Scientific Report

regarding the implementation of the project between January - December 2019 (Stage II)

The objective of the project is to generate antimicrobial compounds by exposing drugs to laser radiation and to identify compounds with antimicrobial properties resulting from irradiation, with a densitometric analysis system of HPTLC plates developed.

This stage aimed to design, conduct, test and validate the experimental system for HPTLC (laser induced fluorescence and fluorescence lifetime) studies of thioridazine (unirradiated and irradiated) and ciprofloxacin (unirradiated) solutions. The purpose was to validate the method for both unirradiated thioridazine and ciprofloxacin. Only irradiated 120 min thioridazine solution was investigated because it presented in stage I the best antimicrobial effect against Gram-positive bacteria. Also, densitometry studies have been compared with alternative methods of analysis, such as QTOF LC-MS and JustTLC. Photoproducts of the thioridazine solution were identified by QTOF LC-MS and their molecular structures were confirmed by Gaussian09.

Within this stage the following were realized:

1. Design, implementation and testing of the experimental system for HPTLC densitometry - LIF analysis and LIF lifetime.
2. Establishing the linearity, precision and repeatability of the method for the unirradiated solutions of TZ and CIP, at different concentrations, by recording the LIF spectra and the LIF lifetime.
3. Performing and analyzing quantitative measurements of HPTLC plates using the JustTLC program.
4. Carrying out QTOF LC-MS experiments on non-radiated solutions of TZ and CIP.
5. Carrying out experiments using HPTLC system developed for TZ solutions, those for which the best antimicrobial effect was obtained.
6. Carrying out QTOF LC/MS experiments for selected irradiated TZ solutions.
7. Determination of the molecular structure of the photoproducts.
8. Comparison of the results obtained with JustTLC software, HPTLC densitometry and with QTOF LC-MS and method validation.

1. Design, implementation and testing of the experimental system for HPTLC densitometry - LIF analysis and LIF lifetime.

The compounds studied are ciprofloxacin CIP (99.5%) and thioridazine TZ (99.8%), in hydrochloride form.

The TZ powders were dissolved in ultrapure water at a concentration of 2 mg/mL. The irradiation of the compounds was performed for different exposure times, respectively 1, 15, 30, 60, 120, 180, and 240 min. The samples were irradiated with a laser beam emitted at 266 nm (6.5 mJ energy) by an Nd:YAG laser (Excel Technology, Surelite II model, 6 pulse width and 10 Hz repetition rate).

The analysis of the generated photoproducts was performed by the chromatographic technique called thin layer chromatography (HPTLC). The samples were applied to the HPTLC plate using the Linomat 5 semi-automatic system (CAMAG). An amount of 4 μL of solution was applied to the plate in the form of a band (5 mm) at a dosing rate of 20 nl/s. The mobile phase consisted of a mixture of acetone: methanol: 25% ammonia (50:50:1, V:V:V) for TZ and dichloromethane: methanol: 25% ammonia (4:2:0.85, V:V:V) in the case of CIP. The chromatogram obtained was viewed at 256 nm using the Chromo-Vue® Cabinet C-65 (UVP) development chamber and then photographed and analyzed using the JustTLC program (Sweday).

The experimental system required for HPTLC densitometry measurements consisted of a laser control unit, laser diode, XY translational stage, fiber optic, spectrograph, photomultiplier and oscilloscope. HPTLC plates were excited with the picosecond laser diode (Alphals, PicoPower LD-

37550) emitting at 375 nm, at 30 mHz, with a pulse duration of 87.7 ps and a measured power of 490 μW.

Laser induced fluorescence (LIF) was recorded in real time. The spectra were recorded with a spectrograph (Acton Research, SpectraPRo SP-2750) coupled with an ICCD camera used to detect and analyze the radiation emitted. For fluorescence lifetime studies it was recorded with a photomultiplier (Hamamatsu H-6780-02) whose output signal is coupled to a Tektronix DPO 7254 digital oscilloscope.

Fluorescence spectra were recorded along the OX direction with a 1 mm pitch. The laser spot was placed on the center of the band containing the drug. Finally, the fluorescence maxima were extracted and plotted as a function of the distance, obtaining the horizontal chromatogram of the investigated drugs.

