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# Utility of a novel turn-off fluorescence probe for the determination of tranilast, an adjunctive drug for patients with severe COVID-19

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Tranilast (TR) could be investigated as a suitable anti-inflammatory and NLRP3 inflammasome inhibitor medication for the treatment of COVID-19 acute patients. Owing to its importance, our study was constructed for the determination of TR using a new, fast, sensitive, and reliable green spectrofluorimetric method. TR was quantified in this study by forming a complex with the acriflavine (AC) reagent. The reaction between TR and AC quenched the fluorescence of AC through the formation of an ion-association complex and the response was measured at 493 nm after excitation at 263 nm. It was observed that the quenching of the fluorescence of AC was linear ( $r = 0.9998$ ) with the concentration of TR in the range of 1.0–15.0  $\mu\text{g mL}^{-1}$ . The limit of detection was 0.224  $\mu\text{g mL}^{-1}$ , and the limit of quantification was 0.679  $\mu\text{g mL}^{-1}$ . The fluorescence quenching mechanism was carefully studied and was confirmed to be able to analyze TR in its pure form and its prepared pharmaceutical dosage form. To validate the method, the international conference of harmonization (ICH) Q<sub>2</sub>R<sub>1</sub> guidelines were followed. The statistical assessment of the proposed and comparison methods revealed no significant differences between them. Moreover, the green criteria of the method were evaluated and confirmed.

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## 1 Introduction

Coronavirus disease (COVID-19), which is caused by the SARS-CoV-2 strain,<sup>1</sup> was first reported in humans in China in December 2019. Subsequently, it spread worldwide and infected millions of people; therefore, it has been considered a global pandemic. A primary reason for death in people with COVID-19 is a cytokine storm that causes acute inflammatory acute pneumonia.<sup>2</sup> As a result of the acute inflammation and cytokine storm acute lung injury and respiratory distress syndrome have been reported.<sup>3</sup> There is an immediate need to develop a treatment for COVID-19. Several antiviral, antibacterial, antiparasitic, and anti-inflammatory medications have been utilized to counter its global spread. SARS-CoV-2 has been previously shown to activate the NLRP3 inflammasome and cause acute inflammation.<sup>4</sup> Tranilast (TR), as an anti-inflammatory drug, has the potential to prevent the exacerbation of COVID-19 by influencing a variety of pathways, including the inhibition of the NLRP3 inflammasome; cytokines (IL-5, IL-13, IL-17, IL-33, and TGF- $\beta$ ), signaling pathways (NF- $\kappa$ B); chemokines (CCL5 and CXCL9); and cell adhesion molecules (ICAM1). Therefore, along with other antiviral medications, TR could be investigated as a possible adjuvant treatment for people with acute COVID-19.<sup>4,5</sup>

TR (Fig. 1A) is chemically named 2-[[*(E)*-3-(3,4-dimethoxyphenyl)prop-2-enoyl]amino]benzoic acid. Initially, TR was discovered as an antiallergic agent prescribed for diseases as bronchial asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, hypertrophic scars, and keloids.<sup>6–11</sup> Its mechanism of action involves the suppression of the release of chemical mediators, such as histamine, as well as leukotriene from mast cells.<sup>12,13</sup> Subsequently, TR is effective in the management of various types of cancer, such as pancreatic, gastric, prostate, and uterine leiomyoma cell cancers.<sup>14–17</sup> Therefore, it is important to find a suitable method for its determination.

Acriflavine (AC) (Fig. 1B) is chemically named 3,6-diamino-10-methylacridin-10-ium chloride.<sup>18</sup> It is a dye that is considered a local antiseptic, anticancer, and antibacterial agent.<sup>19</sup> It has recently been utilized as a fluorescent reagent for the estimation of a variety of drugs, such as ascorbic acid,<sup>20</sup> ketoprofen, and diclofenac sodium.<sup>21</sup> This chemical reaction is based mainly on the quenching of its fluorescence in a quantitative way that could permit drug determination.

The literature survey represented limited methods for the determination of TR, including spectrophotometry<sup>22</sup> and high-performance liquid chromatography (HPLC).<sup>23–25</sup> These methods suffer from many limitations, such as low sensitivity,<sup>22–25</sup> long chromatographic run times,<sup>23–25</sup> complicated sample preparation<sup>25</sup> and using non-green solvents.<sup>23–25</sup> For example: the reported methods<sup>23,25</sup> use high methanol percentage up to 60% and the reported method<sup>24</sup> use high percentage of acetonitrile which is considered a toxic and non-

