Vol. (2), No. (1), January-March; 2025. DOI: -10.21608/MERMJ.2025.374529.1024

Densitometric HPTLC Method for the Simultaneous Determination of Novel 1-Coumarinyl-1,2,3-Triazole Derivatives.

Gehan A. Abdel-Hafez [1*], Ahmed S. Aboraia [2]

[1] Medicinal Chemistry Department, Faculty of Pharmacy, Merit University, Sohag 82755, Egypt.
[2]Medicinal Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt.
*Corresponding author

ARTICLE INFO

Article history:
Received 13 April 2025
Accepted 1 June 2025
Available online 18 June 2025

Keywords:
3-Azidocoumarin; HPTLC.
Spectrophotometric.
Spectrofluorometric.
propargyl derivatives.

Abstract

HPTLC-densitometric techniques show considerable promise as alternative chromatographic models for determining partitioning parameters in complex mixtures comprising inorganic, organic, and biomolecular components. In this study, five newly synthesized 1-coumarinyl-1,2,3-triazole derivatives were effectively separated using silica gel 60 F254 plates as the stationary phase. Three mobile phase systems, chloroform: methanol (9:1 v/v), hexane: ethyl acetate (3:2 v/v), and hexane: ethyl acetate (1:1 v/v), were employed, resulting in well-resolved compounds with R_f values ranging from 0.27 to 0.66. Validation of the developed methods was carried out in compliance with ICH standards. Linearity was observed across the range of 50–500 ng/band, with correlation coefficient (R^2) values between 0.9923 and 0.9991, indicating excellent linearity. The high selectivity and specificity of the methods ensured the accurate separation of the synthesized compounds in their pure form, free from any interference by starting materials or intermediates. Limits of detection (LOD) for the target compounds ranged from 17.5 to 47.8 ng/spot, while limits of quantification (LOQ) were 52.6 to 144.8 ng/spot using spectrophotometric detection and 47.5 to 96.8 ng/spot using spectrofluorometric detection. These findings highlight the applicability of the developed HPTLC methods for both qualitative and quantitative analysis of this compound series, even at concentrations as low as 10^{-9} g in biological fluids.
.

1. Introduction

Coumarins (2H-chromen-2-one) are naturally occurring compounds found in significant quantities in plants, though they have also been identified in certain microorganisms and animal sources. This diverse group of compounds has attracted significant scientific attention because of their broad spectrum of biological activities. Coumarins are widely recognized for their diverse pharmacological activities, including antibacterial, antifungal,

anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticancer effects. [1–5] In addition to their therapeutic applications, coumarins are employed as additives in food and cosmetic products and are also used in optical brightening agents. [6, 7] Their anticancer potential is attributed to multiple mechanisms, such as telomerase inhibition,[8] suppression of protein kinase activity, downregulation of oncogene expression, and activation of caspase-9-mediated apoptotic pathways. Furthermore, coumarins have been shown to impede cancer cell proliferation by inducing cell cycle arrest at both the G0/G1 and G2/Ms (9), as well as by modulating P-glycoprotein (P-gp) activity in cancer cells. The incorporation of triazole rings, with their extended π-conjugation systems, can also introduce fluorescence into otherwise non-fluorescent coumarin structures, offering a strategic advantage for labeling and tracking synthesized derivatives.

In this context, our study focuses on the synthesis and optimization of 1,4-disubstituted-1,2,3-triazole derivatives **VI–X** via a click reaction involving cycloaddition of various bioactive subunits—6-methyluracil, uracil, phenytoin, naproxen, and valproic acid **I–V** with 3-azidocoumarin, as outlined in **Scheme 1**. The structures of the synthesized compounds were confirmed through elemental analysis and a variety of spectroscopic techniques, including FT-IR, ^{1}H-NMR, ^{13}C-NMR, HMBC, and mass spectrometry.[10]A primary goal of this study was to unequivocally identify compounds **VI–X**, ensuring no interference from starting materials or intermediates. Interestingly, both 3-azidocoumarin and the propargyl derivatives are inherently non-fluorescent; however, upon formation of the triazole ring, the resulting compounds **VI–X** displayed pronounced fluorescence, as illustrated in **Figure 1**. [11] This transformation provides a simple and effective means of detecting structurally related analogs and evaluating the presence of residual precursors or intermediates.

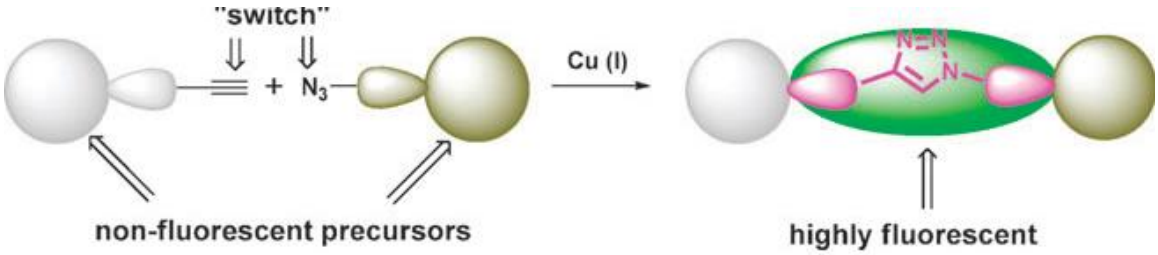
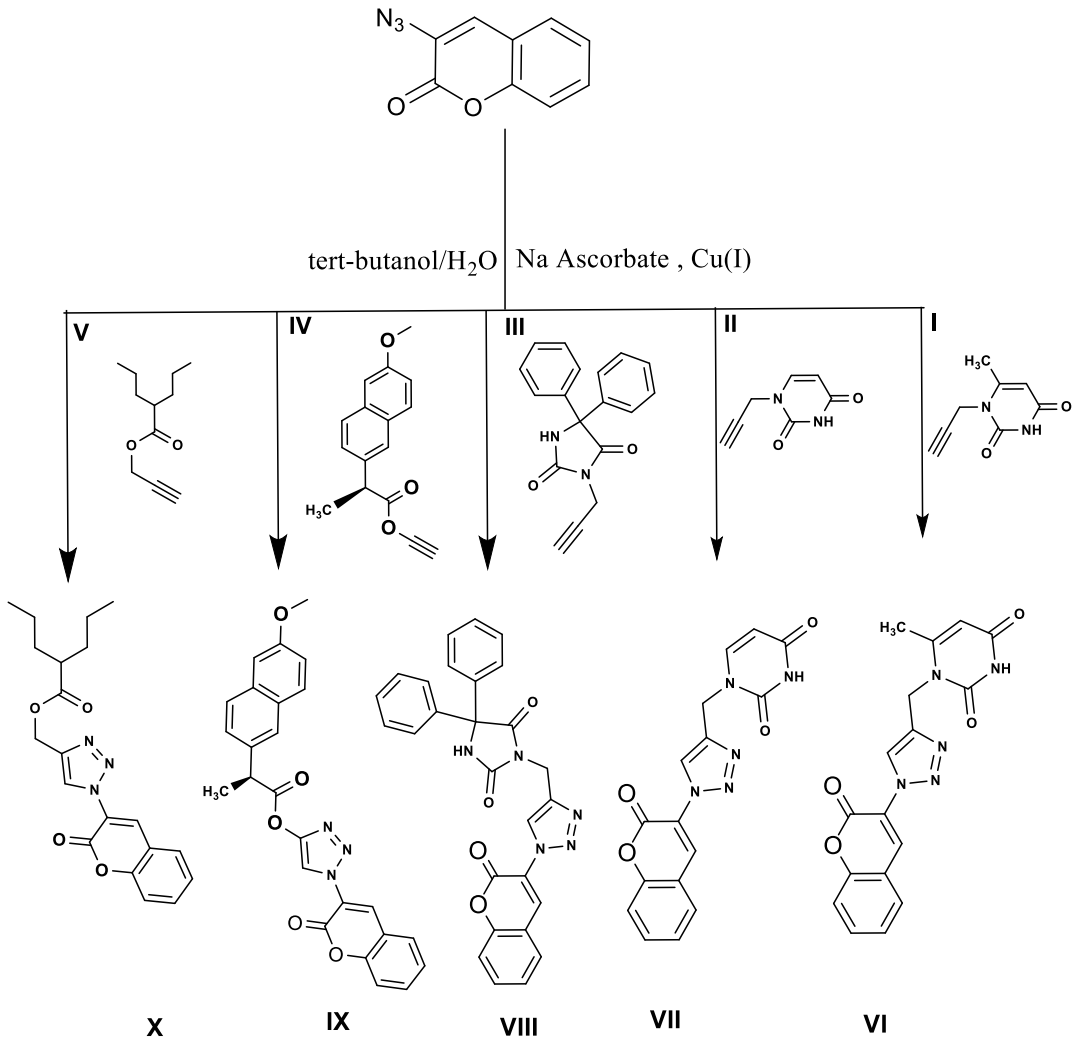


