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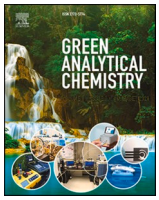

Smart sequential spectrophotometric analysis of Fluconazole and its two toxic official impurities having superimposed spectra; Greenness, whiteness, blueness assessment and *in silico* toxicity profiling

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ABSTRACT

Owing to the health hazards of impurities presence in active pharmaceutical ingredients, even in minute amount. A smart green sustainable spectrophotometric method was developed for simultaneous determination of Fluconazole (FLU) along with its official impurities B and C. The novel sequential dual amplitude difference (SDAD) technique succeeded in resolving the severe overlapping between the three compounds and restoring their respective mother spectrum. According to international Council of Harmonization (ICH) guidelines, the developed method is accurate, precise, and selective with linearity range (50–500 μg/mL) for FLU, (0.5–6 μg/mL) for impurity B and (0.05–0.6) for impurity C. The method was successfully applied to three pharmaceutical preparations without interference from excipients. In addition, the proposed method was assessed and compared with reported chromatographic methods for its green character, sustainability, and practicality, using five tools. The spectrophotometric method was found to be the greenest with the least volume of solvents used and waste produced, moreover, it is the most practical/sustainable method. Besides, the toxicity profiling of the two impurities was predicted using two computational databases: preADMET and pKcsm. After reviewing literature, no previous analytical method to determine FLU and its two official impurities using spectrophotometry was reported.

1. Introduction

Impurities control in Active Pharmaceutical Ingredients (API) represents a serious issue for pharmaceutical manufacturers in terms of quality, efficacy, and safety. The impurity may has health hazards if its content exceeds the safety limits set by health authorities [1]. Accordingly, impurity profiling is one of the most important fields of activities in pharmaceutical analysis which is the process of detection, structure elucidation and quantitative determination of impurities in bulk drugs and pharmaceutical formulations [2].The impurity may be a starting material, by-product, intermediate compound due to side reaction during synthesis or degradation products due to poor storage conditions [3]. Fluconazole (FLU), 2-(2,4-difluorophenyl)−1,3-bis-(1H-1,2,4-triazole-1-yl)−2-propanol [4], a synthetic imidazole antifungal drug commonly used for treatment of candidiasis, cryptococcal infection and fungal infection [5,6]. It acts by interfering the action of cytochrome enzyme P450 and so decreases ergosterol synthesis, thus inhibiting cell membrane formation of susceptible fungi [7]. According to European Pharmacopoeia (EP) [4] and United states Pharmacopoeia (US) [8], FLU has three potential specified impurities. The specified impurities are described and limited by a specific acceptance criterion [9]. This work will deal with the determination of FLU and two specified impurities B and C which have minimum accepted limits of 0.3 % and 0.1 %, respectively, their chemical structures are presented in Fig. 1. On reviewing literature, zero-order UV-spectrophotometric methods for determination of FLU alone in dosage forms [10–14] and with the oxidative degradation product [15] were reported. However, there are no reported spectrophotometric methods for determination of FLU with its impurities. On the other hand, FLU was assayed with its impurities by two main techniques; HPLC/UV [9,16–18] and HPTLC [17,19]. Spectrophotometry is an alternative greener, easily implemented, faster and low-cost technique when compared to the conventional

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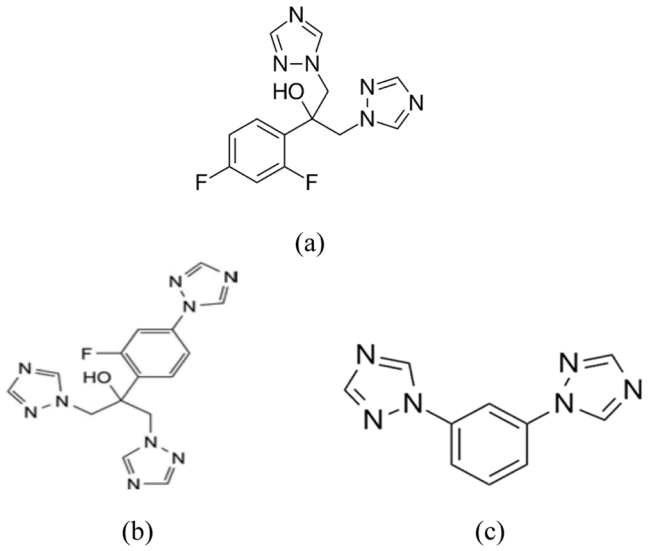


Fig. 1. Chemical structures of (a) Fluconazole, (b) Impurity B and (c) Impurity C.

chromatographic methods. Besides, no toxicity profiling study was published for FLU impurities. Nowadays, computational tools (as PreADMET and pkCSM software) are widely used to predict and estimate the toxicity profile of drugs while reducing the cost, time and ethical concerns of practical tests.