2. Establishing the linearity, precision and repeatability of the method for the non-radiated solutions of TZ and CIP, at different concentrations, by recording the LIF spectra and the LIF lifetime.

The validation of the proposed method was performed in terms of linearity, limit of detection (LOD), limit of quantification (LOQ) and accuracy in accordance with the Guide of the International Conference on Harmonization (ICH) [1] and validation guides published by Ferenczi-Fodor et al. [2,3].

For the evaluation of linearity, seven different concentrations (2.5, 5, 10, 20, 40, 80, 160 μg/band) were applied to the HPTLC plate, in triplicate, and the fluorescence spectra were recorded. The calibration curve was found to be polynomial in the range of 2.5–160 μg/band for thioridazine, with a correlation coefficient of 0.99799. For CIP, it was found that the calibration curve is polynomial in the range of 2.5–80 μg/band, with a correlation coefficient of 0.997. For time-resolved fluorescence measurements, linearity determination is not possible because the fluorescence lifetime does not depend on the concentration of the tested compound.

The sensitivity measure of each analytical method including HPTLC densitometry is represented by LOD and LOQ. The LOD and LOQ values were determined based on the specific calibration curve using samples containing the following concentrations: 1.25, 0.6, 0.3, 0.15, 0.5 μg/band. For TZ it was found that the smallest detectable drug amount was 0.59 μg/band, and the smallest quantifiable drug quantity was 1.77 μg/band. And for CIP, it was found that the smallest detectable drug amount was 0.91 μg/band, and the smallest quantifiable drug quantity was 2.75 μg/band.

For an analytical method, precision is an assessment of the consistency of the results obtained with several measurements in the same sample [4,5]. Thus, 4 HPTLC plates containing unirradiated TZ or CIP were performed. Each plate contains six applications of the two solutions.

For TZ, when investigated by LIF, it was obtained a % RSD <2. A value less than 2 represents the desired level of accuracy for pharmaceutical analyzes. The% RSD value is often used in method validation evaluations because it normalizes the standard deviation to the average [6].

Table 1. Representative parameters for determining the precision, for each plate investigated containing unirradiated TZ solutions, resulted from the LIF densitometric analysis of HPTLC plates.

Plate	**Average fluorescence** (unit. arb.)	**Standard error** (unit. arb.)	**Relative standard error (%**RSD**)** (%)
1	1.91×10^5	5.85×10^2	0.31
2	2.39×10^5	5.42×10^3	2.26
3	1.9×10^5	2.61×10^3	1.38
4	2.08×10^5	3.57×10^3	1.72

When fluorescence lifetime was investigated for TZ, it was observed an RSD <2% for each plate investigated, which represents the desired level of accuracy for pharmaceutical analysis (Table 2).

Table 2. Representative parameters for determining the precision, for each plate investigated containing unirradiated TZ solutions, resulted from the fluorescence lifetime densitometric analysis of HPTLC plates.

Plate	**Average life-time** (ns)	**Standard error** (ns)	**Relative standard error (%**RSD) (%)
1	2.3	0.025	1.12
2	2.3	0.03	1.35
3	2.3	0.037	1.64
4	2.29	0.044	1.96

As for CIP, the LIF and LIF lifetime densiometric analyses showed also %RSD smaller than 2. In Table 3 and Table 4 are presented the representative parameters for the establishment of the precision.

Table 3. Representative parameters for determining the precision, for each plate investigated containing unirradiated CIP solutions, resulted from the LIF densitometric analysis of HPTLC plates.

Plate	**Average fluorescence** (unit. arb.)	**Standard error** (unit. arb.)	**Relative standard error (%**RSD) (%)
1	3.56×10^6	5.67×10^4	1.59
2	3.1×10^6	5.82×10^4	1.88
3	3.09×10^6	6.12×10^4	1.98
4	3.22×10^6	4.11×10^4	1.28

Table 4. Representative parameters for determining the precision, for each plate investigated containing unirradiated CIP solutions, resulted from the fluorescence life time densitometric analysis of HPTLC plates.

