statistical significance of the model was checked using the variance analysis (ANOVA).

22 The relationship between the response and the experimental variables was graphically  
 23 illustrated by plotting the three-dimensional response surface and the two dimensional  
 24 isoresponse curves. NEMRODW Software [25] was used for data calculation and treatment.

### 1 3. Results and Discussion

#### 2 3.1. Determination of the optimal conditions for the removal of TMP

3 The performances of the electro-Fenton process depend on various operating parameters, like  
4 current intensity, initial ferrous ions concentration and electrolysis time [26]. The optimal  
5 conditions of these variables, for the removal of trimethoprim, have been investigated by the  
6 use of Doehlert matrix. The experimental design is represented in Table 1.

7 The obtained responses (TMP removal (%)) were used to compute the polynomial model  
8 coefficients, leading to propose the following model equation:

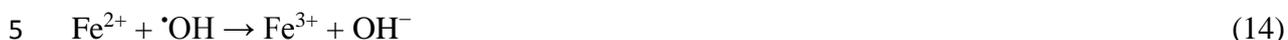
$$9 \quad Y = 98.9 + 2.7 X_1 + 1.5 X_2 + 13.2 X_3 - 2.2 X_1^2 - 3.4 X_2^2 - 13.2 X_3^2 + 0.8 X_1X_2 + 0.7 X_1X_3 - \\ 10 \quad 2.6 X_2X_3 \quad (13)$$

11 The variance analysis for the fitted model indicated that the model was statistically  
12 meaningful (P-value < 0.01) (Table 2). Moreover, the correlation coefficients ( $R^2 = 0.98$  and  
13  $R^2_{Adjusted} = 0.95$ ) were high, implying that in the studied domain more than 95% of the  
14 response variability was explained by the second-order polynomial predicted equation (Eq.  
15 (13)). Consequently, one can conclude that the response Y is adequately described by the  
16 polynomial model, and so the generated equation can be used to predict the Y-values in the  
17 studied domain.

18 Furthermore, the isoresponse curves of TMP removal and the corresponding three-  
19 dimensional representations can be seen in Fig. 1. The graphic analysis of these figures  
20 showed that increasing electrolysis time enhanced the removal of trimethoprim. Indeed, a  
21 higher rate of trimethoprim removal could be reached when the reaction time would vary  
22 from 20 to 30 minutes (Fig. 1a-d).

23 It can also be seen that TMP removal rate was improved when the initial  $Fe^{2+}$  concentration  
24 was located between 0.64 and 0.74 mM (Fig. 1c and d), most likely due to a higher production  
25 of hydroxyl radicals in the presence of more  $Fe^{2+}$  ions in the electro-Fenton reaction [27].

1 However, the use of high ferrous ions concentration ( $[\text{Fe}^{2+}]_0 > 0.74 \text{ mM}$ ) led to a decrease of  
 2 the removal efficiency, probably due to the consumption of  $\cdot\text{OH}$  by the excessive ferrous ions  
 3 according to equation (14), decreasing the quantity of this radical and hence inhibiting the  
 4 degradation reaction [8,27].



6 Moreover, it can be noted that an increase of the applied current intensity would increase the  
 7 removal efficiency; the maximum TMP removal rate was obtained when the current intensity  
 8 was ranging between 455 and 477 mA (Fig. 1e and f). This trend can be explained by the  
 9 excess production of hydrogen peroxide and an increased regeneration rate of  $\text{Fe}^{2+}$  that would  
 10 promote hydroxyl radical production [28]. Besides, at a higher current intensity ( $I > 477 \text{ mA}$ )  
 11 the TMP removal rate decreased; this behavior can be attributed to the  $4e^-$  reduction of  $\text{O}_2$   
 12 leading to the formation of  $\text{H}_2\text{O}$  (Eq. (15)), which inhibits  $\text{H}_2\text{O}_2$  formation reaction (Eq. (4))  
 13 [29]. In addition, at current values higher than 477 mA, the hydrogen gas evolution, at the  
 14 cathode (Eq. (16)), compete with the formation of  $\text{H}_2\text{O}_2$  [30]. This situation reduces the  
 15 production of hydroxyl radicals and consequently, decreases the removal efficiency of  
 16 trimethoprim.



19

### 20 *3.2. Electro-Fenton pretreatment of TMP*

21 The removal of trimethoprim was carried out in the optimal operating conditions deduced  
 22 from the Doehlert matrix, namely  $[\text{Fe}^{2+}]_0 = 0.69 \text{ mM}$  and  $I = 466 \text{ mA}$ , leading to a rapid and  
 23 total TMP removal within 30 min pretreatment (Fig. 2a). Contrarily, mineralization and  
 24 oxidation yields remained low, 12 and 21% for 30 and 60 min electrolysis times for the  
 25 former (Fig. 2a), and 29 and 56% from an initial amount of  $86 \text{ mg L}^{-1} \text{ O}_2$  for the latter after 30  
 26 and 60 min pretreatment respectively (Fig. 2b). This suggested the formation of organic

1 intermediate products, as confirmed at the examination of the HPLC profiles (data not  
2 shown), showing that the disappearance of TMP was accompanied by the formation of several  
3 intermediates products. This behavior is coherent with the fast destruction of TMP and TOC  
4 decrease and can be explained by the oxidation of generated products by hydroxyl radicals  
5 [31].

### 6 *3.3. Improvement of the biodegradability*

7 In order to follow the evolution of the biodegradability profile during the electro-Fenton  
8 pretreatment, the samples were analyzed for BOD<sub>5</sub> and COD, showing a ratio of 0.11 for the  
9 target molecule confirming its recalcitrance and the need for its oxidation prior to a biological  
10 treatment (Fig.2b), while the BOD<sub>5</sub>/COD ratio increased to 0.32 and 0.52 after 30 and 60 min  
11 electrolysis times, namely above the limit of biodegradability (0.4 [32]) after 60 min  
12 pretreatment. From this and owing to the total TMP removal after only 30 min electrolysis, in  
13 addition to TMP removal part of by-products was also oxidized after 60 min pretreatment.