Figure 1: Schematic representation of a fluorogenic CuAAC reaction.



Scheme 1: The copper(I)-catalyzed 1,3-dipolar cycloaddition between non-fluorescent 3-azidocoumarins and terminal alkynes resulted in the formation of highly fluorescent 1,2,3-triazole derivatives **VI-X.**

2. Material and methods

2.1. Instrumentation

A CAMAG TLC Scanner 4, which can scan by absorbance and fluorescence over a wavelength range of 190–900 nm, was used to create the method. With its deuterium, tungsten-halogen, and mercury vapour lamps, the system offers a variety of detecting choices. Data resolution can be set to 100 µm per step, and the scanning speed can be adjusted up to 20 mm/s. The winCATS program (version 1.4.10, CAMAG, Switzerland/Germany) was used for data collection and analysis. The CAMAG Linomat 5 (230V), was used for the sample application, making it possible to apply samples in spot or band format both qualitatively and quantitatively. For accurate sample dosage, a 100 µL Hamilton glass syringe was utilized. Development of the chromatogram was carried out in a CAMAG twin-trough chamber(20 × 10 cm or 10 × 10 cm) fitted with a stainless-steel lid.

2.1. Materials and Reagents

Every reagent and solvent used was of the analytical reagent grade. We acquired HPLC-

grade methanol from Sigma-Aldrich Co. in Germany. Fluka Co. provided pure ethyl acetate, hexane, and chloroform. Pure compounds **VI–X** were synthesized by using the method shown in Scheme 1. Merck Darmstadt, Germany, provided high performance thin-layer chromatography aluminum sheets precoated with silica gel plates 60 F254 (20 x 20 cm, 6–8 µm particle sizes, 250 µm thickness).

2.2. Preparation of sample solutions

In a 100-milliliter volumetric flask, 10 milligrams of each of compounds **VI–X**, carefully weighed, were added and dissolved in around 15 milliliters of methanol. The mixture was shaken for five minutes and then sonicated for five to ten minutes. The solution was then filled to volume with methanol to provide a stock solution containing 100 µg/ml of any of the compounds being studied. The working solutions were prepared in individual 10 ml volumetric flasks by further diluting suitable volumes (0.5–10 ml) of stock solutions with methanol to reach concentrations between 5 and 100 µg/ml, or 10 and 500 ng/spot, for UV and fluorescence measurements, respectively, in accordance with the ranges shown in **Table 1.**

Comp. No	Concentration ranges for UV measurements		Concentration ranges for Fluorescence measurements	
	(µg/ ml)	(ng /band)	(µg /ml)	(ng /band)
VI	15 -100	75 -500	15 -100	75 -500
VII	20-100	100 -500	15 -100	75 -500
VIII	20 -100	100 - 500	20 -100	100 – 500
IX	10 -100	50 - 500	10 -100	50 – 500
X	5 -100	100- 500	5 -100	50 -400

Table 1: Various concentrations of the compounds under study **VI-X** employed for fluorescence and UV measurements, along with the corresponding concentrations in ng/spot values.

2.3. Chromatographic conditions.

Ten milliliters of the suitable mobile phase were added to a TLC development tank lined with thick filter paper to aid in chamber saturation in order to perform the HPTLC–densitometric procedure. Chloroform: methanol (9:1, v/v) was used as the mobile phase for compounds **VI** and **VII**, hexane: ethyl acetate (3:2, v/v) was used for compounds **IX** and **X**, and hexane: ethyl acetate (1:1, v/v) was used for compound **VIII**. Before plate development, the HPTLC tank was covered and allowed to pre-saturate with mobile phase vapors for half an hour at room temperature. HPTLC plates (20 × 10 cm) precoated with silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) were used for the analysis. After being taken out and allowed to dry air for five minutes, the produced plates were examined under a UV lamp. For compounds (**VI–X**), a CAMAG TLC Scanner was used to perform densitometric evaluation in reflectance–absorbance mode at 306 nm and in fluorescence mode at 350 nm. WinCATS software was used to handle data collection and processing. Peak areas derived from diffusely reflected light intensity were used to calculate compound concentrations. Regression models that were built into WinCATS, supported by Microsoft Excel 2016, were used to connect the reported peak regions

with analyte concentrations for additional statistical analysis.