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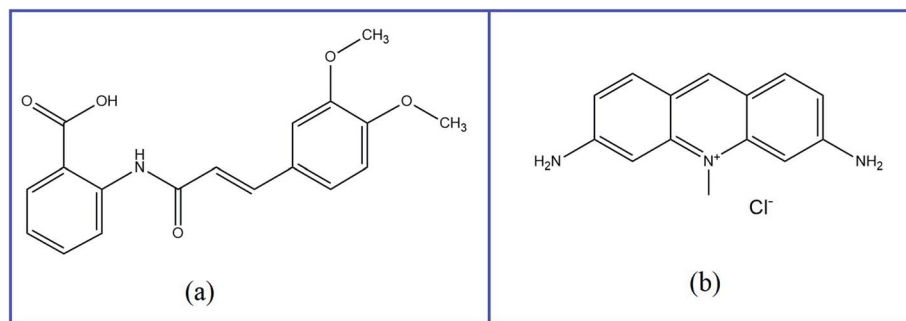



Fig. 1 Structural formulae of tranilast (a) and acriflavine (b).

green solvent. Our study aimed to examine AC quenching for the quick spectrofluorimetric determination of TR depending on the carboxylic group in TR that could interact with AC at an appropriate pH. As far as we know, there have been no previous published spectrofluorimetric methods for the determination of TR in its pure and pharmaceutical forms. Furthermore, the accessibility and usability of the spectrofluorimetric approach could be advantageous in quality control labs with inadequate sophistication or costly techniques. The proposed approach offers other merits, such as sensitivity, selectivity, low cost, and time saving. The green criteria of the proposed approach were assessed using the two following different tools: the analytical ecoscale<sup>26</sup> and Green Analytical Procedure Index (GAPI).<sup>27</sup>

## 2 Experimental

### 2.1 Instrumentation

- To measure the fluorescence intensity, a Cary Eclipse fluorescence spectrophotometer was used. It was equipped with a xenon flash lamp that was adjusted to use a voltage of 700 V, a slit width of 5 nm, and 1 cm quartz cell to record the fluorescence in the wavelength range of 200–550 nm. The quenching of AC fluorescence was measured at 495 nm after excitation at 263 nm and smoothing factor of 20.

- Absorbance measurements used in the official method were scanned using a Shimadzu dual-beam UV-Visible Spectrophotometer model 1650, Tokyo, Japan.

- A Sonic IV model-SS101H 230 (USA) was used for sonication.

- To adjust the pH, A Jenway 3510 pH meter was used.
- A water bath from England Cambridge Ltd (Shaker) was used to monitor the temperature.

### 2.2 Chemicals and materials

- TR was bought from Xiamen Keerda Bio-Tech Co., Ltd (Fujian, China), with a purity of 98%.

- AC was supplied from Sigma-Aldrich, Germany.
- Organic solvents (HPLC grade) such as methanol, ethanol, propanol, and n-butanol were purchased from Fisher Scientific UK, Loughborough Leics (UK).

- Different surfactants including carboxy methylcellulose (CMC), sodium dodecyl sulphate (SDS), Tween 80, and cetrimide were bought from El-Nasr Pharmaceutical Chemicals Co., Abu Zaabal, Egypt.

- Talc, calcium stearate, crystalline cellulose, and maize starch were provided from El-Nasr Pharmaceutical Chemicals Co., Abu Zaabal, Egypt.

- Acetic acid, boric acid, phosphoric acid, and sodium hydroxide were used for the preparation of the Britton–Robinson (BR) buffer and were all supplied from Sigma-Aldrich, Germany.

### 2.3 Samples and standard solutions preparation

- A stock solution of TR was prepared by dissolving 10 mg of TR in 100 mL of ethanol to obtain a concentration of 100  $\mu\text{g mL}^{-1}$  for the drug.

- An aqueous solution of AC was prepared at a concentration of  $2 \times 10^{-6}$  M by transferring 2.5 mL of a  $2 \times 10^{-4}$  M AC stock solution (prepared by dissolving 0.026 g of AC in 500 mL distilled water) into a 250 mL volumetric flask and completing it to the mark with distilled water.

- BR buffer was prepared over a pH range of 2–12 by mixing 0.04 M acetic acid, 0.04 M phosphoric acid, and 0.04 M boric acid and adjusting the pH by 0.2 M sodium hydroxide.

- The surfactants were prepared as 1.0% w/v solutions in distilled water.

### 2.4 Procedures for constructing a calibration graph

Aliquots of TR ( $1.0$ – $15.0 \mu\text{g mL}^{-1}$ ) were sequentially transferred into 10- mL volumetric flasks. Thereafter, 1.2 mL of the BR buffer solution (pH 7) and 1.0 mL of AC solution ( $2 \times 10^{-6}$  M) were added, and the solution was diluted to 10.0 mL with ethanol, shaken well, and set aside for 5 min. The fluorescence quenching was recorded at 495 nm after excitation at 263 nm, as shown in Fig. 2. The study was conducted against a blank measurement simultaneously. The calibration graph was constructed by plotting the decrease in the fluorescence intensity,

$\Delta F$  ( $\Delta F = \text{FI (blank)} - \text{FI (TR - AC ion - pair complex)}$ ), versus the final drug concentration in  $\mu\text{g mL}^{-1}$ , thereafter, the regression analysis was carried out.