In the present study, a novel green cost-effective spectrophotometric method was developed for simultaneous determination of FLU and its official impurities B and C in laboratory prepared mixtures and pharmaceutical preparations. The toxicity profile of the two impurities was estimated using two open-access database servers, PreADMET and pkCSM. Additionally, in the light of implementation of green analytical chemistry (GAC) and sustainability, the developed method was evaluated and compared to reported chromatographic methods by applying five tools; analytical Eco-Scale (AES), Green Analytical Procedure Index (GAPI), Analytical GREEnness calculator (AGREE), Red-Green-Blue model (RGB 12) and The Blue Applicability Grade Index (BAGI).

1.1. Theoretical background

Sequential dual amplitude difference (SDAD) is a smart technique that could be applied for the resolution and determination of components of a complex ternary mixture (X, Y, and Z) exhibiting no intrinsic resolution tools as isoabsorptive points or extension of one drug over the other. SDAD encompasses successive and progressive steps that end up finally by restoring the original mother spectra of each component enabling their determination at their corresponding λ_{max} with minimum error and maximum sensitivity. restoration of the original mother spectra serves both quantitative and qualitative purposes where it is considered a fingerprint for cited components.

Step 1: Select a suitable divisor and obtain the ratio spectra of the whole mixture.

$$Am = X + Y + Z$$

$$Am/Z' = X/Z' + Y/Z' + Z/Z'$$

$$Am/Z' = X/Z' + Y/Z' + Constant$$

Step 2: Select a pair of wavelengths (λ_1, λ_2) where the amplitude of Y/Z′ exhibits equal values. Then record the amplitude difference at the selected wavelengths.

$$\Delta Pm_3 = \{(X/Z')_{\lambda1} + (Y/Z')_{\lambda1} + Constant\} - \{(X/Z')_{\lambda2} + (Y/Z')_{\lambda2} + Constant\}$$
$$= \{(X/Z')_{\lambda1} - (X/Z')_{\lambda2}\}$$

$$\Delta Pm_3 = \{(X/Z')_{\lambda1} + (Y/Z')_{\lambda1} + Constant\} - \{(X/Z')_{\lambda2} + (Y/Z')_{\lambda2} + Constant\}$$

Step 3: multiply the amplitude difference of the ternary mixture (ΔPm_3) by the factorized spectrum of X $\left(\frac{\frac{X}{Z'}}{\Delta Pm3}\right)$ to obtain its ratio spectrum (X/Z′).

Step 4: subtract the recovered ratio spectra (X/Z′) from that of the total mixture to regain the ratio spectra of components Y and Z as a binary mixture.

$$Am_3/Z' - (X/Z') = X/Z' + Y/Z' + Z/Z' - (X/Z').$$
$$= Y/Z' + Z/Z'$$

Step 5: The recovered ratio spectrum (X/Z′) is then multiplied by the used divisor to restore the D^0 spectrum of X and determine its concentration by recording the absorbance values at its λ_{max}.

Similarly, for determination of component Y at its λ_{max}, the same steps (2–5) are applied to the regained ratio spectrum of the binary mixture (Y/Z′+Z/Z′), where another pair of wavelengths (λ_3, λ_4) at which the amplitude of Z/Z′ (constant) exhibits equal values was selected. Then the amplitude difference at the selected wavelengths was recorded.

$$\Delta Pm_2 = \{(Y/Z')_{\lambda3} + Constant\} - \{(Y/Z')_{\lambda4} + Constant\}$$
$$= \{(Y/Z')_{\lambda3} - (Y/Z')_{\lambda4}\}$$

Then the amplitude difference of the regained binary mixture (ΔPm_2) is then by the factorized spectrum of Y $\left(\frac{\frac{Y}{Z'}}{\Delta Pm2}\right)$ to obtain its ratio spectrum (Y/Z′). Then the recovered ratio spectrum (Y/Z′) is subtracted from that of the binary mixture to finally obtain the constant (Z/Z′).

$$Am_2/Z' - (Y/Z') = Y/Z' + Z/Z' - (Y/Z').$$
$$= Z/Z'$$

While for determination of component Z, the obtained constant (Z/Z′) is multiplied by the divisor Z′ to restore its D^0 spectra.

2. Experimental

2.1. Instruments and software

Shimadzu UV–visible 1800 dual beam spectrophotometer (Kyoto, Japan) connected to UV-probe 2.32 software.

2.2. Chemicals and reagents

Pure sample of FLU of purity (99.57± 0.84) according to EP [4], impurity B and C were obtained as a gift from Sunny medical group company, Egypt. Methanol Analar (Fisher Scientific, USA).

