Scientific Report

regarding the implementation of the project between January – April 2020 (Stage III)

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: optical characterize (laser-induced fluorescence and lifetime of fluorescence) of irradiated ciprofloxacin solutions between 1 min and 240 min with 266 nm laser, * LC-TOF / MS measurements for the irradiated ciprofloxacin solutions, and * determine the molecular structure of the photoproducts resulting from the irradiation process of ciprofloxacin solutions.

Within this stage the following were realized:

1. Experiments using the HPTLC developed system on irradiated CIP, those for which the best antimicrobial effect was obtained.
2. LC-TOF/MS experiments on selected irradiated CIP solutions.
3. Determine the molecular structure of the photo-products resulted from CIP irradiation.

1. Materials and Methods

The compound studied was ciprofloxacin - CIP (99.5%) in hydrochloride form. The CIP powders were dissolved in ultrapure water at a concentration of 2 mg/mL. The irradiation 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 ns pulse width and 10 Hz repetition rate).

The analysis of the generated photoproducts was performed by HPTLC densitometry. 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 dichloromethane: methanol: 25% ammonia (4:2:0.85, V:V:V).

The experimental system required for HPTLC densitometry measurements consisted of a laser control unit, laser diode, XY translational stage, optical fiber, 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.

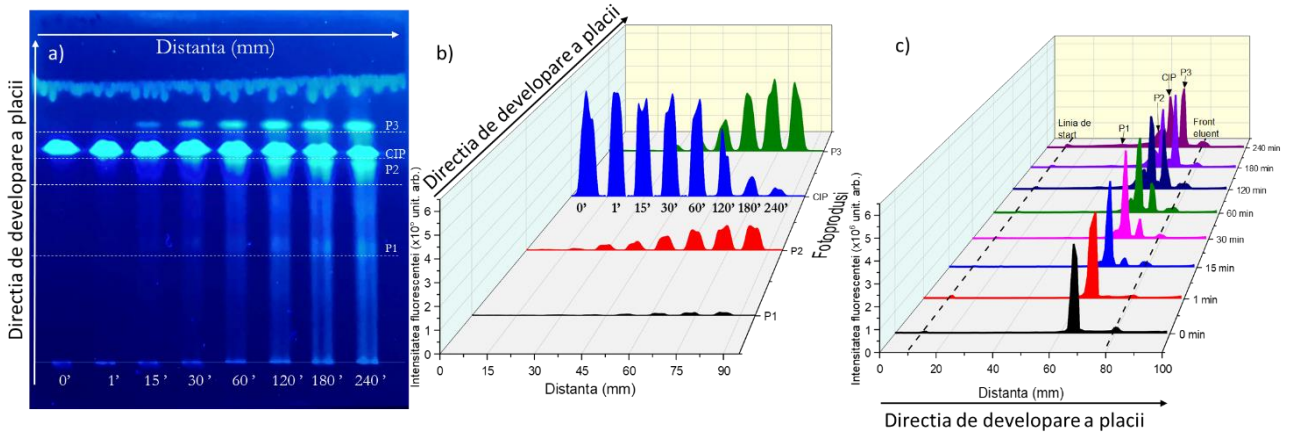
Laser induced fluorescence (LIF) was recorded with a spectrograph (Acton Research, SpectraPRo SP-2750) coupled with an ICCD camera used to detect and analyze the radiation emitted. For fluorescence lifetime studies it was recorded with a photomultiplier (Hamamatsu H-6780-02) whose output signal is coupled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

Fluorescence spectra were recorded along the OX and OY direction with a 1 mm pitch. The fluorescence maxima were extracted and plotted as a function of the distance, obtaining the horizontal chromatogram of the investigated drugs.

2. Experiments using the HPTLC developed system on irradiated CIP, those for which the best antimicrobial effect was obtained.

For this experiment, unirradiated and irradiated CIP 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 from phase II of the project. The plate was investigated by time-resolved fluorescence and fluorescence spectroscopy. All the photoproducts were investigated and the horizontal and vertical chromatograms were obtained.

The HPTLC plate containing bands with unirradiated and irradiated CIP 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 CIP solution with 266 nm was observed. For this analysis, three photoproducts were viewed and analysed with the densitometric system. The horizontal and vertical chromatograms of CIP and its photoproducts are shown in Figure 1b and 1c.



Figur2 1. HPTLC plate containing the unirradiated and irradiated CIP, viewed at 254 nm. b) Horizontal chromatogram for CIP and its photoproducts, which resulted from laser induced fluorescence analysis of HPTLC plate. c) Vertical chromatogram for CIP and its photoproducts, which resulted from laser induced fluorescence analysis of HPTLC plate.

In Figure 1b it is observed that as the CIP fluorescence decreases, respectively the concentration, the fluorescence of the photoproducts changes. Further, the spectral characteristics of the photoproducts were studied. After the first minute of irradiation the photoproduct P3 is formed in the irradiated solution. After 15 min of exposure at 266 nm, P2 is beginning to form and after 30 min P1. The wavelengths of the fluorescence maxima are as follows: P1 - 477 nm, P2 - 486 nm, and P3 - 493 nm.

