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EVALUATION OF THIN LAYER CHROMATOGRAPHY IMAGE ANALYSIS METHOD FOR IRRADIATED CHLORPROMAZINE QUANTIFICATION

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Abstract. We report the use of TLC image analysis software, JustTLC, for identification and quantitative evaluation of the photoproducts generated by the irradiation of Chlorpromazine water solutions with a 266 nm laser beam. The percent concentrations resulting for photoproducts from TLC analysis were compared with the relative concentrations of those identified by LC-TOF/MS measurements. The spots on the analyzed TLC plate were attributed to the corresponding photoproducts. Although LC-MS methods are more accurate, TLC can be successfully used to quantify the photoproducts obtained after exposure to UV laser radiation.

Key words: TLC, Chlorpromazine, photo-degradation, image analysis.

1. INTRODUCTION

A solution that contains a mixture of compounds is difficult to analyze by spectroscopic methods such as UV-Vis-NIR spectrophotometry and FTIR spectroscopy, because individual separation is required in order to perform a complete analysis. If liquid chromatography-mass spectrometry methods are not available, so that the individual compounds cannot be separated, an alternative that offers quantitative and qualitative measurements is Thin Layer Chromatography (TLC).

TLC was traditionally considered a simple and inexpensive separation method and is used to visualize and identify substances. From the methodological standpoint, TLC is the simplest conventional chromatographic technique and the materials can be obtained at a relatively small cost [1–3]. TLC is a subset of liquid chromatography, where the mobile phase is a liquid and the stationary phase

consists of a thin layer of absorbent surface. The solvent or mobile phase is the transport medium for the samples to separate when the substance migrates along the stationary phase due to capillary forces [4]. Image analysis software used in analyzing the pictures of fluorescent TLC plates proved to be fast and to offer a quantitative and qualitative evaluation of the UV-Vis absorbing compounds [5, 6]. In Ref [5] is reported the evaluation of four different image analysis software packages out of which JustTLC proved to be the most sensitive and precise.

Phenothiazines belong to the neuroleptic drugs class and are used in the therapy of mental disorders, mainly in the treatment of psychoses such as schizophrenia and mania, as well as in disturbed behavior [7], modulating in the nervous system the activity of the membrane receptors. Many of the drugs used nowadays in medicine originate from the chemical manipulation of Phenothiazines [8]. Apart of these effects, biological activities of Phenothiazines have been identified in mammalian cancer cells [9] as well as in pathological microorganisms like bacteria [10, 11]. Also, due to their cationic and amphiphilic characteristics, the Phenothiazines can easily penetrate into artificial and natural membranes and change their physico-chemical properties [12]. Recent studies show that exposure of Phenothiazines and of other different compounds to UV laser beams induces modifications of the parental compound [13–21], generating photoproducts with biological activity against bacteria and cancer cells [22, 23].

This paper presents an evaluation of the JustTLC image analysis software in the TLC quantitative evaluation of irradiated Chlorpromazine with a 266 nm laser beam up to 120 min. The results were compared with previous data concerning the relative concentrations of the photoproducts obtained by LC-TOF/MS methods.

2. MATERIALS AND METHODS

The investigated compound is the phenothiazine derivative Chlorpromazine (CPZ) having a purity of 98.9% (Sigma Aldrich). The chemical structure of CPZ is depicted in Fig. 1; it has a chlorine atom at the 2nd position of the Phenothiazine ring and an alkyl-amino side chain at the 10th position.

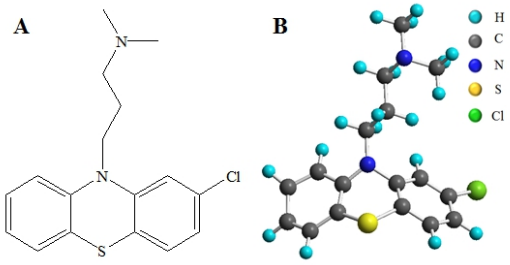

Fig. 1 – Chemical structure of Chlorpromazine: A) 2D; B) 3D.