2.3. Pharmaceutical formulations

Three formulations were purchased from the Egyptian market. Fungican® Capsules (Batch No. 184,001) (150 mg FLU), Amoun company, Flucoral® Capsules (Batch No. 0420242), (150 mg FLU), SEDICO

Company and Diflucan® IV infusion (Batch No. 18,051,002) (2 mg/mL FLU), Pfizer Company.

2.4. Standard solutions

Stock solution of FLU was prepared in concentration of (500 μg/mL) and the two impurities B and C were prepared in concentration (20 μg/mL) using methanol as solvent.

2.5. Laboratory prepared mixtures

Five mixtures having different ratios of FLU and the two impurities were prepared from their respective standard solutions.

2.6. Procedure

2.6.1. Construction of calibration graphs

For FLU, accurate aliquots equivalent to 500–5000 μg were transferred from its respective stock standard solution into a set of calibrated 10-mL volumetric flasks and methanol was used to complete the volume. The zero-order absorption spectra (D^0) were scanned from 200 to 400 nm and the absorbance values at 266 nm (A_{266}) were recorded using the same solvent as blank. The recorded A_{266} values were plotted against the corresponding concentrations (50–500 μg/mL) and the regression equation was computed.

While for impurities B and C, two separate calibration graphs were constructed between the absorbance values of each impurity in their D^0 spectra at 245.5 nm versus the corresponding concentrations ranging between 0.5–6 μg/mL and 0.05–0.6 μg/mL, respectively. A separate regression equation was computed for each impurity.

2.6.2. Preparation of decoding or resolution spectra

The Average Normalized spectrum of impurity B (NS_B) was prepared by separately dividing each of the D^0 spectra of impurity B by its respective concentrations and the average value was recorded and stored.

The factorized spectrum of FLU (FS_{FLU}) was prepared by dividing each of the ratio spectra of FLU (FLU/NS_B) by its amplitude difference between 214.0 nm and 233.0 nm and the average value was recorded and stored.

The factorized spectrum of impurity C (FS_C) was prepared by dividing each of the ratio spectra of impurity C (C/NS_B) by its amplitude difference between 276.0 nm and 288.4 nm and the average value was recorded and stored.

2.6.3. Spectral manipulation for determination of laboratory prepared mixtures

The spectra of the previously prepared mixtures were separately divided by (NS_B). the amplitude values at 214 nm and 233 nm were recorded and the amplitude differences were calculated ($\Delta P_{214–233}$). The FS_{FLU} was then recalled and multiplied by the ($\Delta P_{214–233}$) to recover the ratio spectra of FLU/NS_B. Upon subtracting the recovered ratio spectra of FLU/NS_B from the total ratio spectra of the mixture, the ratio spectra of both impurity B and C would be regained as binary mixtures ($B/NS_B+C/NS_B$). Similarly, the amplitude values at 276 nm and 288.4 nm were recorded, and the amplitude differences were calculated ($\Delta P_{276–288}$). The calculated ($\Delta P_{276–288}$) were then multiplied by the stored FS_C for recovering the ratio spectra of impurity C (C/NS_B) present in the mixtures. Upon subtracting the recovered ratio spectra of C/NS_B from the regained binary mixture ratio spectra ($B/NS_B+C/NS_B$), the ratio spectra of B/NS_B would be obtained as a pure single component. The three recovered separate ratio spectra were then multiplied by the used divisor (NS_B) to obtain the parent zero-order spectra of each component.

2.6.4. Application to pharmaceutical formulations

Ten capsules of Fungican® and Flucarol® were emptied, and powdered, separately. Accurately weighed amount of each powder equivalent to 12.5 mg were dissolved in methanol then filtered, into two separate volumetric flasks and the volumes were completed with methanol, to prepare stock solutions having concentration of (500 μg/mL). For Diflucan® IV infusion, a stock 500 μg/mL was prepared by transferring 6.25 mL into 25-mL volumetric flask and the volume was completed with methanol. The same procedure, detailed under spectral manipulation, was applied for determination of FLU and its two impurities in the three pharmaceutical formulations solution.

3. Results and discussion

On reviewing literature, no spectrophotometric methods were found for the analysis of FLU with its official impurities. Spectrophotometry has the advantages of being cost-effective, easy to apply, rapid and eco-friendly as it uses a small volume of solvents and consequently a small amount of waste is produced when compared to chromatographic methods. As a result, the goal of this research was the application of spectrophotometry for the simultaneous analysis of FLU and its two toxic official impurities B and C which have a challenging overlapped spectrum.

Upon scanning the spectra of FLU and its impurities (B and C), a sever overlapping was noticed between their spectra, in addition to a remarkable similarity between the spectra of both impurities, Fig. 2, making their determination highly challengeable. Sequential dual amplitude difference (SDAD) technique was applied to resolve each component separately as pure single component after regaining their original mother spectra which is considered as a fingerprint.