3. Results and Discussion

3.1. Spectral analysis

The absorption spectra of the compounds under investigation were recorded in the 200–400 nm range. A maximum absorption wavelength (λ_{max}) of 306 nm was observed for 3-azidocoumarin as well as for compounds **VI–X**. The excitation and emission wavelengths for 3-azidocoumarin and its derivatives **VI–X** were identified as 306 nm and 350 nm, respectively. Notably, compound **X** exhibited a distinct excitation-emission behavior, with measurements recorded at 306 nm and 311 nm, corresponding to the "0-0" or resonance band where the absorbed energy is re-emitted with little or no change in frequency as presented in **Table 2** and **Figure 2**. In the present study, complete resolution of all analytes was achieved using HPTLC plates, allowing for accurate quantification of each compound at its specific R_f and λ_{max} values

.

Comp. No	λ_{max} for UV measurements	Fluorescence measurement	
		λ_{ex}	λ_{em}
3-azidocoumarin	306	306	350
VI	306	306	350
VII	306	306	350
VIII	306	306	350
IX	306	306	350
X	306	306	311

Table 2: Wavelengths of excitation and emission are used to measure fluorescence.

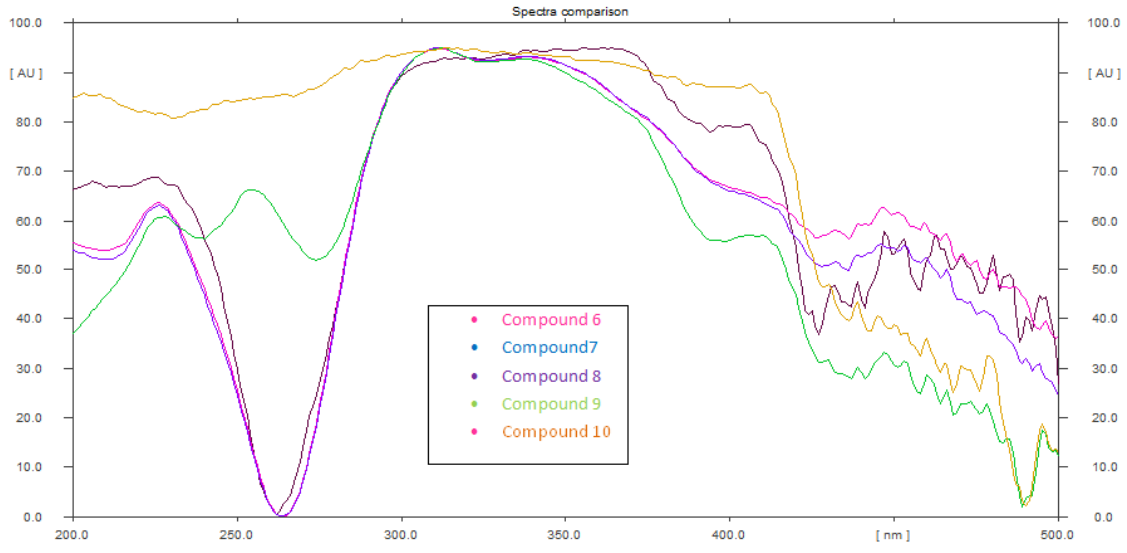


Figure 2: Spectrum of emissions for compounds **VI–X**.

3.2. Optimization of the proposed chromatographic conditions

3.2.1. Mobile phase composition

Owing to the significant differences in polarity and functional groups among the studied compounds, a single mobile phase could not achieve effective separation. Therefore, various mobile phase systems with different solvent ratios were employed to optimize the separation of each compound. The selected mobile phases, detailed in **Table 3** and illustrated in **Figure 3**, provided well-defined spots and satisfactory separation with appropriate R_f values for all analyzed compounds.

Compound	Mobile phase	ratio	R_f
3-azidocoumarin	Hexane: ethylacetate	3: 2	0.82
VI	Chloroform: methanol	9: 1	0.27
VII	Chloroform: methanol	9: 1	0.29
VIII	Hexane: ethylacetate	1: 1	0.35
IX	Hexane: ethylacetate	3: 2	0.47
X	Hexane: ethylacetate	3: 2	0.66

Table 3: Various solvent solutions were utilized to separate the studied compounds **VI-X.**

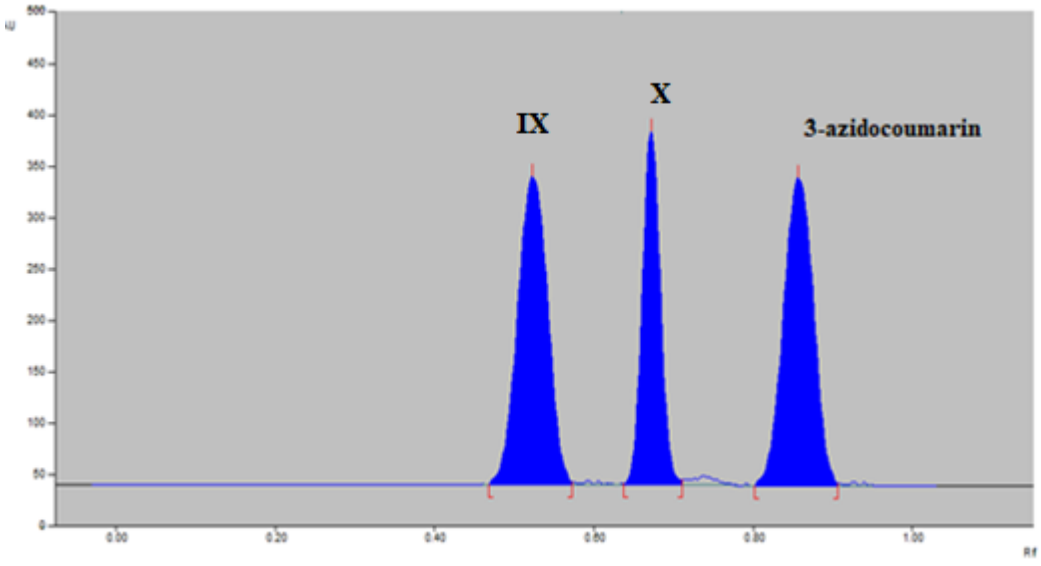


Figure 3: Compounds **3-azidocoumarin**, **IX** and **X** at a concentration level of 600 ng/band in a two-dimensional TLC densitogram using a mobile phase (hexane: ethyl acetate 3:2).

3.2.2. Saturation time

Saturation times ranging from 15 to 60 minutes were evaluated to achieve well-defined and distinctly separated spots for all tested compounds. The optimal results, characterized by high optical intensity and compact spot formation, were observed at saturation times between 25 and 30 minutes. Consequently, a 30-minute saturation time was chosen for all subsequent analyses.