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

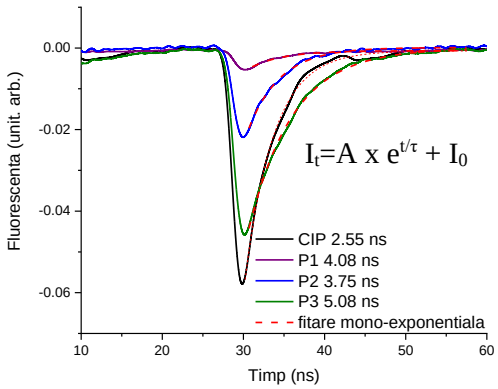


Figure 2. Transient fluorescence signals in the case of CIP and its photoproducts resulting from 375 nm excitation.

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 CIP is 2.55 ns and is approximately the same as that of CIP obtained during stage II of the project. This confirms the fact that the study of fluorescence lifetime of the compounds in the HTPLC plates provides reliable results.

3. LC-TOF/MS experiments on selected irradiated CIP solutions.

The irradiated CIP solution with a laser beam emitted at 266 nm for 30, 60, 120, and 240 minutes were analysed by LC-DAD-FLD to optically characterize the photoproducts (absorbance and fluorescence spectra).

Based on the absorbance measurements, the chromatograms of the samples were obtained and the retention times were extracted for CIP and its photoproducts (Figure 3)

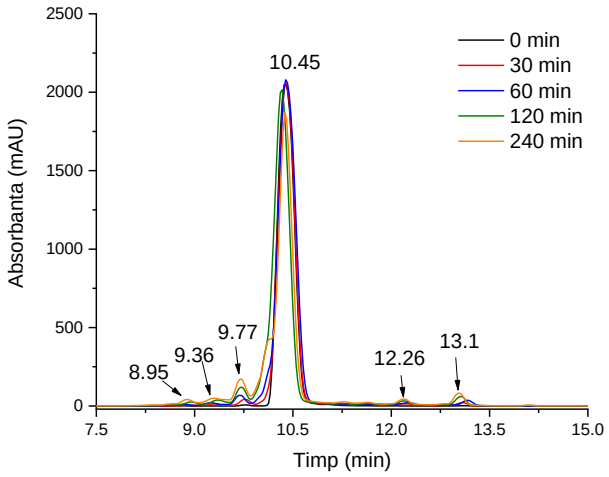


Figure 3. HPLC-DAD chromatograms at 278 nm for unirradiated CIP, irradiated CIP for 30, 60, 120, and 240 minutes.

A total of 5 photoproducts with retention time between 8.5 and 13.1 min were identified. Their absorbance and fluorescence spectra are presented in Figure 4.

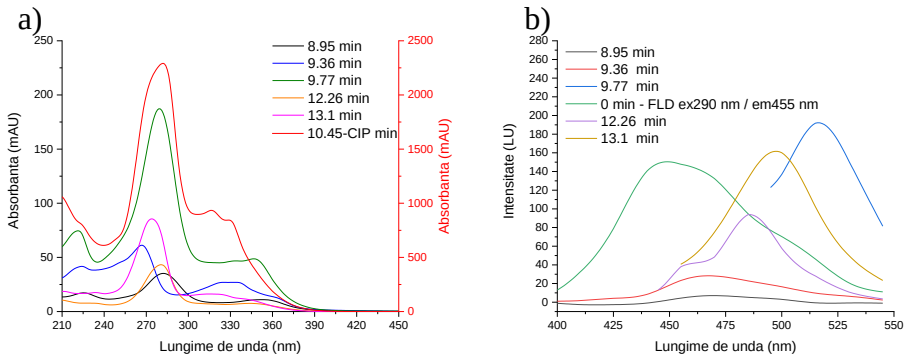
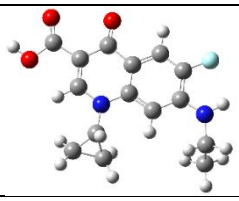
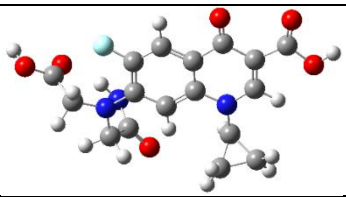
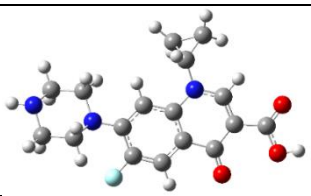


Figura 4. a) Absorption spectra of CIP and its photoproducts. b) Fluorescence spectra of CIP and its photoproducts.

4. Determine the molecular structure of the photo-products resulted from CIP irradiation.

Table 1 presents the proposed molecular structures of the photoproducts resulting from irradiation of CIP with 266 nm pulsed laser beam. Molecular structures were confirmed by Gaussian09 and GaussView. 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 1. Proposed molecular structures of optimized photoproducts using Gaussian09. Caption: grey - C, white - H, blue - N; light blue - F and red - O.

290	**377**	**331CIP**
		

5. Conclusions

During this stage, the unirradiated and irradiated CIP solutions were applied on HPTLC plates using Linomat 5 and their spectral characteristics were determined by recording LIF spectra and LIF lifetime. By processing the fluorescence spectra, the horizontal and vertical chromatograms were obtained.

More, the solutions were analysed by QTOF LC/MS and the absorbance and fluorescence spectra of the photoproducts were determined and 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.

Principal Investigator,

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