Only one divisor was used throughout the whole technique, namely the normalized spectra of impurity B. Using only one divisor has a significant advantage where the optimization step for choosing the divisor became of no importance.

Upon dividing the ternary mixture by the normalized spectrum of B, the ratio spectrum was obtained, and the amplitude difference 214.0 nm and 233.0 nm was recorded as demonstrated in Fig. 3.

The amplitude difference was then multiplied by the factorized spectrum of FLU, previously stored, to restore the ratio spectrum of FLU (FLU/NS_B) as shown in Fig. 4**(a)**. The original mother spectrum of FLU could be restored after multiplying its ratio spectra by the used divisor as shown in Fig. 4**(b)**.

The restored (FLU/NS_B) was then subtracted from the total ratio spectrum of the whole mixture to obtain that of both impurities as binary

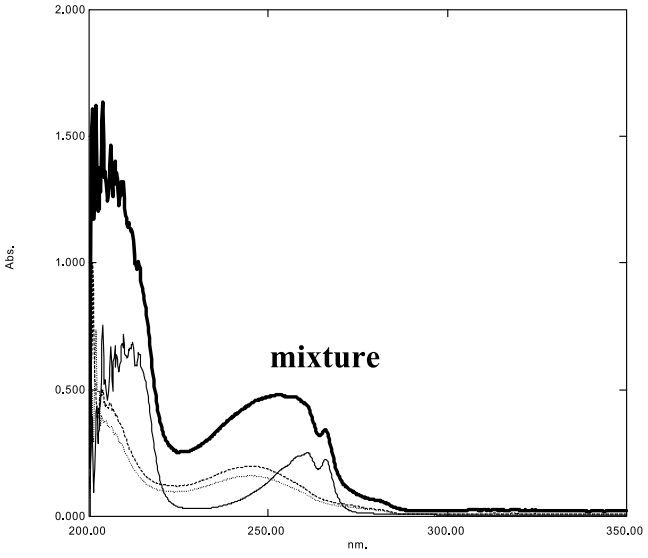


Fig. 2. Original zero order absorption spectra of 100 μg/mL FLU ( ) , 2 μg/mL Impurity B (……) and 0.1 μg/mL Impurity C (——) and their Total mixture, in methanol solvent.

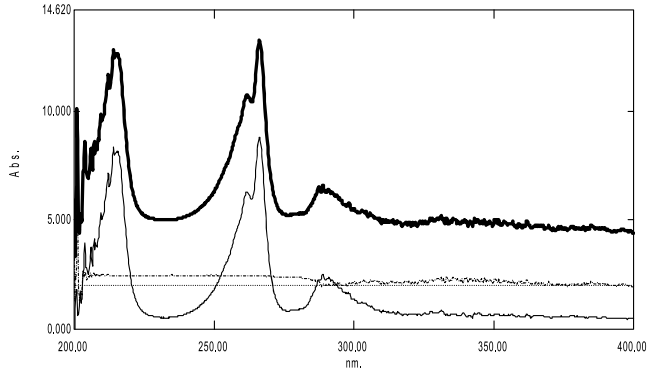


Fig. 3. Ratio spectra of 100 μg/mL FLU ( ) , 2 μg/mL Impurity B (.....) and 0.1 μg/mL Impurity C (——) and their mixture, all divided by the normalized spectrum of impurity B, in methanol solvent.

mixture ($B/NS_B+C/NS_B$) as demonstrated in Fig. 4**(c)**, and the same steps were repeated to restore the ratio spectra of impurity C (C/NS_B) where another pair of wavelengths were selected (276.0 nm and 288.4 nm), as shown in Fig. 4**(d)**. upon multiplying the spectrum (C/NS_B) by its factorized spectrum, the whole mother spectrum of impurity C will be restored as shown in Fig. 4**(e)**.

(C/NS_B) was subtracted from ($B/NS_B+C/NS_B$) to finally obtain a spectrum representing a straight line parallel to zero -axis (constant) with an amplitude resembling the concentration of impurity B is obtained (B/NS_B) as presented in Fig. 4**(f)**. Upon multiplying the later spectrum by NS_B, the original mother spectrum of impurity B was obtained as presented in Fig. 4**(g)**.

It is worthy to mention that the three components under study was determined at their λ_{max} which guaranteed maximum sensitivity and minimum error. Moreover, selecting the wavelength pairs (214.0 nm and 233.0 nm) and (276.0 nm and 288.4), where the ratio spectra of impurity C (C/NS_B) and impurity B (B/NS_B) showed equal absorbance values wasn't critical where both spectra exhibited prominent constant regions along the wavelength range (200 - 400 nm), as demonstrated in Figs. 3 **and** 4.

In addition, the toxicity profiling for impurities B and C was conducted using two online software and the developed method was assessed for the green, white, and blue characters using five tools.