Plate	**Average life-time** (ns)	**Standard error** (ns)	**Relative standard error (%**RSD) (%)
1	2.5	0.02	0.82
2	2.51	0.04	1.78
3	2.49	0.01	0.56
4	2.58	0.05	2.06

2. Performing and analyzing quantitative measurements of HPTLC plates using the JustTLC program.

All plates that were used to determine precision, LOD/LOQ and linearity of the HPTLC densitometry method were photographed and analyzed by JustTLC. JustTLC cannot export the horizontal chromatogram, respectively volume as a function of distance, offering only the volume value for the entire band.

The accuracy of this method was determined and Table 5 presents the results obtained for TZ and Table 6 for CIP. It was observed that% RSD has values much greater than 2%, being above the desired level of accuracy.

Table 5. Representative parameters for determining the precision, for TZ, resulted from the investigation of the plates with JustTLC software.

Plate	Average Volume	Standard error	Relative standard error (%RSD)
1	377.5	35.73	9.47%
2	276.1	16.67	6.04%
3	320.4	17.7	5.52%
4	319.75	24.09	7.53%

Table 6. Representative parameters for determining the precision, for TZ, resulted from the investigation of the plates with JustTLC software.

Plate	Average Volume	Standard error	Relative standard error (%RSD)
1	1614.77	47.13	2.92%
2	1485.84	66.73	4.49%
3	2127.10	93.74	4.41%
4	2007.1	57.97	2.89%

Thus, JustTLC analysis of HPTLC plates that do not have a fluorescence marker cannot be used for analysis of CIP solutions due to a greater than 2 %RSD.

3. Carrying out QTOF LC-MS experiments on unirradiated solutions of TZ and CIP.

The unirradiated TZ and CIP solutions were investigated by QTOF LC/MS using a LC 1260 Infinity Series system (DAD detector and fluorescence detector) coupled with the Agilent 6530 Accurate Mass Q-TOF Mass Spectrometer (G6530). Chromatographic separation was performed using an Eclipse Plus C18 3X150 column, 3.5 μm for both TZ and CIP. For TZ, the solvents for the mobile phase were acetonitrile and water (0.1% formic acid) and for the isocratic separation the following parameters were used: 0-1 min, 80% acetonitrile and 20% water; 1-9 min, 50% acetonitrile and 20% water; and 9-10 min, 80% acetonitrile and 20% water. The flow rate was set to 1 mL/min. For CIP, the solvents for the mobile phase were acetonitrile and water (0.1% formic acid) and for the isocratic separation the following parameters were used: 0-1 min, 5% acetonitrile and 95% water; 1-10 min, 100% acetonitrile and 0% water. The flow rate was set at 0.3 mL/min.

The fluorescence spectra resulted from LC-DAD-FLD investigation were compared with the fluorescence spectra of TZ and CIP in droplet form and applied as bands on the HPTLC plate. For the excitation of the droplet and the band it was used the same laser diode that emitted at 375 nm (υ = 30 MHz, pulse duration of 87.7 ps and measured power of 490 μW).

For TZ, comparing the absorbance spectra obtained by LC-DAD-FLD with those obtained by classical absorption spectroscopy, a 2 nm blue shift of the maxima was observed due to the mobile phase used in liquid chromatography. In the case of fluorescence, there are differences of the fluorescence maxima, respectively 471 nm by FLD, 476 nm for the plate and 481nm for the drop.

For the CIP, comparing the absorbance spectra obtained by LC-DAD-FLD with those obtained by classical absorption spectroscopy, a 6 nm red shift of the 277 nm maxima was observed due to the mobile phase used in liquid chromatography. In the case of fluorescence, there are differences of the fluorescence maxima, respectively 457 nm by FLD, 442 nm for the plate and 457 nm for the drop. The fluorescence is strongly influenced by the environment of the molecules, thus this difference in fluorescence maxima.

As for the MS spectra, the following values could be detected from the ion chromatogram: TZ = 371.1638 aum and CIP = 332.1285 aum.

4. Carrying out experiments using HPTLC system developed for TZ solutions, those for which the best antimicrobial effect was obtained.