14

### 15 *3.4. Identification of intermediate products and pathway of TMP degradation*

16 The electro-Fenton degradation of 0.2 mM trimethoprim aqueous solution containing  
17 0.69 mM ferrous ions and 50 mM Na<sub>2</sub>SO<sub>4</sub> at pH 3 and 466 mA constant current led to the  
18 formation of aromatic by-products and short-chain carboxylic acids. The identification of the  
19 main aromatic by-products was carried out by UPLC-MS/MS; it was based on the analysis of  
20 the Total Ion Chromatogram (TIC) and the corresponding mass spectra. The proposed  
21 structures were also determined according to the structure of the parent compound and the  
22 molecular weight (MW) of the by-products. Among the degradation products, six compounds  
23 were confirmed by comparing their retention time and their molecular weight with those of  
24 commercially available standard compounds (Table 3); their evolution during electrolysis was

1 presented in Fig. 3. Besides, generated carboxylic acids were identified by ion  
2 chromatography and their retention times were compared with standard compounds (Table 3).  
3 The obtained results allowed suggesting a degradation pathway for the electro-Fenton  
4 oxidation of trimethoprim (Fig. 4). As can be seen, the cleavage of the central methylene  
5 group of TMP, at the beginning of electrolysis, was accompanied by the formation of 1,2,3-  
6 trimethoxybenzene (MW 168) and 2,4-diamino-5-pyrimidinemethanol (MW 140) which has  
7 already been reported during the TiO<sub>2</sub> photocatalysis of TMP [33]. The compound MW 168  
8 was further transformed to 3,4,5-trimethoxyphenol (MW 184) through hydroxylation reaction.  
9 In addition, the compound MW 140 was converted into 2,4-diaminopyrimidine-5-  
10 carbaldehyde (MW 138) which was presumably further oxidized by  $\cdot\text{OH}$  to give 2,4-  
11 diaminopyrimidine-5-carboxylic acid (MW 154). These latter by-products have been proven  
12 by earlier studies [21,33]. Furthermore, the hydroxylation of the 2,4-diaminopyrimidine  
13 portion of TMP yielded the phenolic product 6-hydroxytrimethoprim (MW 306) which has  
14 also been identified during the  $\cdot\text{OH}$  oxidation of TMP [20]. 2,4-diamino-6-hydroxypyrimidine  
15 (MW 126) and (3,4,5-trimethoxyphenyl)methanol (MW 198) were formed during the  
16 cleavage of the central methylene group of MW 306 by the attack of  $\cdot\text{OH}$ . The latter  
17 compound (MW 198) has already been reported during the electrochemical degradation of  
18 TMP at boron-doped diamond electrode [34]. Moreover, we presume that 2,6-  
19 diaminopyrimidine-4,5-diol (MW 142) was the result of hydroxylation of the compound MW  
20 126. In addition, the product (MW 198) was attacked by  $\cdot\text{OH}$  to form the 3,4,5-  
21 trimethoxybenzaldehyde (MW 196). The latter degradation product, which has been proven  
22 by earlier study [33], can also undergo oxidation leading to 3,4,5-trimethoxybenzoic acid  
23 (MW 212).

24 The observation of Fig. 2a and Fig. 3 shows that the disappearance of TMP and the formation  
25 of intermediate compounds took place simultaneously. As can be seen, the major aromatic by-

1 products were 2,4-diaminopyrimidine-5-carbaldehyde and 3,4,5-trimethoxybenzaldehyde,  
2 their formations were very fast, they reached their maximum concentrations (196.5 and  
3  $91 \mu\text{g L}^{-1}$  respectively) at 5 min and completely disappeared within 40 and 50 min,  
4 respectively. Whereas, 3,4,5-trimethoxyphenol and 2,4-diamino-6-hydroxypyrimidine  
5 reached their maximal accumulation values (lower than  $2 \mu\text{g L}^{-1}$ ) after 10 min of treatment  
6 and then their concentrations decreased until complete disappearance. It was found that the  
7 concentration of 1,2,3-trimethoxybenzene remained at small steady-state values during the  
8 electrolysis. On the other hand, the 3,4,5-trimethoxybenzoic acid concentration increased  
9 rapidly attaining a maximum concentration of  $4 \mu\text{g L}^{-1}$  at 5 min of electrolysis and then  
10 followed a slow degradation kinetic. Finally, all aromatic by-products would be further  
11 oxidized by  $\cdot\text{OH}$  through ring cleavage reactions into short-chain aliphatic carboxylic acids,  
12 such as acetic acid (MW 60), glyoxylic acid (MW 74), oxalic acid (MW 90) and succinic acid  
13 (MW 118). The time-course evolution of some carboxylic acids was followed; the obtained  
14 results showed that glyoxylic and succinic acids appeared during the first minutes of  
15 treatment, reaching concentrations of  $11.5$  and  $2 \text{ mg L}^{-1}$  after 60 min of electrolysis.  
16 According to Haidar *et al.* [35], the action of hydroxyl radicals on the aromatic intermediates  
17 is easier than on the carboxylic acids, which have a good resistivity and are hardly oxidizable  
18 by  $\cdot\text{OH}$ .

19

### 20 3.5. Biological treatment

21 Owing to biodegradability improvement, activated sludge cultures of pretreated solutions of  
22 TMP were carried out in duplicates for 20 days (Fig. 5). 37 and 41% mineralization were  
23 obtained after 4 and 6 days of culture for the solutions pretreated during 30 and 60 minutes,  
24 respectively (Fig. 5). This behavior can be attributed to a readily assimilation of some of the  
25 degradation products by microorganisms [36]. Low mineralization rates can be then observed

1 beyond this time until reaching 47 and 59% on the 20<sup>th</sup> day for the solutions electrolyzed  
2 during 30 and 60 minutes, respectively (Fig. 5). From this, the level of mineralization  
3 observed during activated sludge culture increased with the pretreatment time. It is  
4 noteworthy that in the absence of pretreatment, no noticeable TMP biodegradation was  
5 observed (Fig. 5), in agreement with its low BOD<sub>5</sub>/COD ratio, 0.11 (Fig. 2b).