A 2 ml volume of CPZ dissolved in ultrapure water at 2 mg/ml was irradiated with a 266 nm pulsed laser beam (fourth harmonic of the Nd:YAG laser, Excel Technology, Surelite II) at an average energy of 6.5 mJ per pulse. The characteristics of Nd:YAG laser pulses are 6 ns FWHM and 10 Hz pulse repetition rate. The experimental set-up and the irradiation protocol is presented in detail elsewhere [24]. The irradiation was carried out for different exposure times: 1, 5, 15, 60, and 120 min.

For application of the TLC technique, it was used a 0.25 mm aluminum plate pre-coated with silica gel $60F_{254}$ (DC-Alufolien silica gel $60F_{254}$, Merck Darmstadt, DE) as stationary phase; as mobile phase a mixture of acetone: methanol: 25% ammonia (50:50:1, V:V:V) was used. To facilitate a saturated vapor atmosphere, filter paper was placed along the walls of the developing thank. In order to be able to view as many photoproducts as possible, 1 ml of CPZ aqueous solution, unirradiated as well as irradiated, was placed in a desiccator which contains silica gel obtaining finally a CPZ concentration 10-fold greater than the initial one. A total amount of 1µl of concentrated solution was applied on the plate. The TLC plate was developed at room temperature until the mobile phase traveled about 8 cm from the starting line. After the chromatographic plate was dried it was visualized under UV light at 254 nm using a darkroom viewing cabinet (Chromo-Vue®, Cabinet C-65, UVP®) equipped with two lamps, UVLS-28 EL (365/ 254 nm) and UVLS-18 EI (254 nm/white light), then photographed using a digital camera Nikon D80 and analyzed using JustTLC (Sweday) image analysis package. The data extracted using JustTLC was compared with the relative concentrations of the photoproducts identified by LC-TOF/MS, the detailed analysis of LC-TOF/MS being presented elsewhere [24].

3. RESULTS AND DISCUSSIONS

TLC allows the visualization, identification and direct comparisons of the photoproducts generated by the irradiation of CPZ to 266 nm laser beam and JustTLC offers a qualitative and quantitative analysis opportunity of each photoproduct. Fig. 2 shows the migration and the separation of irradiated CPZ solution in individual compounds or classes of compounds having the same polarity on the TLC plate, visualized at 254 nm. Each column of the TLC plate corresponds to a specific irradiation time, the first column representing the unirradiated sample followed by CPZ irradiated for 1, 5, 15, 60 and 120 minutes.

All the identified photoproducts are much polar than CPZ, their polarity increasing from top to bottom, as highlighted by the arrow in Fig. 2. The photoproducts with the highest polarity are those found near the origin (dotted line) and can be observed in the samples irradiated from 5 up to 120 min. After only 1 min of exposure to UV laser radiation three photoproducts can be visible on the

TLC plate. Also, after irradiating CPZ for 60 min, the parental compound is no longer present in solution and, therefore, CPZ is photo-degraded completely. The generation of photoproducts is not linear and depends on the irradiation time interval. The number and concentration (intensity) of each photoproduct is increasing or decreasing during irradiation due to the competition between the various photo-chemical processes which occur during irradiation.