3.3. Validation of HPTLC method

The method was validated in accordance with ICH (Q2R1) guidelines[12] and was also consistent with the requirements of USP 31-NF 26 (13, 14), covering parameters such as linearity, range, limits of detection and quantification, accuracy, selectivity, and specificity. Most calculations and statistical analyses were performed using Microsoft Excel 2016.

3.3.1. Linearity and range of calibration

According to ICH (Q2R1) guidelines on the "Validation of Analytical Procedures," the Analytical range refers to the interval between the lowest and highest concentrations at which the method maintains acceptable precision, accuracy, and linearity. The capacity of the method to yield findings that are exactly proportionate to the analyte concentration within the specified range is known as linearity. To assess linearity, six or more concentration levels analyzed in triplicate were used to construct calibration curves. The concentration range tested was 50 to 500 ng/spot for both UV absorbance and fluorescence detection, as presented in **Table 4.** Linear regression analysis revealed a strong correlation between analyte concentration and peak area, with correlation coefficients (r) ranging from 0.9923 to 0.9991, confirming excellent linearity across the evaluated range. **Figures 4-13** show the three-dimensional representations of the calibration levels for the substances under study as well as the corresponding linear calibration graphs.

Compound No.	Range of linearity (ng/spot)		Correlation coefficient (r)		Intercept (a)		Slope (b)	
	UV	Fluoro.	UV	Fluoro.	UV	Fluoro.	UV	Fluoro.
VI	75-500	75-500	0.9988	0.9991	1935.47	1328.27	12.35	17.14
VII	100-500	75-500	0.9974	0.9987	2959.74	1415.46	19.03	19.02
VIII	100-500	100-500	0.9980	0.9966	86.94	206.23	17.17	14.60
IX	50-500	50-500	0.9979	0.9990	334.46	344.28	18.80	13.34
X	100-500	50-400	0.9923	0.9981	1211.88	1008.41	18.09	30.48

Table 4: Calibration data and analytical parameters of the proposed HPTLC densitometric method for the studied compounds **VI-X.**

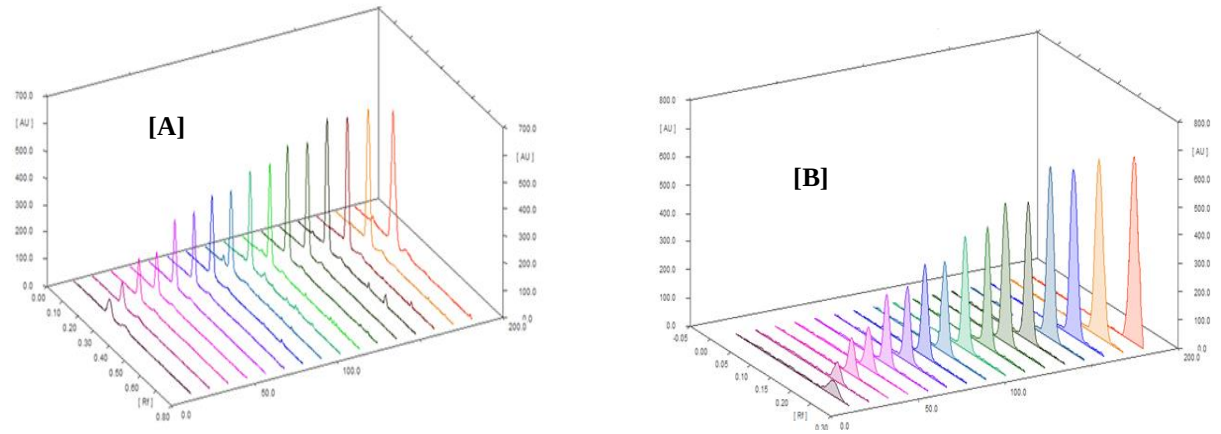


Figure 4: Compound **VI** calibration ranges in 3D HPTLC densitograms utilizing ultraviolet [A] and fluorescence [B] measurements

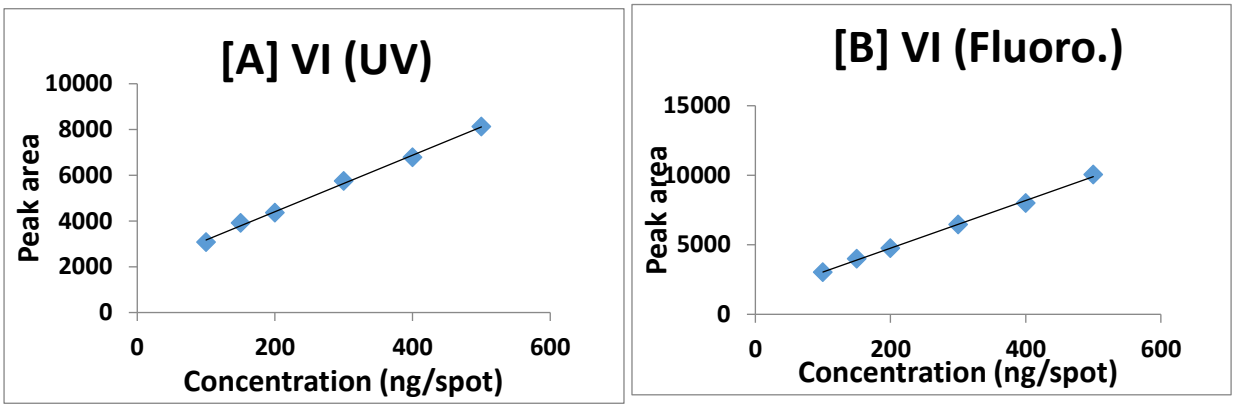


Figure 5: The spectrophotometric [A] and spectrofluorometric[B] linear calibration curves for compound **VI**.