4. Validation of the proposed method and application to pharmaceutical formulations

The proposed method was validated as per ICH guidelines [20] for the linearity and range, accuracy, precision, LOD, LOQ and specificity parameters, results are shown in Table 1. There was a linear relationship between the absorbance and the corresponding concentration of FLU, and the two impurities and the ranges were (50–500 μg/mL) for FLU, (0.5–6 μg/mL) for impurity B and (0.05–0.6 μg/mL) for impurity C. The method accuracy was confirmed by measuring the recovery% and SD for five concentrations within the linearity range, the recoveries of the three compounds were found within the accepted range. The method is precise by measuring the RSD% of three concentrations for each compound, in triplicate, intra-daily and inter-daily, the resulted RSD% values were <2. The specificity was proved by the analysis of laboratory prepared mixtures and showed no interference from the two impurities spectra, results are displayed in Table 2. The developed method was applied to three pharmaceutical formulations (Fungican® and Flucarol® capsules and Diflucan® IV infusion) and the recoveries of FLU were accepted and no interference from excipients was found. The validity of the application was confirmed by the standard addition technique, Table 3.

4.1. In silico toxicity profiling for the impurities

The results for examining the toxicity profiling of FLU and its impurities B and C using two databases; preADMET and pKcsm are presented in Tables 4 **and** 5 respectively. The preADMET results revealed that impurity B showed positive AMES mutagenicity to two salmonella strains, while impurity C showed mutagenicity to three strains. Moreover, impurity C showed the highest toxicity against algae and daphnia.

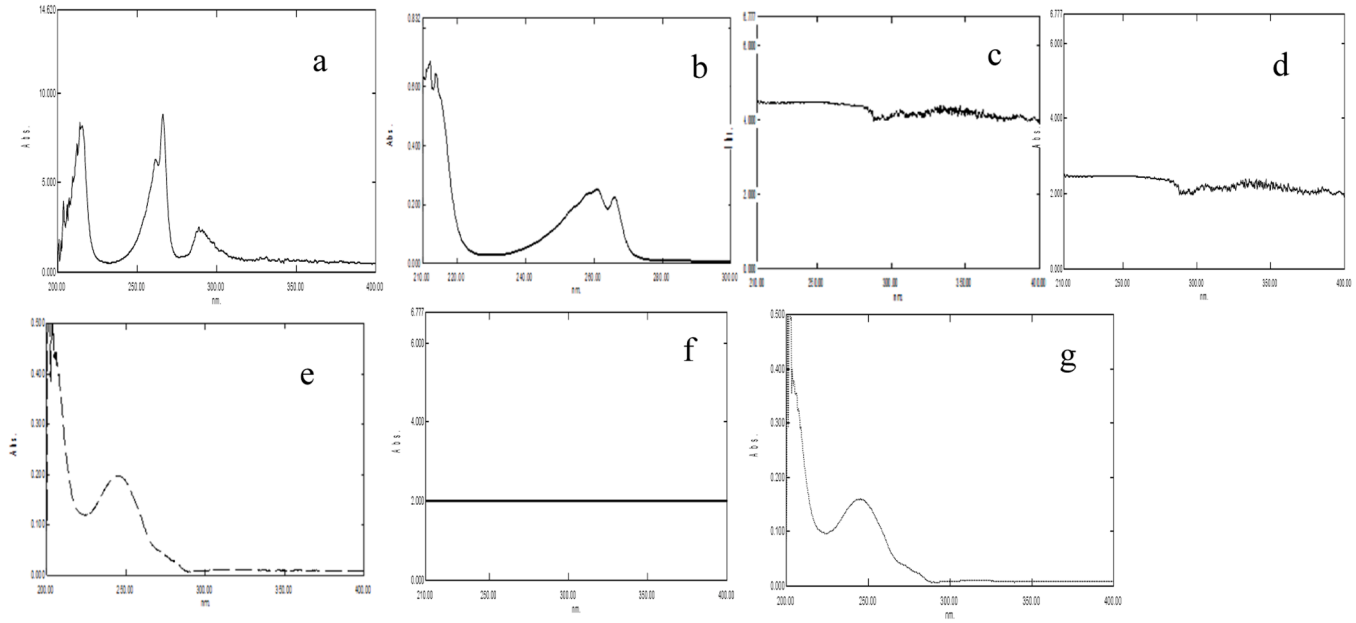


Fig. 4. (a) Restored ratio spectrum of FLU (100 μg/mL) after multiplication by its factorized spectrum. (b) Restored parent zero order spectrum of FLU (100 μg/mL) as a single component after multiplication by the normalized spectra of impurity B. (c) Regained ratio spectra of impurities B (2 μg/mL) and C (0.1 μg/mL) as binary mixture after subtraction of the ratio spectrum of FLU from that total mixture. (d) Restored ratio spectra of impurity C (0.1 μg/mL) after multiplication by its factorized spectra. (e) Restored parent zero order spectrum of impurity C (0.1 μg/mL) as a single component after multiplication by the normalized spectra of impurity B. (f) Regained ratio spectra of impurity B (2 μg/mL) as a single component after subtraction of the ratio spectrum of impurity C from the binary mixture. (g) Restored parent zero order spectrum of impurity B (2 μg/mL) as a single component after multiplication by its normalized spectrum, in methanol solvent.