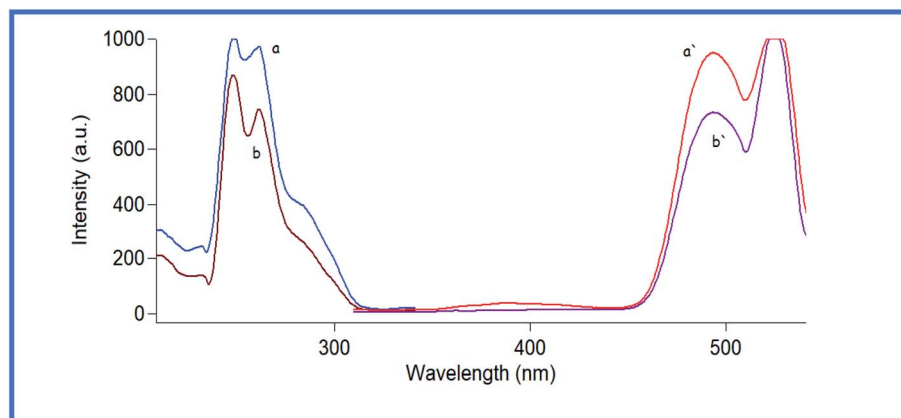


Fig. 2 Fluorescence excitation and emission spectra of: (a and a')  $2 \times 10^{-6}$  M acriflavine. (b and b')  $2 \times 10^{-6}$  M acriflavine with  $8 \mu\text{g ml}^{-1}$  tranilast.

### 2.5 Procedures for laboratory-prepared mixture content of capsules

TR is available under the trade name of Rizaben® capsules. These capsules are not available in Egypt; therefore, the mixture contents were prepared in the laboratory. The proposed method could determine the active ingredient in the presence of pharmaceutical excipients. TR capsules with a concentration of 100 mg per capsule were prepared using 20 mg of talc, 7 mg of calcium stearate, 15 mg of crystalline cellulose, and 15 mg of maize starch.<sup>28</sup> Upon mixing, a precise weight of the drug equivalent to 10 mg TR was transferred into a 100 mL volumetric flask. Thereafter, 70 mL of ethanol was added, followed by 20 min of sonication, and the flask was completed to the mark with ethanol. The solution was filtered using a  $0.45 \mu\text{m}$  syringe filter; the filtrate was diluted with ethanol, and the general procedures described under “constructing a calibration graph” were followed.

## 3 Results and discussion

According to a review of the existing literature, no spectrofluorimetric methods for determining TR have been published. Any process that causes a decrease in the fluorescence intensity of an analyte is known as fluorescence quenching.<sup>29</sup> Different molecular interactions involving energy transfer, ground-state complex formation, excited-state reactions, molecular rearrangements, and collisional quenching can cause the quenching process.<sup>29</sup> In this study, a rapid, facile, sensitive, selective, and green spectrofluorimetric method was used for the estimation of TR using AC dye depending on the fact that AC exhibits native fluorescence at 493 nm ( $\lambda_{\text{ex}}$ , 263 nm), and this fluorescence intensity decreased after the addition of TR to AC at a pH of 7, as shown in Fig. 2.

### 3.1 Optimization of experiment parameters

Various criteria that could influence the reaction were studied to determine optimal conditions for the formed ion-association complex. The parameters investigated include the following: pH

and volume of buffer, volume of reagent, diluting solvents, and type of surfactants.

**3.1.1 Effect of pH and buffer volume.** The study was performed over a pH range of 3.5–12.0 using the BR buffer. It was observed that the highest values of  $\Delta F$  were obtained when the pH ranged from 6.5 to 7.5; therefore, pH 7.0 was chosen as the optimal pH for the study, as shown in Fig. 3a. This pH of the solution is well recognized to have a considerable impact on the formation and stability of the ion-association complex. After pH 8.5, the solution was precipitated such that measurements could not be applied. Moreover, different volumes of the buffer (pH 7.0) ranging from 0.5 to 1.5 mL were examined, and it was found that  $\Delta F$  remained constant when the volume ranged from 1.0 to 1.3 mL, so the ideal volume for proceeding with the reaction was 1.2 mL, as shown in Fig. 3b.

**3.1.2 Effect of AC volume.** By increasing the volume of the AC solution ( $2 \times 10^{-6}$  M) up to 1.2 mL, it was observed that the maximum values of  $\Delta F$  were obtained when using a volume ranging from 0.9 to 1.1 mL so the ideal volume was 1 mL, as shown in Fig. 3c.

**3.1.3 Effect of diluting solvent.** Several solvents were investigated, such as methanol, ethanol, propanol, n-butanol, and water. The best solvent was ethanol as it exhibited the highest  $\Delta F$ , as shown in Fig. 3d. Methanol, propanol and water exhibited lower values of  $\Delta F$  rather than ethanol. *N*-Butanol caused precipitation so it could not be measured.