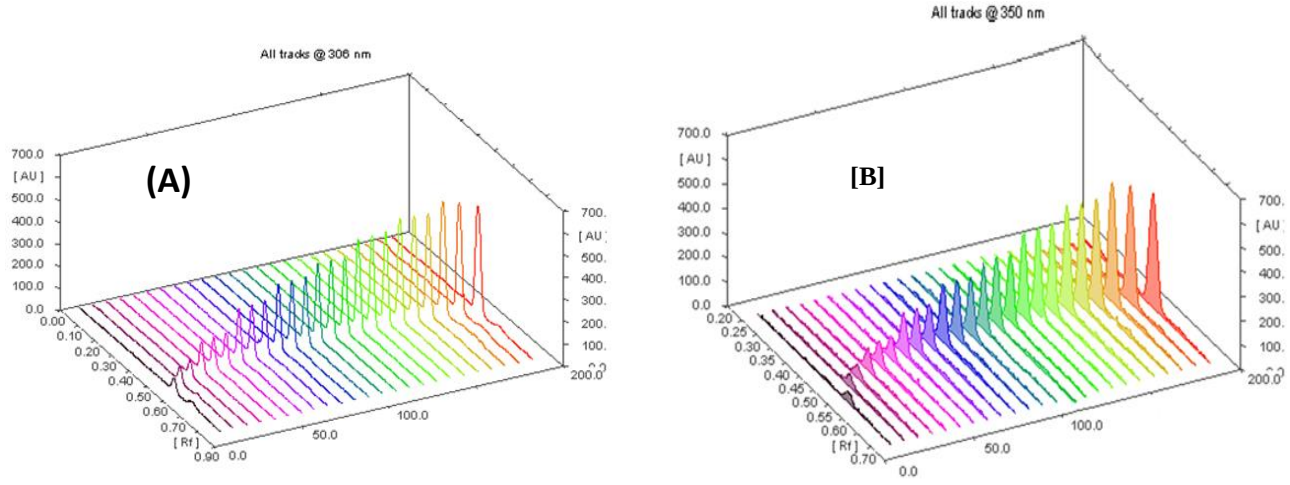


Figure 6: Compound **VII** calibration ranges in 3D HPTLC densitograms utilizing ultraviolet [A] and fluorescence [B] measurements

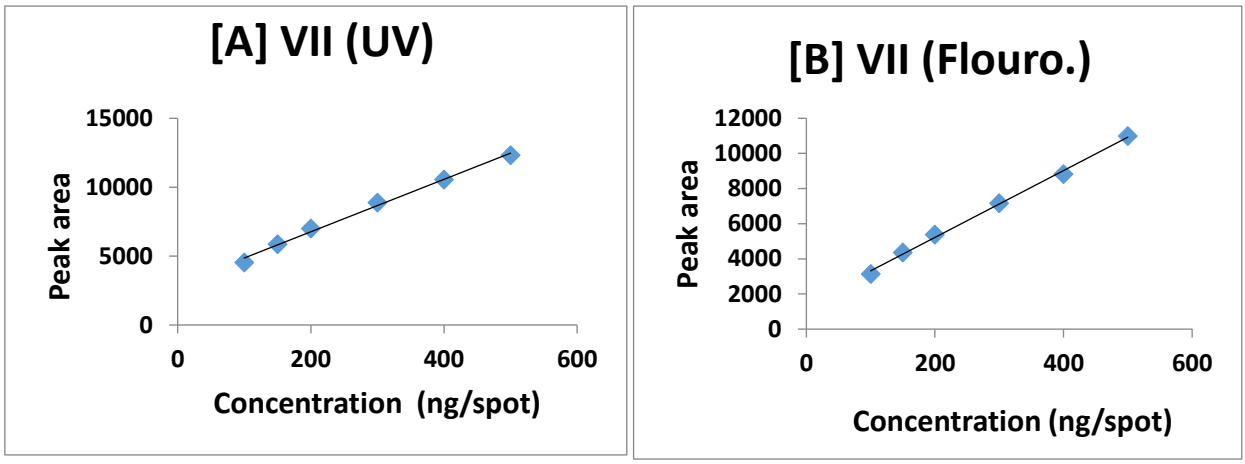


Figure 7: The spectrophotometric [A] and spectrofluorometric [B] linear calibration curves for compound **VII.**

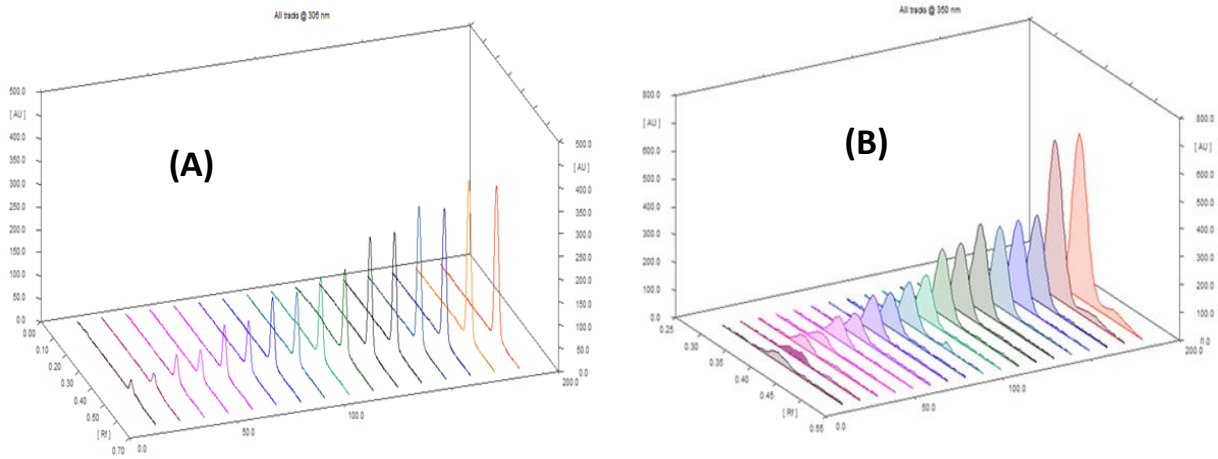


Figure 8: Compound **VIII** calibration ranges in 3D HPTLC densitograms utilizing ultraviolet [A] and fluorescence [B] measurements.

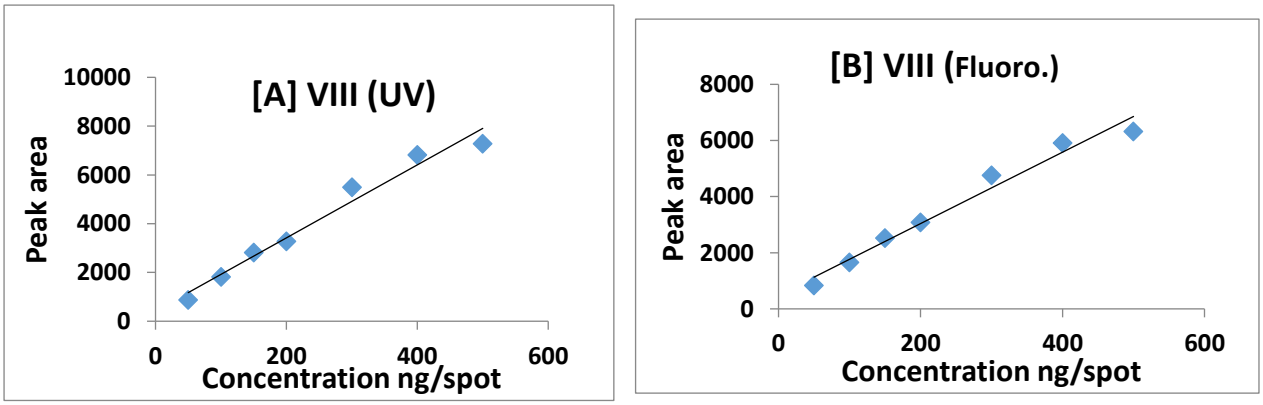


Figure 9: The spectrophotometric [A] and spectrofluorometric[B] linear calibration curves for compound **VII.**