Table 1
Validation parameters of the proposed spectrophotometric method for determination of fluconazole and its impurities B and C.

Parameter	FLU	Impurity B	Impurity C
Range (μg/mL)	50–500	0.5–6	0.05–0.6
Slope	0.0022	0.0799	1.975
Intercept	0.0009	0.0014	0.0009
Correlation coefficient (r)	0.9999	0.9998	0.9998
[ab]Accuracy (mean recovery% ± SD)	99.66 ±1.138	100.36 ±.779	99.79 ±0.781
[ac]Precision intra-day (RSD)	0.581	0.533	0.598
[ac]Precision Inter-day (RSD)	0.839	0.761	0.496
[d]LOD (μg/mL)	0.008	0.005	0.001
[d]LOQ (μg/mL)	0.049	0.032	0.007

[a] Average of three determinations. Five concentration of FLU (50, 100, 200, 300 and 450 μg/mL), Imp.B (0.5,2.5,3.5,4.5 and 5.5 μg/mL) and Imp.C (0.05, 0.1, 0.2,0.4 and 0.5 μg/mL), 3 replicate each ($n = 15$).[c] Three concentrations of FLU (50, 200 and 400 μg/mL), Imp.B (0.5,3,6) and Imp.C (0.05,0.2 and 0.5 μg/mL), 3 replicate each ($n = 9$).[d] LOD = 3.3 (SD / S), LOQ = 10 (SD / S); where SD is the residual standard deviation of the slope and S is the slope.

Table 2
Selectivity results obtained by the analysis of laboratory prepared mixtures, using the Proposed spectrophotometric method.

Mixtures of FLU: Imp.B: Imp. C	FLU	Impurity B	Impurity C
(μg/mL)	[a] **Recovery%**		
100 :0.5: 0.05	99.28	98.94	101.2
100: 0.5: 0.1	100.91	99.87	100.34
200:0.5: 0.05	99.34	101.1	100.29
200: 2: 0.6	101.03	98.9	99.56
300: 3: 0.1	100.71	98.61	99.12
Mean±SD	100.25 ± 0.869	99.48 ± 1.019	100.10 ± 0.799

[a] Average of three determinations.

Table 3
Application of the proposed spectrophotometric method to the pharmaceutical preparations

Pharmaceutical preparations	[a]Mean recovery% ± SD
Fungican® capsules claimed to contain 150 mg FLU	96.32 ± 1.32
Flucarol® capsules claimed to contain 150 mg FLU	97.47 ± 0.65
Diflucan® IV infusion claimed to contain 2 mg/mL FLU	99.58 ± 0.42
[b]Standard addition technique	97.56 ± 1.37

[a] Average of three determinations. [b] standard addition technique was performed by adding pure FLU equivalent to 10, 20 and 50 μg/mL to 10 μg/mL of the pharmaceutical preparation.

Table 4
Prediction of toxicity profiling of FLU and its two impurities B and C, using preADMET software (https://preadmet.qsarhub.com/toxicity/).

	FLU	Impurity B	Impurity C
Toxcity	**Value**		
algae_at	0.221284	0.133067	0.350869
Ames_test	mutagen	mutagen	Mutagen
Carcino_Mouse	negative	negative	Negative
Carcino_Rat	positive	positive	Positive
daphnia_at	0.690609	0.731932	0.846458
hERG_inhibition	medium_risk	medium_risk	medium_risk
medaka_at	0.675238	0.856351	0.927808
minnow_at	0.363013	0.536076	0.404147
TA100_10RLI	positive	negative	Positive
TA100_NA	positive	positive	Negative
TA1535_10RLI	positive	positive	Positive
TA1535_NA	positive	negative	Positive

Table 5
Prediction of toxicity profiling of FLU and its two impurities B and C, using pkcsm software (https://biosig.lab.uq.edu.au/pkcsm/)

Property	FLU	Impurity B	Impurity C
Toxcity	**Value**		
Ames toxicity	negative	negative	negative
Max. tolerated dose(human)	0.114	−0.047	0.201
hERG I inhibitor	negative	negative	negative
hERG II inhibitor	negative	positive	negative
Oral Rat Acute Toxicity (LD50)	2.328	2.267	2.338
Oral Rat Chronic Toxicity (LOAEL)	1.033	0.965	0.556
Hepatotoxicity	positive	positive	positive
Skin sensitization	negative	negative	negative
T.Pyriformis Toxcity	0.312	0.286	0.331
Minnow toxicity	3.872	4.126	1.236

Additionally, both impurities showed positive carcinogenicity for rats, and negative carcinogenicity for mice. Moreover, FLU and both impurities have a medium risk for hERG inhibition. The pKcsm results showed low MTRD; as the values are <0.447 log (mg/kg/day). The predicated values for hepatotoxicity are likely to be associated with disrupted normal function of the liver while it is not likely to be associated with skin sensitization. The findings revealed that impurity B and C showed mutagenicity and carcinogenicity.