For this experiment, unirradiated and irradiated TZ solutions were applied between 1 min and 240 min on an HPTLC plate under the same conditions as for the study of linearity and precision. The plate was investigated by time-resolved fluorescence and fluorescence spectroscopy. All the photoproducts were investigated and the horizontal chromatograms were obtained.

4.1. Laser induced fluorescence

The HPTLC plate containing bands with unirradiated and irradiated TZ solutions was investigated before and after mobile phase development.

After developing the HPTLC plate, it was viewed at 256 nm and photographed (Figure 1a). Thus, the separation of the photoproducts resulting from irradiation of the TZ solution with 266 nm was observed. For this analysis, eight photoproducts were viewed and analyzed with the densitometric system. The horizontal chromatograms of TZ and its photoproducts are shown in Figure 1b.

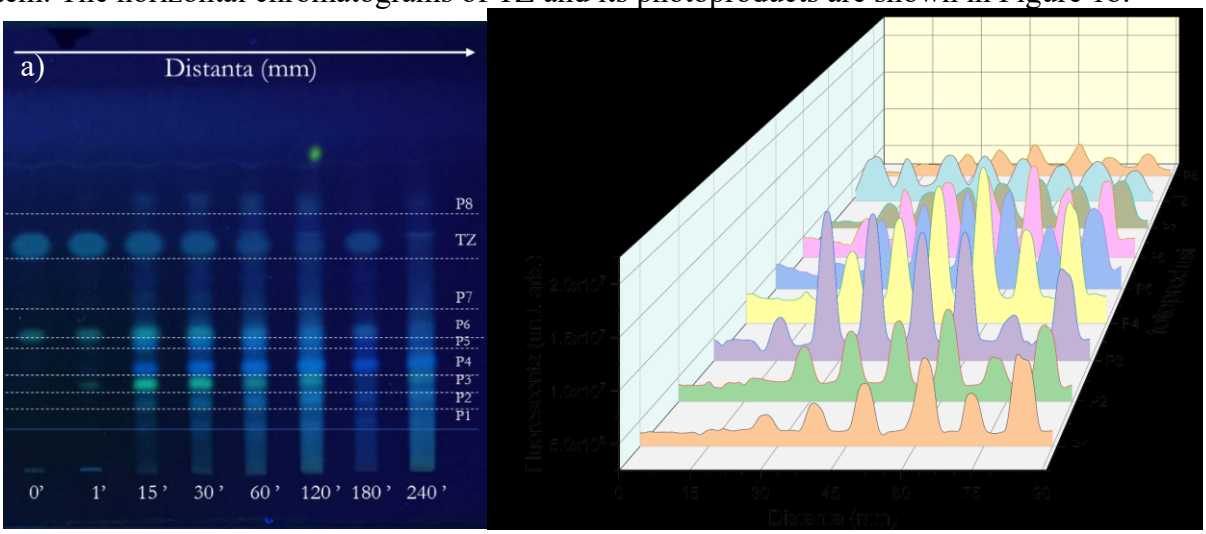


Figure 1. a) HPTLC plate containing the unirradiated and irradiated TZ, viewed at 254 nm. b) Horizontal chromatograms for TZ and its photoproducts, which resulted from laser induced fluorescence analysis of HPTLC plate.

In Figure 1b it is observed that as the TZ fluorescence decreases, respectively the concentration, the fluorescence of the photoproducts changes. It can be observed that the generation of photoproducts is not linear with the irradiation time.

Further, the spectral characteristics of the photoproducts were studied. After the first minute of irradiation the photoproducts P3, P4, P5, P6, P7 and P8 are formed in the irradiated solution. After 15 min of exposure at 266 nm, P1 and P2 also begin to form. The wavelengths of the fluorescence maxima are as follows: P1 - 477 nm, P2 - 486 nm, P3 - 493 nm, P4 - 469 nm, P5 - 479 nm, P6 - 475 nm, P7 - 482 nm, TZ - 476 nm and P8 - 480 nm.

As a result of the experiments performed during stage I of the project, it was established that the 120 min irradiated TZ solution has the best antimicrobial effect against Gram-positive bacteria. For irradiated TZ 120 min it is observed that the photoproduct with the most intense fluorescence is P4, followed by P3 and P5. Compound P8 has been identified in the literature as thioridazine N-desmethyl [7]. In conclusion, it was observed that there is a competitiveness between the photoproduction processes.