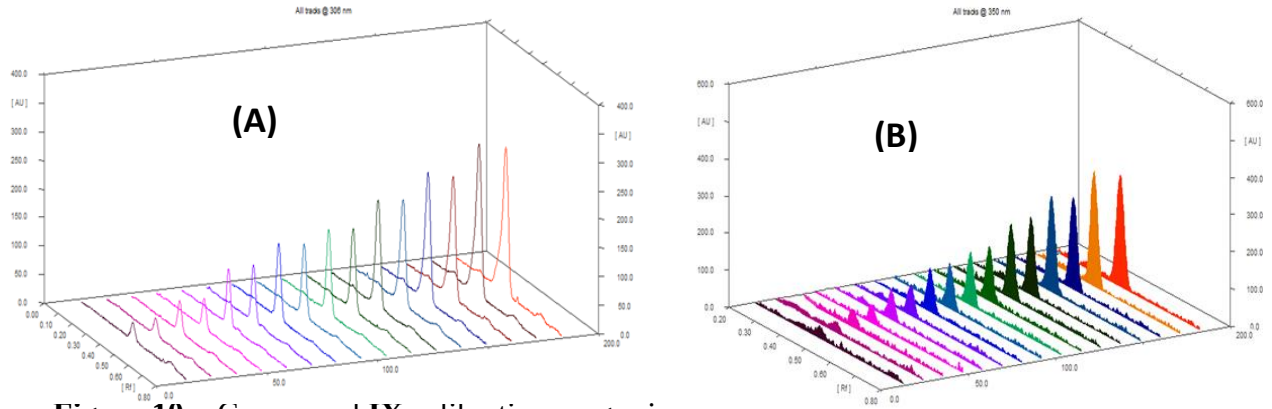


Figure 10: : Compound **IX** calibration ranges in 3D HPTLC densitograms utilising ultraviolet [A] and fluorescence [B]measuremen

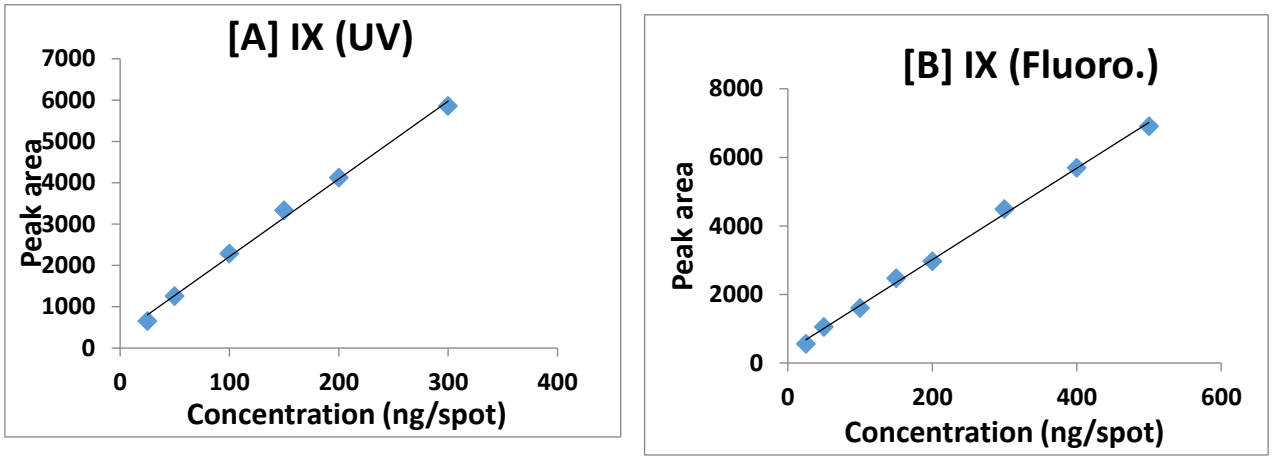


Figure 11: The spectrophotometric [A] and spectrofluorometric [B] linear calibration curves for compound **IX**

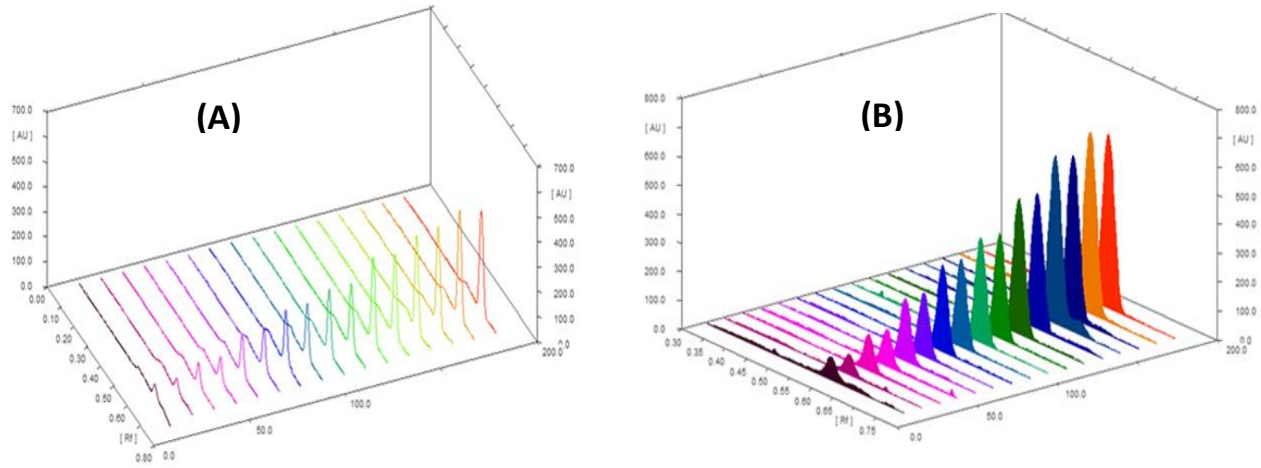


Figure (12): The linear calibration curves for the compound **X** measured spectrophotometrically [**A**] and spectrofluorometrically [**B**].