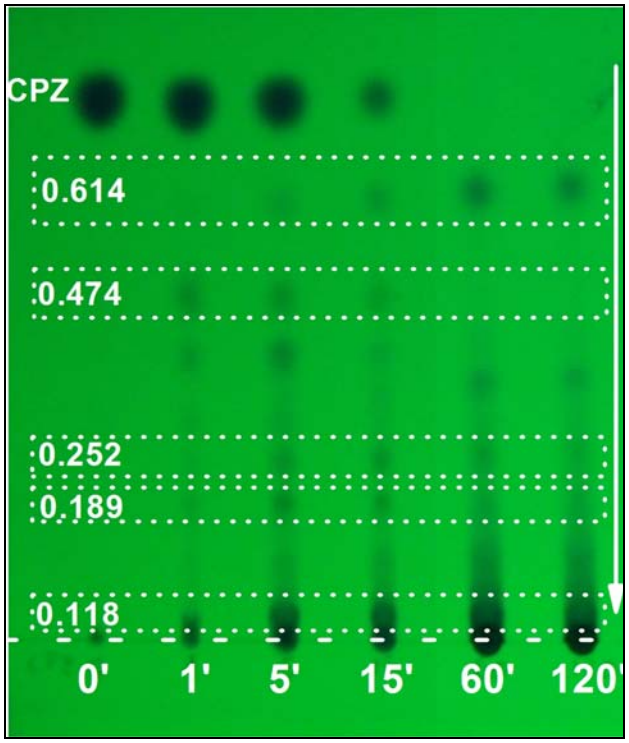

Fig. 2 – TLC plate with unirradiated and irradiated CPZ for 1, 5, 15, 60, and 120 min, visualized at 254 nm (online color).

The image was introduced in the TLC image analysis program JustTLC where the color input image was transformed into an intensity (grayscale) image and a qualitative analysis of the plate was performed. The retention factor values and real volume of the samples were extracted.

Assuming the starting line (origin) as 0 and the front line of the developing solvent as 1, the retention factor, R_f, of photoproducts can be calculated. The retention factor is defined as the distance traveled by each spot divided by the distance traveled by the solvent. Thus, CPZ has an R_f value of 0.744, followed by eight photoproducts having the following values: 0.614, 0.477, 0.376, 0.304, 0.252, 0.189, 0.118 and 0.023. In Ref [17] it was proved that the compound with an R_f

value of 0.614 is Promazine (PZ), which is another phenothiazine derivative formed by the cleavage of the C-Cl bond of CPZ under UV laser radiation [24].

The real volume is obtained by summing up the difference between the estimated background and the noise filtered input image within each defined band region. The level of filtering is based on the noise level given during analysis of the plate [25]. The real volume of unirradiated CPZ was considered the reference (100%) and the relative concentrations (estimated from the real volume of the spots) of photoproducts were determined by comparing it to the parental compound. In order to evaluate the TLC image analysis of the tested samples, a comparative study was performed between the relative concentration of the photoproducts identified *via* LC-TOF/MS in Ref. [24] and the relative concentration of the compounds visualized at 254 nm and determined *via* JustTLC.

Using LC-TOF/MS, extremely precise measurements (< 5 ppm) of molecular masses were obtained by TOF detection and the identified photoproducts were: PZ, promazine sulfoxide (PZ-SO), 2-hydroxy promazine (PZ-OH), 2-hydroxy promazine sulfoxide (PZ-OH-SO), chlorpromazine sulfoxide (CPZ-SO), and three other compounds with m/z values of 292, 300, and 308 amu labeled P1, P2, and P3, respectively [24]. The molecular structure of the compounds are presented in Ref. [24].

In Fig. 3 a comparison is shown between the distribution in time of the relative concentration resulting from LC-TOF/MS measurements (continuous line) and the relative concentration resulting from the JustTLC analysis (dotted line) of the unirradiated CPZ and the CPZ irradiated for 1, 5, 15, 60, and 120 min. The correlation between the known compounds and the spots representing photoproducts separated on the plate is proposed.

In Fig. 3A are presented the dynamics in time of the relative concentrations for the unirradiated CPZ (0 min) and the remaining CPZ in the irradiated sample after different exposure time intervals to UV laser radiation. Thus, the LC-TOF/MS analysis shows that after 120 min of irradiation there is no CPZ left in the sample and the JustTLC analysis shows the same, but for 60 min irradiation time. This difference appears because TLC is not as accurate as LC-TOF/MS, the limit of detection being lower in TLC compared to that of LC-TOF/MS. The dependence in time of concentrations is the same, both decreasing almost in the same manner.

The curves in Fig. 3B suggest that PZ is located at an R_f value of 0.614, both graphs having the same time profile. The same behavior can be observed for all the samples; thus, for every R_f value of each observable individual spot on the TLC plate visualized at 254 nm, the corresponding photoproduct was assigned. The photoproduct CPZ-SO is situated at 0.474, P_1 at 0.252, PZ-OH-SO at 0.189 and PZ-OH/PZ-SO at 0.118. The assignment of the R_f values to these compounds is also supported by the fact that polarity is increased by the addition of OH groups or H atoms to the molecular structure of a substance due to H bonding with the silicagel substrate [26].