**3.1.4 Effect of surfactant.** Various surfactants were investigated to enhance the  $\Delta F$  of ion-association between AC and TR involving; macromolecule such as; (CMC), anionic (SDS), cationic (cetrimide), and nonionic (Tween 80). They were prepared at 1% w/v solutions in water. CMC, SDS, and Tween 80 did not exhibit a significant increase in  $\Delta F$ . In contrast, cetrimide enhanced the fluorescence of AC not quenching. Correspondingly, the study was performed without a surfactant.

### 3.2 Mechanism of the reaction between TR and AC

**3.2.1 Determination of the stoichiometry between AC and TR.** A limiting logarithmic method<sup>30</sup> was applied by the alternative measurement of the  $\Delta F$  of the reaction product at



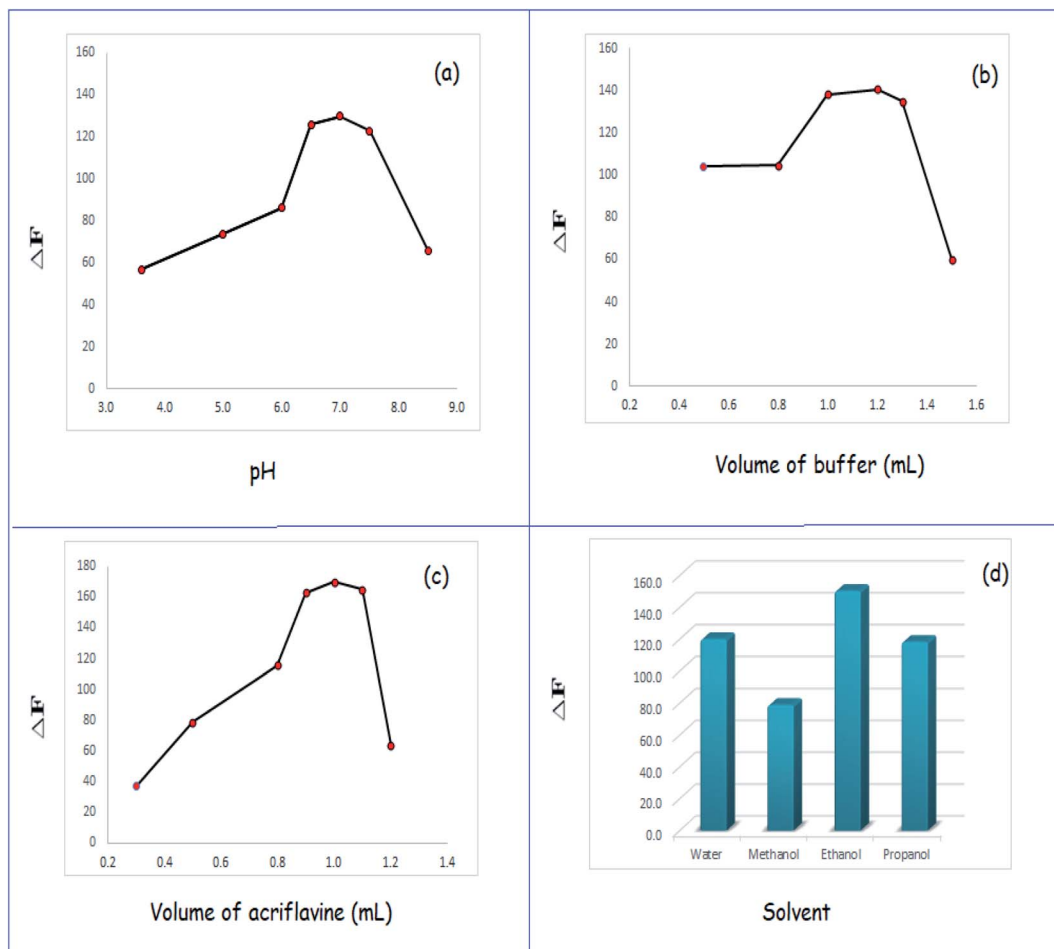


Fig. 3 Effect of different experimentation parameters on the fluorimetric quantification of tranilast using  $2 \times 10^{-6}$  M acriflavine: (a) effect of pH using Britton–Robinson buffer. (b) Effect of volume of buffer. (c) Effect of volume of  $2 \times 10^{-6}$  M acriflavine. (d) Effect of different diluting solvents.

increasing concentrations of either AC or TR. A plot of  $\log F$  vs.  $\log[AC]$  and  $\log[TR]$  exhibited straight lines, and the values of the slopes were 0.59 and 0.55, respectively, as shown in Fig. 4a and b. It was concluded that the molar reactivity of the reaction was 0.59/0.55, *i.e.* the reaction between AC and TR proceeded at a ratio of 1 : 1. The ratio could be deduced by the presence of one carboxylic group in TR. At a specific pH, an AC positively charged nitrogen atom reacted with a TR negatively charged carboxylate group. Therefore, electrostatic forces contributed to the formation of an ion-association complex, as shown in Scheme 1.