4.2. Time-resolved fluorescence

The time-resolved fluorescence signals for TZ and the resulting photoproducts from the HPTLC plate are shown in Figure 2.

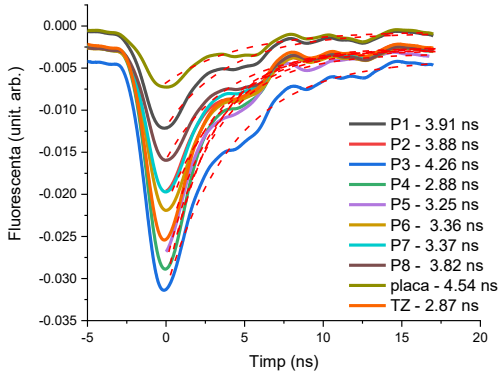


Figure 2. Transient fluorescence signals in the case of TZ and its photoproducts resulting from 375 nm excitation of the HPTLC plate.

From the kinetics of fluorescence signal, using the exponential fit function, the fluorescence lifetime values were extracted and are shown in Figure 2. The fluorescence lifetime for TZ is 2.87 ns and is approximately the same as that of TZ obtained in stage I, in liquid, respectively 3.02. This confirms the fact that the study of fluorescence lifetime of the compounds in the HTPLC plates provides reliable results.

5. Carrying out QTOF LC-MS experiments for selected irradiated TZ solutions.

The irradiated TZ solution with a laser beam emitted at 266 nm for 120 min was analyzed by LC-DAD-FLD to optically characterize the photoproducts (absorbance and fluorescence spectra) and by QTOF LC (DAD)/MS to obtain the molecular mass of photoproducts.

Based on the mass measurements of the generated ions and the chromatograms of the extracted ions (Figure 3), the photoproducts were identified for TZ irradiated 120 min with 266 nm.