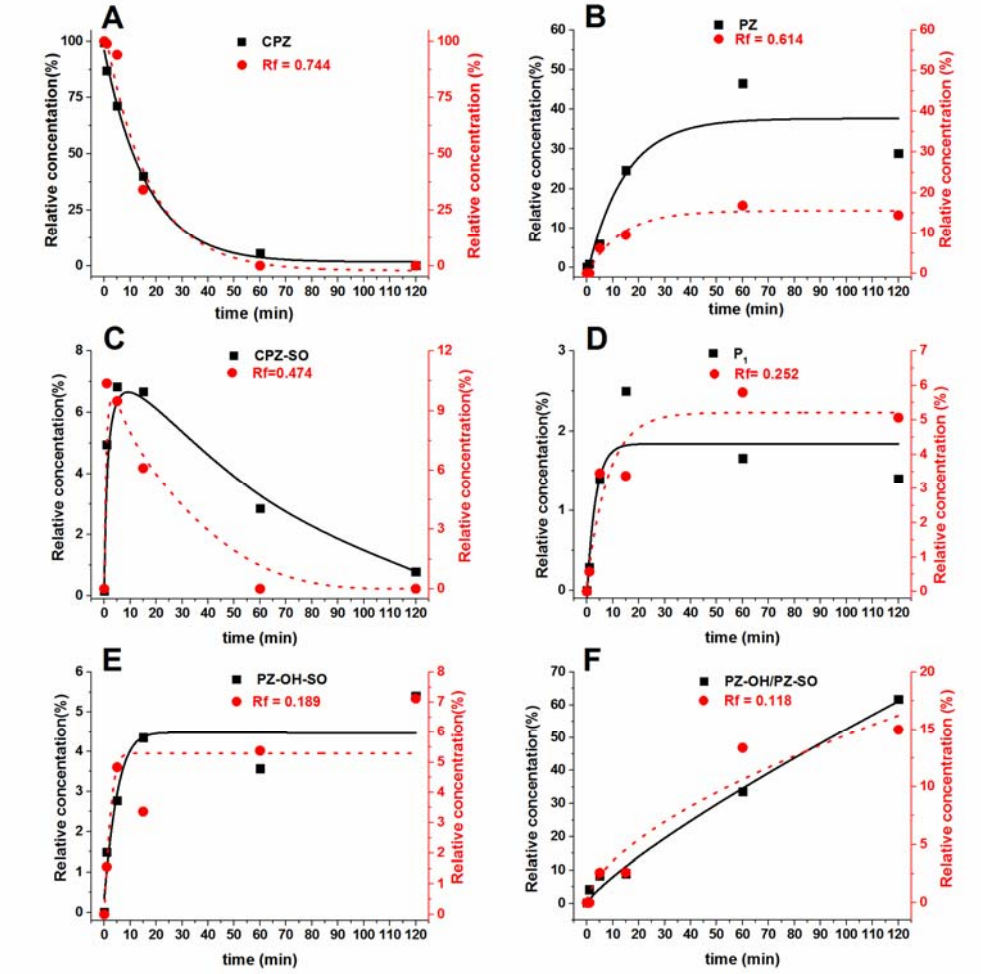

Fig. 3 – The evolution of the relative concentration of the photoproducts identified *via* LC-TOF/MS (continuous line) and *via* JustTLC (dotted line); online color.

4. CONCLUSIONS

The TLC plate visualization at 254 nm coupled with image analysis software was used to obtain a qualitative determination of the photoproducts generated by the irradiation of a water solution containing CPZ with a laser beam at 266 nm. A corresponding structure for each spot from the TLC plate, having a specific R_f value was proposed.

Comparing the results obtained in this paper using TLC image analysis, with those from the LC-TOF/MS analysis, one can validate the use of TLC image analysis as a method for the qualitative evaluation of compound mixtures resulted after the interaction of CPZ solution with the UV laser beam.

The paper reports that it is possible to use TLC image analysis software, like JustTLC, to get reliable qualitative results.

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