**3.2.2 Quenching mechanism.** Static and dynamic quenching, energy transfer, excited-state interactions, and molecular rearrangement are examples of molecular interactions that cause fluorescence quenching.<sup>29</sup> Consequently, static and dynamic quenching processes can be explained using the Stern–Volmer method.<sup>31,32</sup> The ratio between the fluorescence intensities in the absence ( $F_0$ ) and presence ( $F$ ) of the quencher was plotted against the molar concentration of the quencher  $[Q]$  to create a Stern–Volmer plot, as shown in Fig. 4c. The obtained straight line was fitted to the Stern–Volmer equation as follows:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (1)$$

The intercept of the straight line is one and the slope is Stern–Volmer constant ( $K_{sv}$ ). The linear relationship ( $r = 0.997$  and  $0.991$ ) between  $F_0/F$  and  $[Q]$  indicated the presence of a single type of quenching process, either dynamic or static quenching. Fig. 4c shows an increase in  $K_{sv}$  values from  $8.8 \times 10^3$  to  $17.2 \times 10^3$  L mol<sup>-1</sup> with an increase in temperature from 303 to 323 K, indicating dynamic quenching.<sup>31–33</sup>

Additionally, the bimolecular quenching constant ( $K_q$ ) could be calculated from the following equation:<sup>33</sup>

$$K_q = \frac{K_{sv}}{\tau_0}, \quad (2)$$

where  $\tau_0$  is the lifetime of native radiation of AC ( $5 \times 10^{-9}$  s); therefore, the calculated values of  $K_q$  were  $1.76 \times 10^{12}$  and  $3.44 \times 10^{12}$  L mol<sup>-1</sup> s<sup>-1</sup> within a temperature range of 303–323 K. Moreover, the calculation of the binding site number and rate constant of the reaction between AC and TR was performed according to the modified Stern–Volmer plot (Fig. 4d) from the following equation:<sup>34,35</sup>



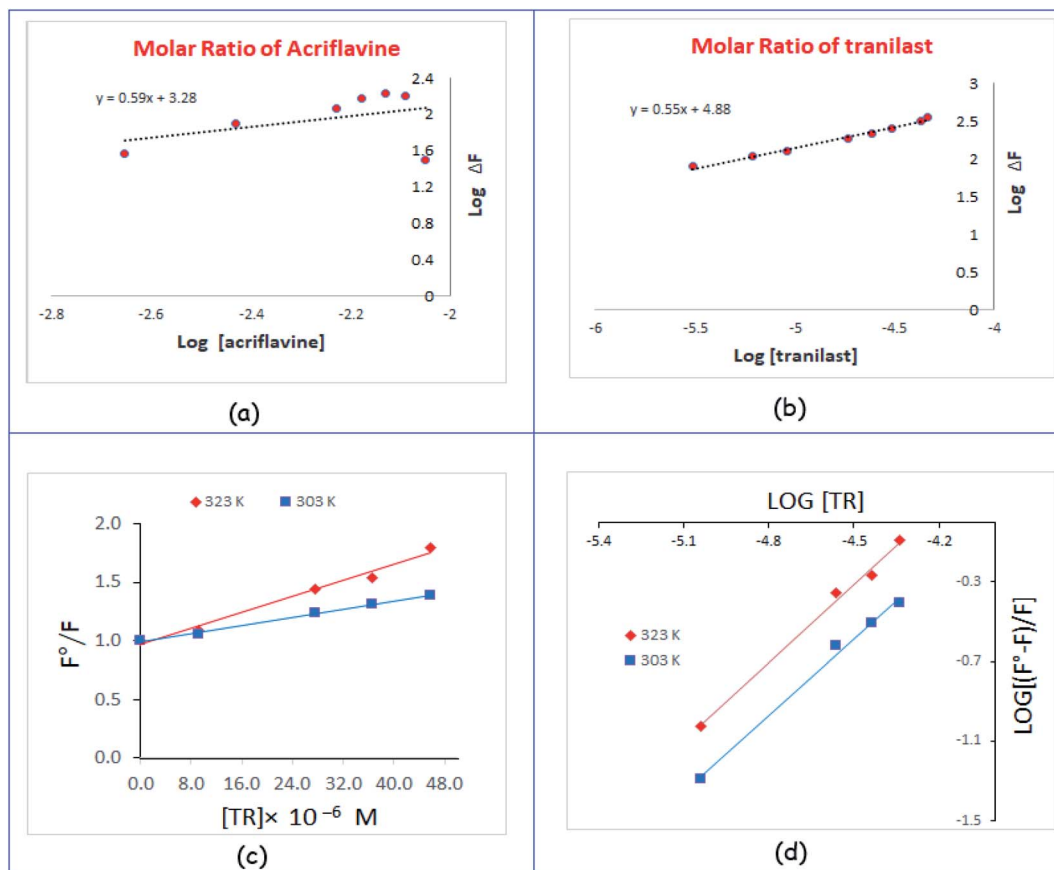


Fig. 4 Stoichiometric ratio of the fluorimetric reaction between tranilast and acriflavine using limiting logarithmic method where: (a) log [acriflavine] against log  $\Delta F$ . (b) log [tranilast] against log  $\Delta F$ . Mechanism of reaction between acriflavine and tranilast explained by: (c) Stern–Volmer plot for fluorescence quenching at 303 K and 323 K using  $2 \times 10^{-6}$  M acriflavine at pH = 7. (d) Modified Stern–Volmer plot for fluorescence quenching at 303 and 323 K using  $2 \times 10^{-6}$  M acriflavine at pH = 7.