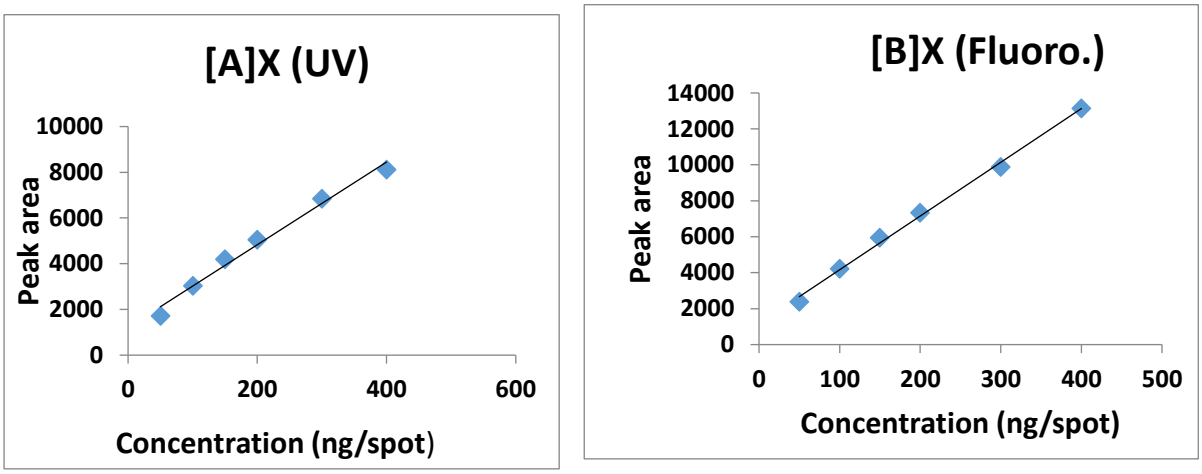


Figure 13: The spectrophotometric [A] and spectrofluorometric[B] linear calibration curves for compound **X.**

3.3.2. Sensitivity

Limits of detection (LOD) and limits of quantitation (LOQ) were used to assess the method's sensitivity. The lowest concentration of an analyte that can be identified but not always precisely quantified is known as the limit of detection. The limit of quantitation is the lowest analyte concentration that can be measured with a respectable level of precision and accuracy. The formula for the compounds being studied was used to determine the (LOD) and (LOQ). LOD or LOQ is equal to K SDa/b. where b is the slope of the calibration curve, SDa is the standard deviation of the blank determination or standard deviation of the calibration curve's intercept, and K is a constant that is equal to 3.3 for LOD and 10 for LOQ. **Table 5** displays the calculated limits of detection (LOD) and quantitation (LOQ) for each of the chemicals under study, demonstrating the excellent sensitivity of the suggested approach

Compound No.	Linearity range (ng/spot)		LOD (ng/spot)		LOQ (ng/spot)	
	UV	Fluoro.	UV	Fluoro.	UV	Fluoro.
VI	75-500	75-500	25.06	21.48	75.95	65.10
VII	100-500	75-500	36.72	25.62	110.20	76.88
VIII	100-500	100-500	24.18	31.95	73.29	96.83
IX	50-500	50-500	17.52	15.84	52.57	47.54
X	100-500	50-400	47.79	19.40	144.82	58.78

Table 5: The suggested HPTLC approach yielded linearity ranges, limits of detection, and quantitation for the compounds VI-X.

3.3.3. Selectivity and specificity

The capacity of an analytical method to precisely quantify a particular compound inside a sample matrix without interference from other components, such as degradation products or matrix constituents, is known as selectivity, and it is a crucial feature in analytical method validation. Clear, well-resolved peaks were produced in every instance, and no interference from the starting materials or intermediates was noted in the data obtained for the separation and quantification of the compounds under study. As a sample example for the complete series, **Figure 14** shows the successful separation of compound **VI** in the presence of its corresponding starting ingredients. Using peak purity analysis, the method's selectivity was further evaluated. By comparing the spectral profiles at the peak onset, apex, and end position, the purity of each peak was assessed. The absence of co-elution was confirmed by the comparison, which revealed a high degree of similarity between the peaks of the starting materials and the compounds under study. Furthermore, as illustrated in Figure 15, the bands that corresponded to each drug were verified by contrasting their peak areas, Rf values, and spectral features. These results validate that the technique is very dependable and selective for identifying the target compounds in both their pure forms and when associated starting materials and intermediates are present

.

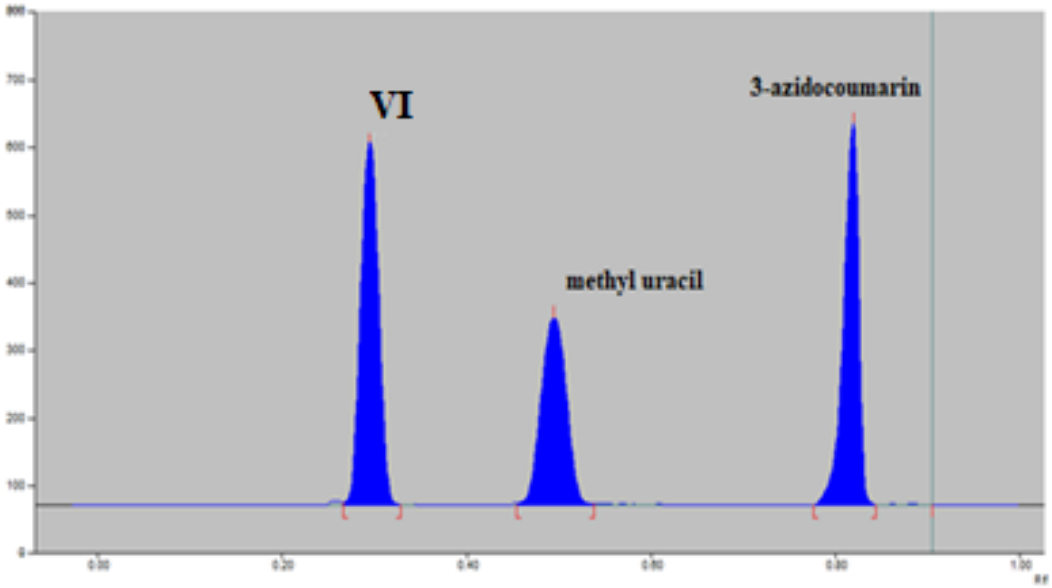


Figure 14: Two-dimensional TLC densitogram of compound **6** and starting material **3-azidocoumarin, 6-methyluracil** at a concentration level of 600 ng/band for all compounds and using mobile phase (chloroform:methyl alcohol 9:1).