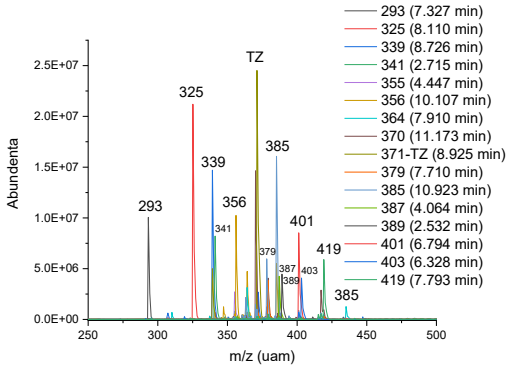


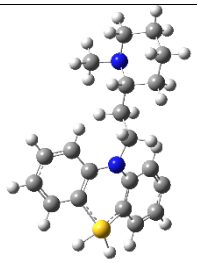
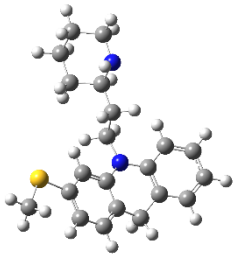
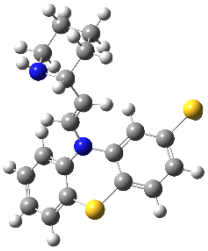
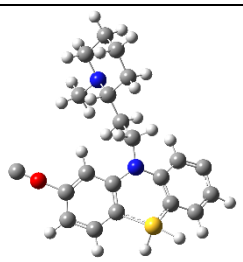
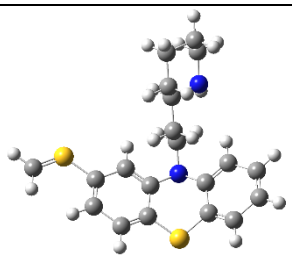
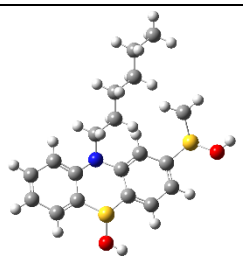
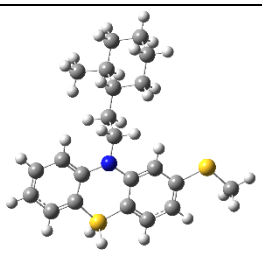
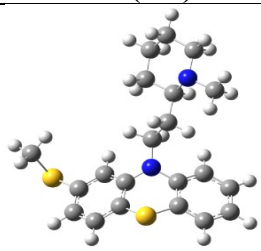
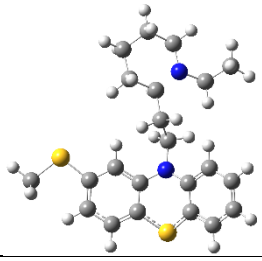
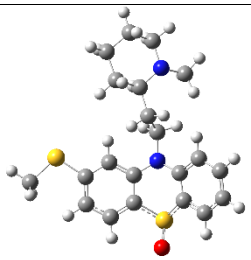
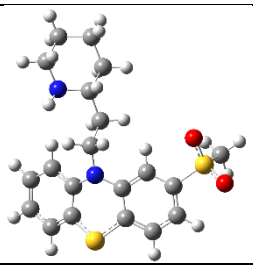
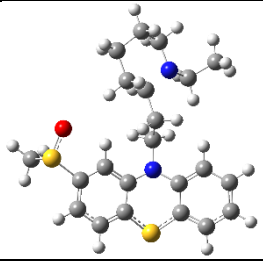
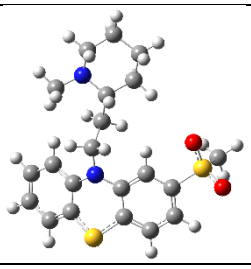
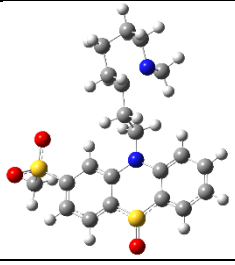
Figure 3. MS spectra of the photoproducts identified in TZ irradiated 120 min.

A total of 15 photoproducts with molecular masses between 290 and 419 aum were identified. Their retention times are found next to their molecular masses in Figure 3. Also, for each photoproduct, the absorbance spectra and the fluorescence spectra were extracted.

6. Determination of the molecular structure of the photoproducts.

Table 7 presents the proposed molecular structures of the photoproducts resulting from irradiation of the 266 nm TZ solution. Molecular structures were confirmed by Gaussian09 and GausView. Each structure was calculated using the Hartree-Fock method with a 3-21G basis set, but with no restrictions on temperature and environment. After the calculation was completed, the density functional theory (DFT) was applied together with the functional basis B3LYP and the basic set 6-31G (d, p), this method being known as a method that is reliable for organic structures of the molecules.

Table 7. Proposed molecular structures of optimized photoproducts using Gaussian09. Caption: gray - C, white - H, blue - N; yellow - S and red - O.

292	**324**	**338**	**340**
Not identified			
354	**355**	**363**	**369**
			
370 (TZ)	**378**	**384**	**386**
	Not identified		
388	**400**	**402**	**418**
			

7. Comparison of the results obtained with the JustTLC program, by HPTLC densitometry and by QTOF LC-MS and method validation.

The unirradiated and irradiated TZ solutions were applied to HPTLC plates in the form of bands and were analyzed by HPTLC densitometry and the JustTLC program. QTOF LC / MS was used to identify the molecular masses of the photoproducts.

The densitometric method proposes the study of the fluorescence of photoproducts from HPTLC plates. This method provides a simple, accurate and reproducible quantitative analysis for determining the photoproducts obtained from the irradiation of TZ with 266 nm.

Figure 4 shows the HPTLC plate containing the unirradiated TZ solution and the irradiated TZ solutions between 1 min and 240 min viewed at 254 nm lamp. Figure 4a shows all the photoproducts that could be analyzed by densitometry, and Figure 4b shows the same plate introduced in the JustTLC software.