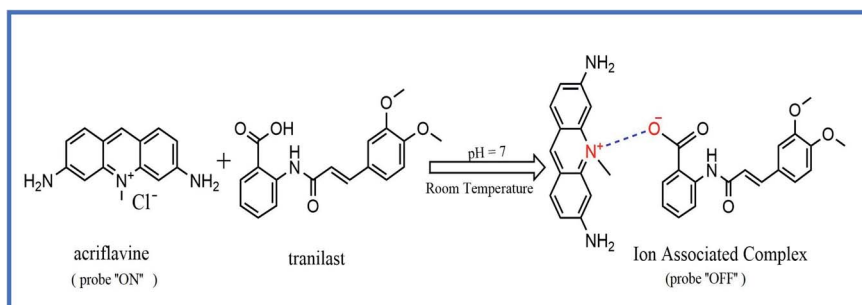
$$\log \frac{F_0 - F}{F} = \log K_b + n \log [D], \quad (3)$$

where  $K_b$  is the reaction rate constant,  $n$  is the number of binding sites,  $[D]$  is the molar concentration of TR, and  $F_0$  and  $F$  are the fluorescence intensities of AC in the absence and presence of TR, respectively. The reaction rate constant ( $K_b$ ) was calculated from the intercept and observed to be  $1.57 \times 10^5$ , and the number of moles of TR was 1.28 (approximately 1), further confirming the molar ratio (1 : 1) between AC and TR obtained from the limiting logarithmic method.

Moreover, the  $K_b$  value was used to calculate Gibb's free energy ( $G^0$ ), as demonstrated in the following equation:

$$\Delta G^0 = -RT \ln K_b, \quad (4)$$

where  $R$  is the gas constant ( $8.314 \text{ J K}^{-1} \text{ mol}^{-1}$ ),  $T$  is the absolute temperature in Kelvin, and  $K_b$  is the reaction rate constant. The calculated  $\Delta G^0$  was  $-30.15 \text{ kJ mol}^{-1}$ . The negative value of  $\Delta G^0$  indicated the reaction spontaneity and feasibility at ambient temperature.



Scheme 1 Mechanism for formation of ion-associated complex between acriflavine and tranilast.



**Table 1** Analytical performance data for the determination of TR by the proposed method

Parameter	TR
Range ( $\mu\text{g mL}^{-1}$ )	0.679–15.0
Linearity range ( $\mu\text{g mL}^{-1}$ )	1.0–15.0
Intercept ( $a$ )	67.98
Slope ( $b$ )	19.61
Correlation coefficient ( $r$ )	0.9998
S.D. of residuals ( $S_{y/x}$ )	2.11
S.D. of intercept ( $S_a$ )	1.331
S.D. of slope ( $S_b$ )	0.149
Percentage relative standard deviation, %RSD	0.993
%Error	0.351
Limit of detection, LOD ( $\mu\text{g mL}^{-1}$ )	0.224
Limit of quantitation, LOQ ( $\mu\text{g mL}^{-1}$ )	0.679

### 3.3 Validation of the proposed method

This method was validated following the international conference of harmonization (ICH) Q<sub>2</sub>R<sub>1</sub> guidelines,<sup>36</sup> as shown in Table 1. The inspected parameters include linearity, range, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, robustness, and selectivity.

**3.3.1 Linearity and range.** Several TR standard solutions in concentrations ranging from 1.0 to 15.0  $\mu\text{g mL}^{-1}$  were used to determine and confirm the linearity of the green spectrofluorimetric method. The calibration graph was constructed by measuring AC fluorescence intensities in the absence and presence of TR. The correlative regression equation was established by plotting the decline in fluorescence intensity ( $\Delta F$ ) against the final TR concentration. The linearity analysis was conducted using eight different concentrations of TR and the resulting regression equation was as following:

$$\Delta F = 67.98 + 19.61C \quad (r = 0.9998)$$

A correlation coefficient with a high value ( $r > 0.999$ ) was used to confirm linearity.<sup>37</sup> The linearity was determined by