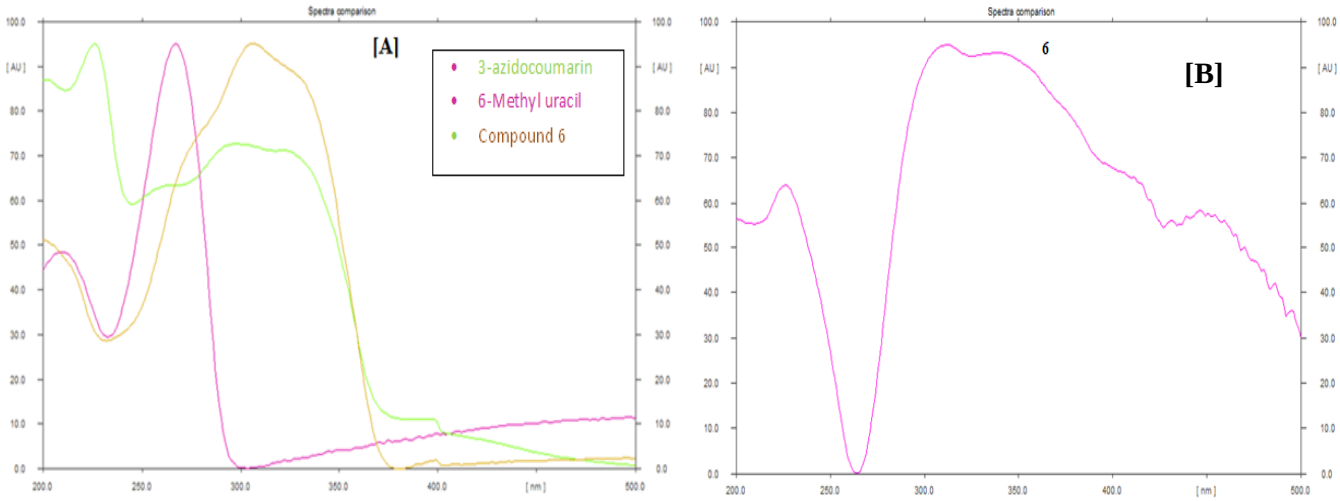


Figure 15: Comparing the spectrophotometric [A] and spectrofluorometric[B] spectra of compound 6 with starting materials chosen from the spectrum list produced by the WinCATS program.

4. Conclusion

For the simultaneous determination of the synthesized compounds VI–X, a novel, straightforward, sensitive, and selective TLC-spectrodensitometric method has been developed. Compared to other chromatographic methods, the proposed method has many advantages, including the ability to detect and quantify the derivatized molecules, uracil, phenytoin, naproxen, and valproic acid at the ng level in bulk and biological environments, as well as improved sensitivity, better recovery, shorter analysis times, lower costs, larger sample capacities, fewer solvents, and easier clean up. The procedure's simplicity makes it suitable for quantitative studies once the specific assay conditions are taken into account. In this study the validated procedure and the low limits of the estimated LOD and LOQ parameters render tracing and location of the fluorescent compounds in biological environment amenable for studying drug / target interaction binding.

Reference

1. Borges F, Roleira F, Milhazes N, Santana L, Uriarte E. Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis, and biological activity. Current medicinal chemistry. **2005**;12(8):887-916.
2. Naik, Reshma J, Kulkarni, Manohar V, Sreedhara Ranganath Pai K, Nayak Pawan G. Click Chemistry Approach for Bis-Chromenyl Triazole Hybrids and Their Antitubercular Activity. Chemical biology & drug design. **2012**;80(4):516-23
3. Kostova I, Bhatia S, Grigorov P, Balkansky S,S Parmar V, K Prasad A, Saso L. Coumarins as antioxidants. Current medicinal chemistry.**2011**;18 (25):3929-51.
4. Li D, Wu L. Coumarins from the roots of Angelica dahurica cause anti-allergic inflammation. Experimental and therapeutic medicine. **2017**;14(1):874-80.
5. Katsori A-M, Hadjipavlou-Litina D. Coumarin derivatives: an updated patent review (2012–2014). Expert opinion on therapeutic patents. **2014**;24(12):1323-47.
6. Jain P, Joshi H. Coumarin: Chemical and pharmacological profile. Journal of Applied Pharmaceutical Science **2012**; 236-240**,** Doi: 10.7324/JAPS.2012.2643.
7. Xu L, Wu Y-L, Zhao X-Y, Zhang W. The Study on Biological and Pharmacological Activity of Coumarins. Asia-Pacific Energy Equipment Engineering Research Conference,**2015;** 135-137.

8. Wu X-Q, Huang C, Jia Y-M, Song B-A, Li J, Liu X-H. Novel coumarin-dihydropyrazole thio-ethanone derivatives: design, synthesis, and anticancer activity. European journal of medicinal chemistry. **2014**; 74:717-25.
9. Chen Y, Liu H-R, Liu H-S, Cheng M, Xia P, Qian K, et al. Antitumor agents 292. Design, synthesis, and pharmacological study of S-and O-substituted 7-mercapto-or hydroxy-coumarins and chromones as potent cytotoxic agents. European journal of medicinal chemistry. **2012**; 49:74-85.
10. Gehan A. Abdel-Hafez, Abdel-Maaboud I. Mohamed, Adel F. Youssef, Claire Simons & Ahmed S. Aboraia (2022) Synthesis, computational study and biological evaluation of 9-acridinyl and 1-coumarinyl-1,2,3-triazole-4-yl derivatives as topoisomerase II inhibitors, Journal of Enzyme Inhibition and Medicinal Chemistry, 202237:1,502-513,DOI: 10.1080/14756366.2021.2021898
11. Droumaguet CL, Wang C, Wang QJCSr. Fluorogenic click reaction. 2010;39 4:1233-9
12. International Conference on Harmonization (ICH) Topic Q2 (R1): Validation of analytical procedures: Text and Methodology, Geneva, Nov **2005,** http: www.ich.org.
13. The United States Pharmacopoeia USP31-NF26, US Pharmacopoeia Convention, Twin brook Parkway, Rockville, MD, **2007**.
14. Abdel-Hafez, G. A., Aboraia, A. S., Mohammad, A. M. I., & Youssef, A. F. Development and validation of a high-performance thin-layer chromatography densitometric method for the simultaneous determination of novel 1-acridinyl-1, 2, 3-triazole derivatives. *JPC–Journal of Planar Chromatography–ModernTLC*, ***2022***, *35(4), 349-362.*

Corresponding author: Gehan Ahmed Abdel-Hafez

Department of Medicinal chemistry, Faculty of Pharmacy, Merit University Egypt (MUE), Sohag, Egypt.

E-mail: Gehan_ahmad@hotmail.com

Phone: 01005794116