6 It should be noted that possible biosorption of TMP or the degradation products from the  
7 pretreatment was checked and was found to be not significant (data not shown).

8 Moreira *et al.* [37], have studied the mineralization of 20.0 mg L<sup>-1</sup> of TMP by electro-Fenton  
9 process, with a 10 cm<sup>2</sup> BDD anode and a 10 cm<sup>2</sup> carbon PTFE air diffusion cathode. The  
10 treatment was performed using a low Fe<sup>2+</sup> dose of 2.0 mg L<sup>-1</sup>, a low current density of 5  
11 mA·cm<sup>2</sup> and a pH of 3.5; it has led to a 14% mineralization after 180 min reaction.

12

### 13 *3.6. Application to an industrial pharmaceutical effluent*

14 The pretreatment of an industrial pharmaceutical effluent was carried out in the same  
15 operating conditions as for the synthetic solution (Fig. 6). As shown in Fig. 6a, continuous  
16 TMP removal was observed during the oxidation of the industrial effluent until reaching 98%  
17 after 180 minutes. The rate of TMP degradation in the industrial effluent was therefore slower  
18 than in the synthetic solution; this behavior may be due to the competitive consumption of  
19 hydroxyl radicals by other organic compounds contained in the real effluent, since TMP  
20 accounted for only 6% of the organic content of the industrial effluent (Tables 1 and 2). A low  
21 level of mineralization was concomitantly observed, 14 and 16% for 180 and 300 minutes  
22 electrolysis times (Fig. 6a), as well as a relatively low level of oxidation, 18 and 20% from an  
23 initial amount of 8770 mg L<sup>-1</sup> O<sub>2</sub> after 180 and 300 minutes of pretreatment respectively (Fig.  
24 6b). These results can be explained by the generation of intermediate compounds  
25 simultaneously with the degradation of the target compound, as confirmed at the examination

1 of the HPLC profiles (data not shown). The low BOD<sub>5</sub>/COD ratio (0.14 – Fig. 6b) of the  
2 industrial effluent confirmed the need for a pretreatment prior to a biological treatment; while  
3 after electrolysis, the solutions became biodegradable since the BOD<sub>5</sub>/COD ratios increased  
4 to 0.45 and 0.47 for 180 and 300 minutes pretreatment (Fig. 6b). The corresponding  
5 biological treatments were therefore carried out and are displayed in Fig. 7. The  
6 mineralization yields observed were close to 70% after about 10 days of culture for the  
7 effluent pretreated during 180 minutes and final mineralization yields were 76 and 87% for  
8 180 and 300 minutes of effluent pretreatment, respectively (Fig. 7). Contrarily, in the absence  
9 of pretreatment, the rate and the yield of mineralization were significantly lower, since only  
10 15% TOC removal was observed after 8 days of culture and about 50% at the end of culture  
11 (15<sup>th</sup> day). Overall removal yields of the industrial effluent during the combined process were  
12 therefore 80 and 89% after 180 and 300 minutes of effluent pretreatment, respectively.

13 It should also be noted that as for the synthetic solution possible biosorption of the organic  
14 content of the industrial effluent or the degradation products from the pretreatment was  
15 checked and was found to be negligible (data not shown).

16 The higher levels of mineralization observed for the industrial effluent if compared to the  
17 synthetic solution should be underlined. Indeed, TMP contributed for only 6% of the carbon  
18 content of the industrial effluent, and hence at least part of this organic content was also  
19 oxidized during pretreatment and mineralized during biological treatment. Indeed, in the  
20 absence of pretreatment, no TMP degradation was observed (Fig. 5), while significant  
21 mineralization was observed at the end of the biological treatment of the industrial effluent  
22 (Fig. 7). Furthermore, for 30 and 180 minutes pretreatment of the synthetic solution and the  
23 industrial effluent, namely the time needed for a total TMP removal (Figs.2a and 6a),  
24 important mineralization was observed for the industrial effluent, 70% after about 10 days

1 biological treatment (Fig. 7), while only 47% after 20 days culture on the synthetic solution  
2 (Fig. 5).

3

#### 4 **4. Conclusions**

5 Doehlert experimental design was used to determine the optimal operating conditions for the  
6 degradation of trimethoprim by the electro-Fenton process. The obtained results showed a  
7 total TMP removal after an electrolysis time of 30 min, for an initial ferrous ions  
8 concentration of 0.69 mM and using a current intensity of 466 mA. The degradation of TMP  
9 was accompanied by the formation of aromatic by-products and short-chain carboxylic acids.  
10 Based on the identified intermediates, a plausible degradation pathway was proposed.

11 Trimethoprim pretreatment was carried out in the optimal operating conditions deduced from  
12 the Doehlert matrix, leading to low levels of mineralization, 12 and 21% after 30 and 60 min  
13 electrolysis times respectively. Simultaneously, an improvement of the biodegradability was  
14 noted, (0.32 and 0.52 from an initial BOD<sub>5</sub>/COD ratio of 0.11). Therefore, biological  
15 treatments were performed during 20 days and showed that the level of mineralization  
16 increased with the electrolysis time, from 47 to 59% for 30 and 60 min pretreatment times.

17 In order to confirm the relevance of the proposed combined process, an industrial  
18 pharmaceutical effluent was electrolyzed under the same conditions, showing an almost total  
19 TMP removal (98%), while the mineralization yields remained low, 14 and 16% after 180 and  
20 300 minutes electrolysis times, respectively. Moreover, the BOD<sub>5</sub>/COD ratio increased from  
21 0.14 to 0.45 and 0.47 proving the enhancement of biodegradability of the pretreated effluent.