5. Greenness, whiteness, and blueness assessment

The green, white and blue profiles of the developed spectrophotometric method were assessed and compared to reported chromatographic methods [17] used for analysis of FLU with its impurities B and C. Three tools were applied to assess the green character; analytical Eco-Scale (AES), Green Analytical Procedure Index (GAPI) and Analytical GREEnness calculator (AGREE). In addition, the newly introduced RGB 12 algorithm and BAGI tool were used to assess the method whiteness and blueness, respectively.

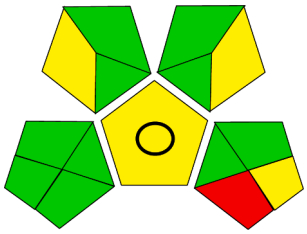
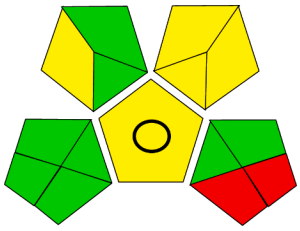
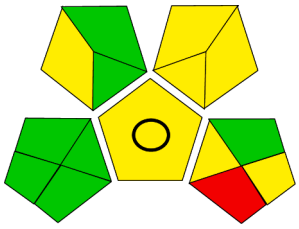
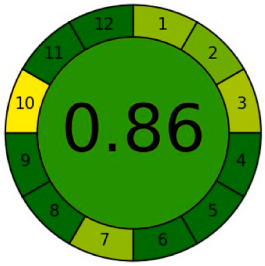
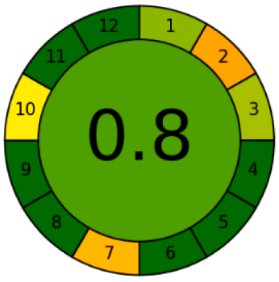
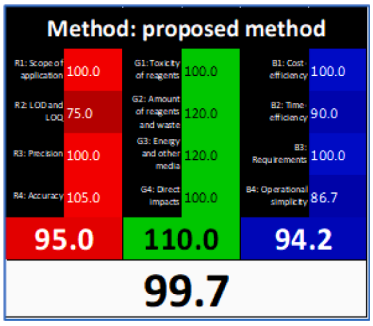
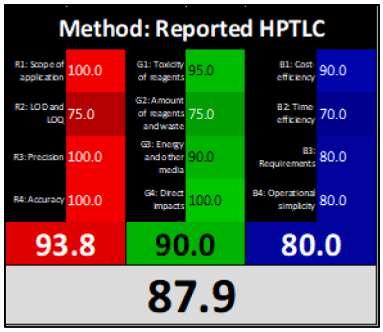
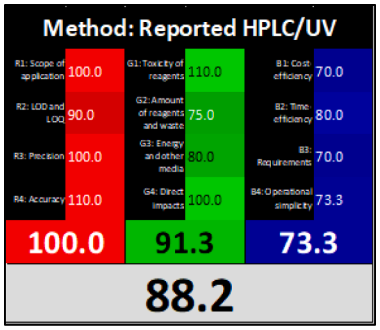
For the AES assessment, a final score out of 100 was given to the method after subtracting the penalty points [21–23]. The proposed method has the highest score (93) owing to the use of methanol only as a solvent in small volume and consequently the waste produced is lowered when compared to the reported chromatographic methods which have score of (83) and (90) for the HPTLC and HPLC/UV, respectively, shown in Table 6.

The GAPI figure has 5 pentagrams with 15 segments, as shown in Table 6. Each segment was colored green, yellow or blue according to its degree of greenness [24,25]. Segments 9 and 10 are green for the proposed method but shaded yellow for the two chromatographic reported methods. This resulted from the volume of reagents used in spectrophotometric method, which is lower. In addition to the lower National Fire Protection Association (NFPA) health hazard score of methanol which is equal to 1 when compared to that of the reagents used in the reported methods (ethyl acetate and ethanol) whose score is equal to 2 for both. For the reported HPLC/UV method, segment 12 is yellow colored due to the high instrumental energy. Contrarily, the proposed and the reported HPTLC methods have segment 12 colored green owing to the amount of consumed energy which is <0.1 kWh per sample. Owing to these reasons, the proposed method has the greatest number of green segments.