**Table 3** Precision data for the determination of TR by the proposed method

Proposed method		Conc. ( $\mu\text{g mL}^{-1}$ )		
		3.0	10.0	15.0
Intraday	% found <sup>a</sup>	100.18	99.16	99.29
		101.71	100.54	100.62
		100.40	100.29	100.02
	Mean	100.77	99.997	99.98
	S.D.	0.83	0.73	0.67
Interday	%RSD	0.82	0.73	0.67
	%Error	0.47	0.42	0.39
	% found	99.12	98.90	98.38
		101.07	100.70	99.84
		99.82	100.40	101.46
	Mean	100.0	100.0	99.9
	S.D.	0.99	0.97	1.54
	%RSD	0.99	0.97	1.54
	%Error	0.57	0.56	0.89

<sup>a</sup> N. B. Each result is the average of three separate determinations.

calculating the percentage relative standard deviation (%RSD) and displaying the variances around the slopes ( $S_b$ ) with a negligible value to designate a small degree of available data around the regression line. Other statistical characteristics include the intercept standard deviation ( $S_a$ ) and standard deviation of the residuals ( $S_{y/x}$ ) were then shown in Table 1.<sup>37</sup>

**3.3.2 Limit of detection (LOD), limit of quantitation (LOQ).** LOD is the lowest concentration of the analyte that can be detected but not necessarily quantified by the analytical method while LOQ is the lowest concentration that can be quantified with acceptable accuracy and precision by the analytical method. According to the ICH Q<sub>2</sub>R<sub>1</sub> guidelines, the LOQ and LOD were calculated using the following formulae:

$$\text{LOD} = \frac{3.3S_a}{b}, \text{LOQ} = \frac{10S_a}{b},$$

where  $S_a$  is the standard deviation of the intercept, and  $b$  is the slope of the calibration curve. The LOD and LOQ values were

**Table 2** Assay results for the determination of the TR in pure form by the proposed and comparison method

Compound	Proposed method			Comparison method <sup>22</sup>	
	Conc. taken ( $\mu\text{g mL}^{-1}$ )	Conc. found ( $\mu\text{g mL}^{-1}$ )	% found <sup>a</sup>	Conc. taken ( $\mu\text{g mL}^{-1}$ )	% found <sup>a</sup>
TR	1.0	0.984	98.4	3.0	99.33
	2.0	2.028	101.4	6.0	100.68
	3.0	3.003	100.1	9.0	99.77
	6.0	6.035	100.58		
	8.0	7.957	99.46		
	10.0	9.95	99.5		
	14.0	14.145	101.04		
	15.0	14.903	99.35		
	Mean			99.98	99.93
± S.D.			0.99	0.69	
$t$ -Test			0.07 (2.26)*		
$F$ -Test			2.06 (19.35)*		

<sup>a</sup> N.B. Each result is the average of three separate determinations. The figures between parentheses are the tabulated  $t$  and  $F$  values at  $P = 0.05$ .<sup>37</sup>



Table 4 Assay results for the determination of the TR in its pharmaceutical prepared capsule

Parameters	Proposed method			Comparison method <sup>22</sup>	
	Conc. taken ( $\mu\text{g mL}^{-1}$ )	Conc. found ( $\mu\text{g mL}^{-1}$ )	% found <sup>a</sup>	Conc. taken ( $\mu\text{g mL}^{-1}$ )	% found <sup>a</sup>
TR	5.0	4.946	98.92	3.0	101.02
	10.0	9.935	99.35	5.0	98.28
	15.0	14.991	99.94	9.0	99.02
Mean			99.40	99.44	
$\pm$ S.D.			0.51	1.16	
<i>t</i> -Test			0.05 (2.78)*		
<i>F</i> -Test			1.0 (19.00)*		

<sup>a</sup> N. B. Each result is the average of three separate determinations. The figures between parentheses are the tabulated *t* and *F* values at  $P = 0.05$ .<sup>37</sup>

0.224 and  $0.679 \mu\text{g mL}^{-1}$ , respectively, indicating the sensitivity of the proposed method,<sup>36</sup> as shown in Table 1.

**3.3.3 Accuracy and precision.** Accuracy was evaluated by comparing the results of the proposed method for TR in pure form with a reported spectrophotometric method.<sup>22</sup> The comparison method<sup>22</sup> involved measurements of the absorbance of TR at a  $\lambda_{\text{max}}$  of 334 nm using methanol as a diluting solvent. The statistical analysis of the results showed no significant difference between the proposed and reported methods regarding accuracy and precision, as assessed by

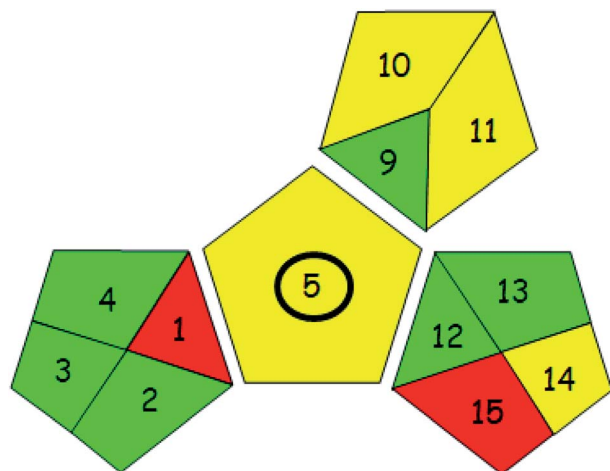
Student's *t*-test and variance ratio test (*F*-test),<sup>37</sup> as shown in Table 2.