22 The corresponding biological treatments led to 76 and 87% of mineralization after 15 days.  
23 Overall removal yields of the industrial effluent during the combined process were therefore  
24 80 and 89% for 180 and 300 minutes of effluent pretreatment, respectively. An increase of the

1 pretreatment time entailed therefore a better biodegradability resulting in a higher  
2 mineralization of the electrolyzed solution during the subsequent biological culture.

3

4

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8

9

1 **Table 1** – Doehlert matrix experiments and experimental results.

Experiment Number	Coded variables			Real variables			Results Y (%)
	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Current intensity:	Fe <sup>2+</sup> concentration:	Electrolysis time:	
				U <sub>1</sub> (mA)	U <sub>2</sub> (mM)	U <sub>3</sub> (min)	
1	1	0	0	500	0.525	18	100
2	-1	0	0	300	0.525	18	93
3	$\frac{1}{2}$	$\frac{\sqrt{3}}{2}$	0	450	1.000	18	100
4	$-\frac{1}{2}$	$-\frac{\sqrt{3}}{2}$	0	350	0.050	18	92
5	$\frac{1}{2}$	$-\frac{\sqrt{3}}{2}$	0	450	0.050	18	97
6	$-\frac{1}{2}$	$\frac{\sqrt{3}}{2}$	0	350	1.000	18	94
7	$\frac{1}{2}$	$\frac{\sqrt{3}}{6}$	$\frac{\sqrt{6}}{3}$	450	0.683	30	100
8	$-\frac{1}{2}$	$-\frac{\sqrt{3}}{6}$	$-\frac{\sqrt{6}}{3}$	350	0.367	6	78
9	$\frac{1}{2}$	$-\frac{\sqrt{3}}{6}$	$-\frac{\sqrt{6}}{3}$	450	0.367	6	76
10	0	$\frac{\sqrt{3}}{3}$	$-\frac{\sqrt{6}}{3}$	400	0.841	6	80
11	$-\frac{1}{2}$	$\frac{\sqrt{3}}{6}$	$\frac{\sqrt{6}}{3}$	350	0.683	30	100
12	0	$-\frac{\sqrt{3}}{3}$	$\frac{\sqrt{6}}{3}$	400	0.209	30	100
13	0	0	0	400	0.525	18	99
14	0	0	0	400	0.525	18	99
15	0	0	0	400	0.525	18	99

2

3

4 **Table 2** – ANOVA result for TMP removal (%) under electro-Fenton treatment.

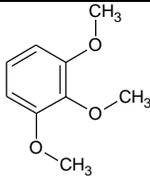
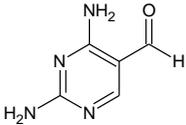
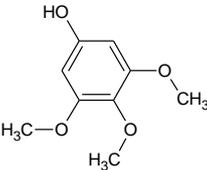
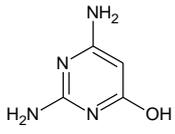
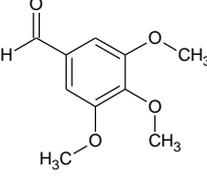
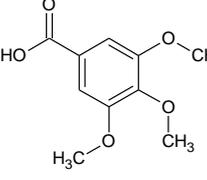
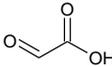
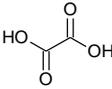
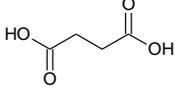
Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio	P-value
Regression	991.773	9	110.197	28.721	0.00088
Residual	19.184	5	3.8368		
Total	1010.957	14			

5  $R^2 = 0.98$ ;  $R^2_{\text{Adjusted}} = 0.95$ .