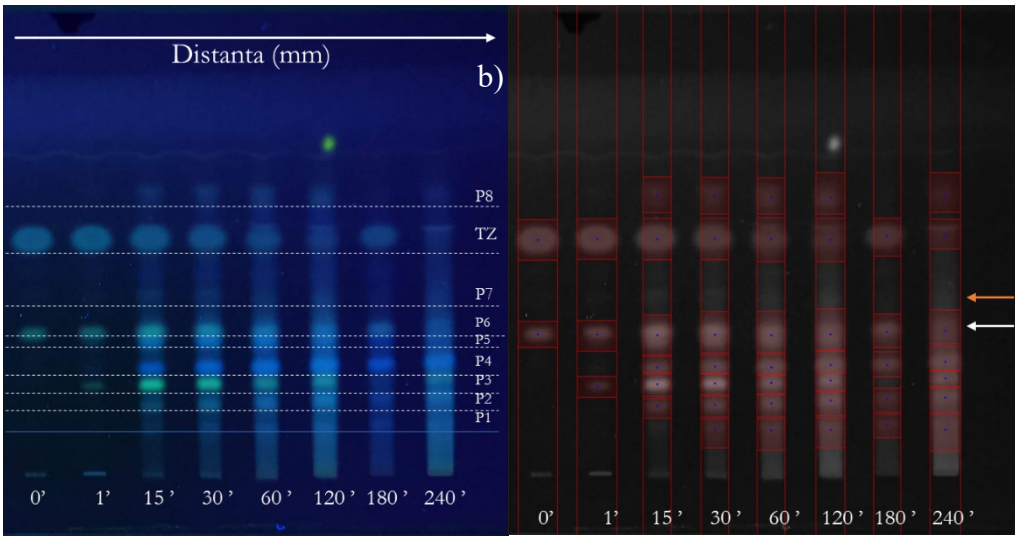


Figure 4. a) HPTLC plate exposed at 254 nm and photographed. b) The same plate introduced in the JustTLC software.

It can be observed that the TLC software is not as accurate as the densitometric method: the P7 product could not be identified (Figure 4, yellow arrow), and the photoproducts P5 and P6 were considered as a single photoproduct (Figure 4b, white arrow).

Also, the precision study for the two methods showed that JustTLC has an %RSD values much greater than 2.

Comparing HPTLC densitometry with QTOF LC/MS it results the following:

1. HPTLC densitometry is more advantageous because of its simplicity, flexibility, accessibility and the fact that is cheaper.
2. QTOF LC/MS is more accurate and can identify more photoproducts than HPTLC densitometry, but the costs are much higher.
3. Through QTOF LC/MS the fluorescence spectra cannot be obtained for photoproducts that do not spend enough time in the FLD detector.
4. HPTLC densitometry offers a much faster discrimination of photoproducts than QTOF LC/MS, where method optimization can take a long time.
5. Even though HPTLC densitometry is not as accurate as QTOF LC/MS, it can be successfully used in the analysis of irradiation solutions if there is no possibility of LC/MS analysis.
6. HPTLC densitometry can be used for any type of photoproducts provided that the excitation source is selected accordingly.
7. HPTLC densitometry can provide fluorescence lifetime information, measurements that cannot be performed by QTOF LC/MS or JustTLC.

8. Conclusion

During this stage, the experimental system for HPTLC densitometry - LIF and LIF lifetime analysis was developed.

The unirradiated TZ and CIP solutions were applied on HPTLC plates using Linomat 5 and linearity, precision and LOD/LOQ were determined by recording LIF spectra and LIF lifetime. The LIF lifetime was determined only for precision because it does not depend on the concentration of the samples. Also, the plates viewed at 254 nm were analyzed with JustTLC program to compare the two methods. HPLTC densitometry has been shown to be superior to that used in the JustTLC program.

Furthermore, TZ irradiated solutions were applied on an HPTLC plate to determine the spectral characteristics of the photoproducts. The fluorescence spectra and fluorescence lifetime were determined for all photoproducts, respectively for all irradiation times. More, the unirradiated TZ and CIP solutions and the 120 min TZ solution were analyzed by QTOF LC/MS and the absorbance, fluorescence and mass spectra of the photoproducts were determined. As a result of mass spectra, possible molecular structures of the photoproducts have been proposed.

In this way the project objectives for this stage have been fulfilled and the estimated results have been obtained.

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