Precision was evaluated by examining different concentrations of TR three times in three replicates in the same day (intraday precision) and on different sequent days (interday precision). Low values of percentage relative standard deviations (%RSD < 2%) and percentage error (%Error < 1%) indicated the repeatability and reproducibility of the proposed method, as shown in Table 3.

Table 5 Results for evaluation of greenness of the proposed method

Item	Penalty points
<b>1. Analytical eco-scale score<sup>26</sup></b>	
(1) Reagent; volume (ml)	
Ethanol; <10 ml	4
Acriflavine; 1 ml	6
BR buffer; 1.2 ml	2
(2) Spectrofluorimeter; <0.1 KW h per sample	0
(3) Occupational hazard	0
(4) Waste	3
Total penalty points	15
Analytical eco-scale score	85

## 2. Green analytical procedure index (GAPI)<sup>27</sup>



**3.3.4 Robustness.** To confirm the robustness of the method, minor alterations of the parameters that affect the  $\Delta F$  were evaluated. These parameters include the pH of BR buffer ( $7 \pm 0.2$ ), volume of BR buffer ( $1.2 \pm 0.1$ ), and volume of the AC reagent ( $1 \pm 0.1$ ). The introduction of these small variations did not cause a significant change in the  $\Delta F$ , thereby affirming the robustness of the method.

**3.3.5 Selectivity.** The proposed method selectivity was assessed to ensure that it could only determine TR in the presence of other excipients in the capsule. These excipients include maize starch, calcium stearate, crystalline cellulose, and talc. They did not affect the fluorescence intensity measured in this reaction, as shown in Table 4, thereby affirming the selectivity.

## 4 Application of the proposed method

### 4.1 Assay of laboratory prepared capsules

As TR could be estimated in bulk powder; therefore, it was relevant to test TR in a laboratory-prepared capsule. The results of the proposed approach were compared with those of the reported method using Student's *t*-test and variance ratio *F*-test.<sup>37</sup> As evidenced by the *t* and *F* values, there were no significant differences between the proposed and reported approaches considering accuracy and precision, as shown in Table 4.

### 4.2 Evaluation of the greenness of the proposed approach

Ecofriendly greenness lies at the core of each analytical procedure. In this study, we employed two techniques; Green Analytical Procedure Index (GAPI) and analytical ecoscale, to evaluate the greenness of this approach.

The analytical ecoscale<sup>26</sup> is a semiquantitative tool that for assessing the greenness of the method. The analytical ecoscale score is achieved by calculating penalty points of the amount of reagent, hazards, instrumentation energy, and waste and subtracting the total penalty points from 100. Herein, the results are ranked on a scale, where scores > 75 rank the approach as excellent green, scores > 50 represent an acceptable green approach, and scores < 50 represent an inadequate green approach. The score of the proposed approach was 85, which revealed an excellent green methodology (Table 5).

Recently, GAPI<sup>27</sup> was developed as a new tool to evaluate the greenness of analytical methods. It uses five pictograms to analyze and calculate the impact of each stage of an analytical process on the environment with three color codes; green, yellow, and red indicating low, medium, and high environmental impact, respectively. New criteria such as health and safety were considered by this tool. When GAPI was applied to our proposed method, we observed that the majority of GAPI criteria were fulfilled. Criteria 1 and 15 were colored in red, while criteria 5, 10, 11, and 12 were colored in yellow, as shown in Table 5.

## 5 Conclusion

In our study, AC was utilized to form an ion-association complex with TR. Sensitivity, selectivity, reliability, precision, cost, and

time saving were the advantages of the proposed method. The quenching of the fluorescence of AC was linear ( $r = 0.9998$ ) with the concentration of TR in the range of 1.0–15.0  $\mu\text{g mL}^{-1}$  with limit of detection = 0.224  $\mu\text{g mL}^{-1}$ . In this method, there was no requirement for sample pretreatment or extraction with organic solvents, which was the most significant advantage of this method, compared with other reported methods. The suggested procedures were used for routine assays with high sample throughput in bulk and pharmaceutical dosage forms. This method is a viable choice for quality control because of its simplicity and low environmental impact. The usage of fluorimetry as a cost-effective technique is also an advantage. The eco-friendly property and dependence on ethanol diluting solvent added to the facility of the method.

## Ethics permission

No study on humans or animals has been included.

## Author contributions

Diaa Dagher carried out the lab work, and statistical calculations. Heba Elmansi, Jenny Nasr, and Nahed El-Enany supervised the whole research and the manuscript. All authors participated in study and approved the manuscript.

## Conflicts of interest

No competing interests.

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