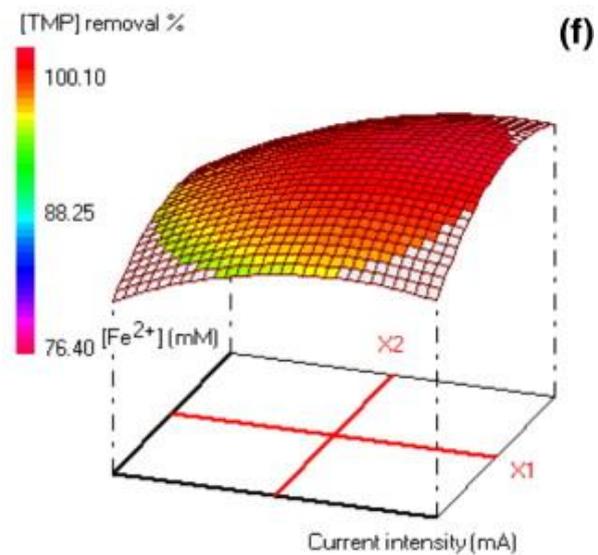
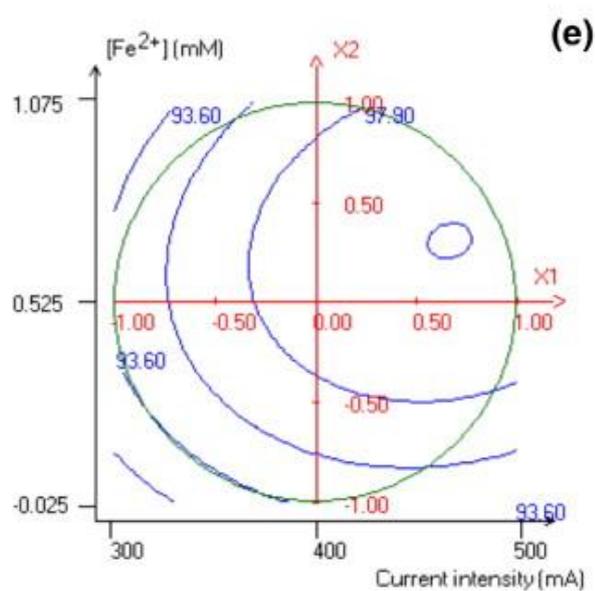
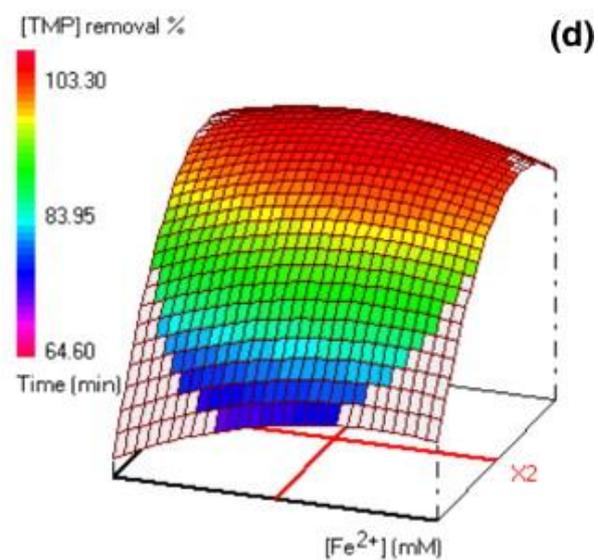
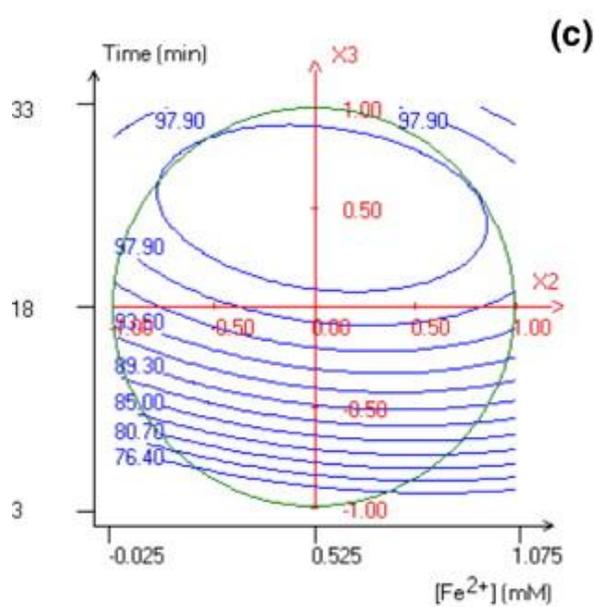
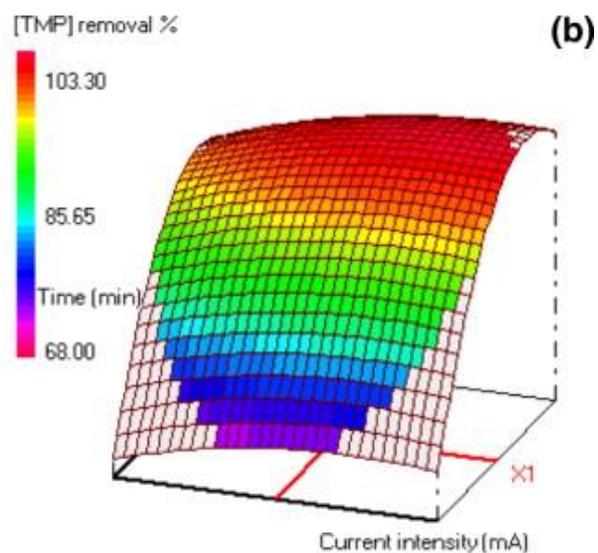
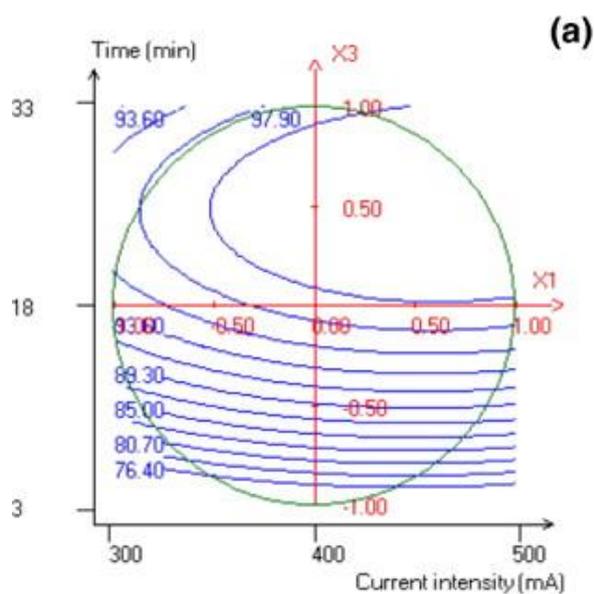
6

7

1 **Table 3** – Chromatographic identification of the products generated during the electro-Fenton  
 2 degradation of trimethoprim.