The AGREE tool is an open-source, user-friendly software which is based on the 12 principles of GAC. It shows a pictogram indicating the final score out of 1.0 and the weights of each evaluated parameter ranged from (0.0–1.0) [26,27]. The proposed method has the highest score of (0.86) which confirms its highest green character, Table 6.

The whiteness assessment is important in order to balance between the greenness of the method and its functionality/usefulness and sustainability [28]. The AES, GAPI and AGREE tools did not evaluate the method performance as accuracy and sensitivity, which are important

Table 6
The greenness, whiteness and blueness assessment of the proposed spectrophotometric method versus the reported chromatographic methods (17).

The tool	Proposed method		Reported HPTLC [17]		Reported HPLC /UV [17]	
AES	- Methanol	6	-Ethyl acetate	8	-Water	0
	- Instrumental energy	0	-Water	0	-Ethanol	4
	-Occupational hazards	0	-Acetic acid	4	-Instrumental energy	1
	-Waste	1	-Instrumental energy	0	-Occupational hazards	0
	-Total PPS	∑ 7	-Occupational hazards	0	-Waste	5
	- Eco-scale score	93	-Waste	5	-Total PPS	∑ 10
			-Total PPS	∑ 17	- Eco-scale score	90
			- Eco-scale score	83		
GAPI						
AGREE						
RGB-12						
BAGI						

parameters to be considered while developing an analytical method. The recently introduced RGB 12 algorithm tool was applied which is based on the 12 principles of White Analytical Chemistry (WAC) [29] . It evaluates the whole methodology; the greenness criteria (green color), the analytical performance (red color) and sustainability/productivity and practicality (blue color). The methods' performance was evaluated in terms of sensitivity, accuracy, and precision; the reported HPLC/UV has the highest red score of 100% versus the proposed spectrophotometric method with score 95 %. For the greenness score, the developed method has the highest score as confirmed by the three other green assessment tools (AES, GAPI and AGREE tools). Regarding the practical side of methods (including cost and time efficiency, operational simplicity and requirements for personal skills, facilities, and advanced instruments); the proposed spectrophotometric technique has the highest score of 94.2 %. followed by the two chromatographic methods with scores of 80% for HPTLC and 73.3 % for HPLC/UV. Obviously, the proposed spectrophotometric method is greener with less amount of solvent used and waste production, less energy consumption, less complicated, quicker, and more affordable than the reported chromatographic methods, earning it a whiteness score of 99.7 % overall versus scores of 87.9 % and 88.2 % for HPTLC and HPLC/UV, respectively.

The recent BAGI tool is used to assess the method blueness or practicality which is a very important parameter when comparing different methods of analysis or it is used to improve the method applicability [30]. It evaluates ten main attributes inspired by the blue color of the RGB model. The results of assessment appear as an asteroid pictogram with 10 sections and an overall score at the center. The sections have different grades for the blue color according to their compliance, the dark blue for high compliance and the faint blue for low compliance. The method is considered practical if its blue score is more than 60 points. The reported HPLC/UV method has a score of 92.5 and the two other methods have the same score of 87.5; the nearby score is owing to the similarity of nine attributes and the only difference is the number of analytes that could be simultaneously determined in one run which is more than 15 compounds for the HPLC technique.

6. Conclusion

The new proposed method (SDAD) was successfully applied and validated for determining FLU in presence of its two official impurities and in different pharmaceutical preparations. The quantitation of impurities is crucial as their presence above certain limit may affect the drug efficacy and safety. According to the literature review, there are no reported spectrophotometric methods for determination of FLU with its impurities. The developed spectrophotometric method has many advantages over the commonly used chromatographic methods for separation and quantitation of mixtures. This technique novelty lays in being able to resolve each compound in the ternary mixture as pure single component without any need for any prerequisite such as extension, isosbestic point or spectral extension. The proposed method is smart, facile, green and economic where it requires neither sophisticated instruments nor expensive software. Additionally, using five recent tools of assessment, the developed method was found to be the most eco-friendly, sustainable, and practical when compared to the reported chromatographic methods used for the same purpose. Besides, the toxicity profiling of the impurities was studied for the first time, using two computational databases. The findings revealed that impurity B and C showed mutagenicity and carcinogenicity which necessities their quantitation in pharmaceutical preparations. The presented spectrophotometric method is suitable to be used for routine quality control tests.

Consent for publication

All authors give consent for the publication of the manuscript in the Journal of Microchemical Journal.

CRediT authorship contribution statement

Christine M. El-Maraghy: Formal analysis, Software, Validation, Writing – original draft. **Mai S. Nour:** Writing – original draft, Software, Resources, Data curation. **Ekram H. Mohamed:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest to declare that are relevant to the content of this article.

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Data availability

Data will be made available on request.

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