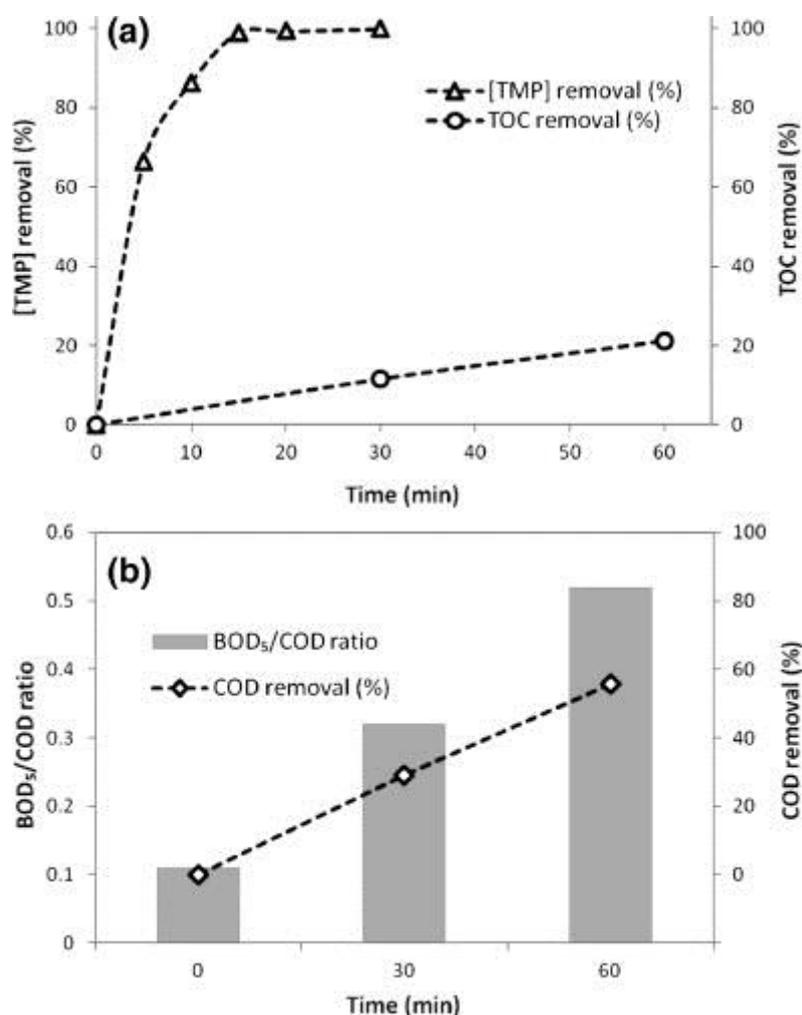
Compound	Chromatography	Chemical structure	Retention time (min)	ESI mode	Precursor ion (m/z)	Product ions (m/z)
1,2,3-trimethoxybenzene (MW 168)	UPLC-MS/MS		1.98	Positive	169	154 138
2,4-diaminopyrimidine-5-carbaldehyde (MW 138)	UPLC-MS/MS		0.69	Positive	139	97 69
3,4,5-trimethoxyphenol (MW 184)	UPLC-MS/MS		0.91	Negative	183	168 153
2,4-diamino-6-hydroxypyrimidine (MW 126)	UPLC-MS/MS		0.58	Positive	127	85 68 60
3,4,5-trimethoxybenzaldehyde (MW 196)	UPLC-MS/MS		1.63	Positive	197	169 138
3,4,5-trimethoxybenzoic acid (MW 212)	UPLC-MS/MS		0.72	Negative	211	167 152
Acetic acid (MW 60)	IC		4.2			
Glyoxylic acid (MW 74)	IC		4.5			
Oxalic acid (MW 90)	IC		5.4			
Succinic acid (MW 118)	IC		14.6			

## 1 **Figure captions**



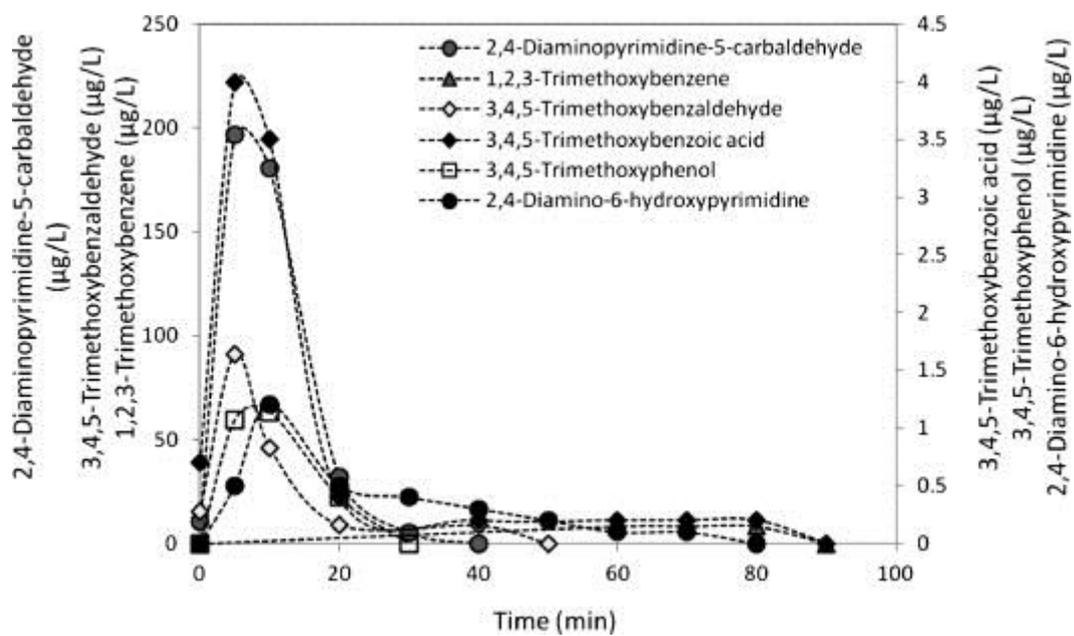
1 **Figure 1.** (a) Contour plots of TMP removal versus the electrolysis time (min) and the current  
 2 intensity (mA); (b) corresponding three-dimensional plot; (c) contour plots of TMP removal  
 3 versus the electrolysis time (min) and the initial  $\text{Fe}^{2+}$  concentration (mM); (d) corresponding  
 4 three-dimensional plot; (e) contour plots of TMP removal versus the initial  $\text{Fe}^{2+}$  concentration  
 5 (mM) and the current intensity (mA); (f) corresponding three-dimensional plot. Results  
 6 obtained from the Doehlert matrix (Table 1).

7  
8



9  
 10 **Figure 2.** (a) Time evolution of TMP and TOC removals; (b) BOD<sub>5</sub>/COD ratio and COD  
 11 removal. Experimental conditions:  $[\text{TMP}]_0 = 0.2$  mM,  $[\text{Fe}^{2+}]_0 = 0.69$  mM,  $[\text{Na}_2\text{SO}_4] = 50$   
 12 mM, pH = 3, T = 18°C, I = 466 mA, V = 1 L.

1

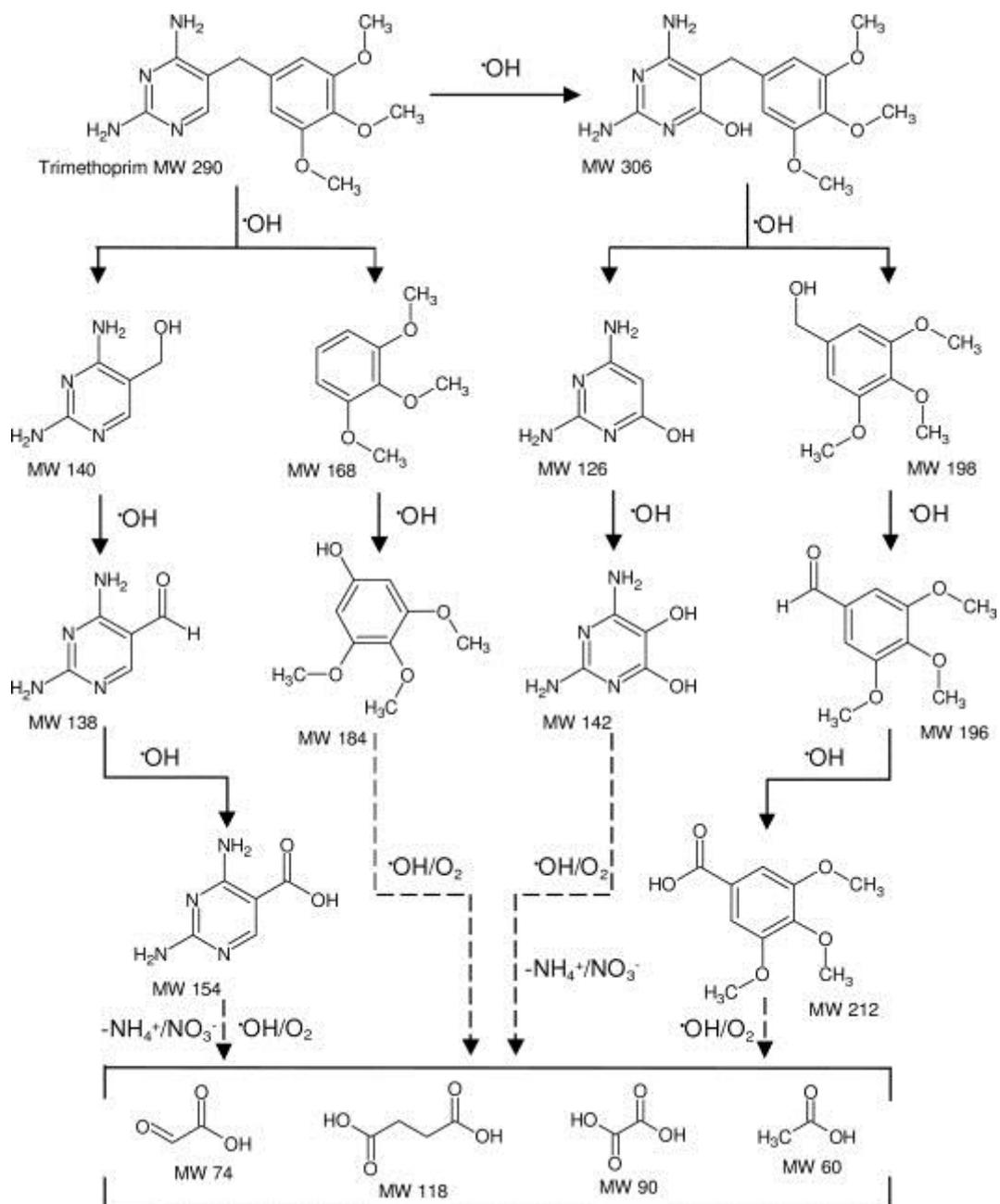


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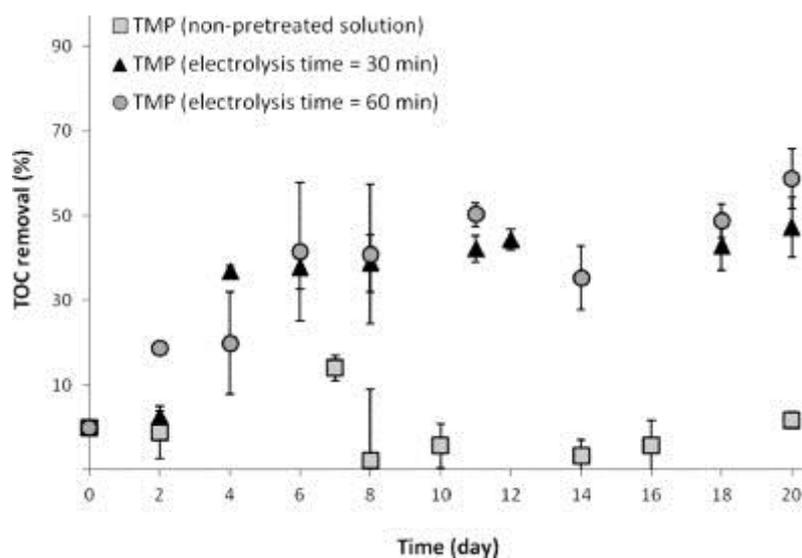
3 **Figure 3.** Time-course of aromatic by-products formed during the electro-Fenton treatment.

4 Experimental conditions:  $[\text{TMP}]_0 = 0.2 \text{ mM}$ ,  $[\text{Fe}^{2+}]_0 = 0.69 \text{ mM}$ ,  $[\text{Na}_2\text{SO}_4] = 50 \text{ mM}$ ,  $\text{pH} = 3$ ,

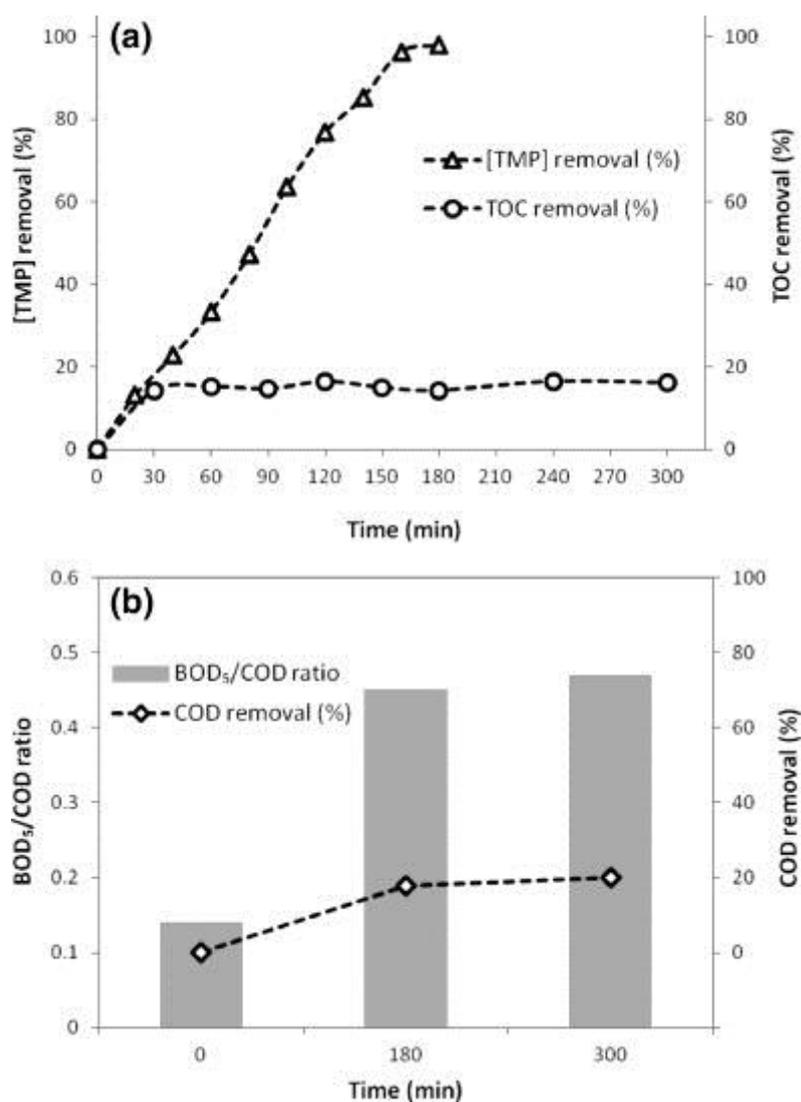
5  $T = 18^\circ\text{C}$ ,  $I = 466 \text{ mA}$ ,  $V = 1 \text{ L}$ .



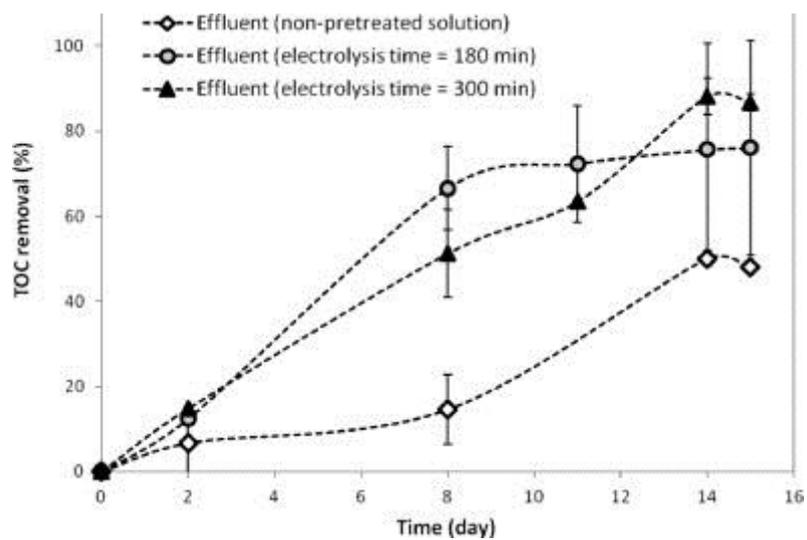
2 **Figure 4.** Pathway proposed for the degradation of TMP by the electro-Fenton process.



1  
2 **Figure 5.** Mineralization during activated sludge cultures of non-pretreated TMP solution and  
3 solutions electrolyzed during 30 min and 60 min. Electro-Fenton pretreatment conditions:  
4  $[\text{TMP}]_0 = 0.2 \text{ mM}$ ,  $[\text{Fe}^{2+}]_0 = 0.69 \text{ mM}$ ,  $[\text{Na}_2\text{SO}_4] = 50 \text{ mM}$ ,  $\text{pH} = 3$ ,  $T = 18^\circ\text{C}$ ,  $I = 466 \text{ mA}$ ,  
5  $V = 1 \text{ L}$ .



1  
2 **Figure 6.** Pretreatment of the industrial pharmaceutical effluent: (a) Time evolution of TMP  
3 and TOC removals; (b) BOD<sub>5</sub>/COD ratio and COD removal. Experimental conditions:  
4 [TMP]<sub>0</sub> = 0.2 mM, [Fe<sup>2+</sup>]<sub>0</sub> = 0.69 mM, [Na<sub>2</sub>SO<sub>4</sub>] = 50 mM, pH = 3, T = 18°C, I = 466 mA,  
5 V = 1 L.



1  
 2 **Figure 7.** Mineralization during activated sludge cultures of non-pretreated industrial effluent  
 3 and effluent electrolyzed during 180 and 300 min. Electro-Fenton pretreatment conditions:  
 4  $[\text{TMP}]_0 = 0.2 \text{ mM}$ ,  $[\text{Fe}^{2+}]_0 = 0.69 \text{ mM}$ ,  $[\text{Na}_2\text{SO}_4] = 50 \text{ mM}$ ,  $\text{pH} = 3$ ,  $T = 18^\circ\text{C}$ ,  $I = 466 \text{ mA}$ ,  
 5  $V = 1 \text{ L}$ .