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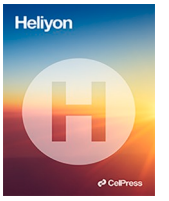

Three new, UV spectrum filtration protocols for the synchronous quantification of ciprofloxacin HCl and ornidazole in the existence of ciprofloxacin-induced degradation compound

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ABSTRACT

Three new spectrum filtration protocols have been developed and adapted to overcome some difficulties in dealing with highly overlapping triple drug mixtures by proposing new smart mathematical techniques that facilitate the resolution of the ternary mixture and the recovery of a filtrated zero-order spectrum (D^0 spectrum) of each component without any overlapping from the accompanying components. The three established spectrophotometric protocols were conducted on the combination of ciprofloxacin hydrochloride and ornidazole as a green alternative to the usual chromatographic technique: the first protocol is ratio difference-isosbestic points coupled with ratio difference-areas under the curve (RD-ISO/RD-AUC); the second protocol is ratio difference-isosbestic points coupled with dual-wavelength equation (RD-ISO/DWE); and the third protocol is signal retrieval by zero-crossing point (SRZ). All three developed protocols have the power to recover a filtrated zero-order spectrum of each ornidazole and ciprofloxacin hydrochloride without any involvement from the ciprofloxacin-induced degradation substance through processing their spectral data either in the zero-order spectrum, ratio spectrum, or derivative spectrum. The correctness of the spectral filtration process for each protocol was checked by involving the spectral print recognition index to ensure the drug's purity and freeness from impurities or degradation products. The validation process was performed as per the directions of ICH, which confirmed the effectiveness of the elaborated protocols and their usability as daily analysis methods with a linearity range of (3.5–15 μg/ml) for ciprofloxacin in (RD-ISO/RD-AUC) and (RD-AUC/DWE) protocols and (1.5–15 μg/ml) in (SRZ) protocol; and a linearity range of (3–20 μg/ml) for ornidazole in (RD-ISO/RD-AUC) and (SRZ) protocols and (3–15 μg/ml) in (RD-ISO/DWE) protocol. A statistical comparison and greenness evaluation utilizing NEMI, AGREE, GAPI, and CALIFICAMET-HEXAGON tools were made with the reference approach, confirming no statistical variations and a better greenness profile for the newly established protocols.

1. Introduction

Bacterial vaginosis and non-specific vaginitis diseases are both related to the progression of diseases like asymptomatic bacteriuria, pyelonephritis, and contagious complications [1]. A recent study demonstrates the therapeutic effect of a dosage form containing ciprofloxacin and ornidazole for the treatment of bacterial vaginosis and vaginal dysplasia associated with the concurrent existence of

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anaerobic and aerobic bacteria [2].

Another study indicates that a combined antimicrobial and antiprotozoal drug containing ornidazole and Ciprofloxacin has established remarkable clinical efficiency and good tolerability in the antibiotic prophylaxis of postoperative complications and in the treatment of cystitis and overactive bladder in women who have undergone sling operations for urinary incontinence with enuresis [3].

Ciprofloxacin hydrochloride (CIPH) [4] Fig. 1, is bactericidal from the fluoroquinolone's family and works by inhibiting bacterial DNA replication [5].

Ornidazole (ORNI), Fig. 1, is an antiprotozoal agent from nitroimidazole derivatives [6], which works by NO_2 reduction, producing poisonous radicals and derivatives [7].

Previous studies include spectroscopic approaches [8–10], HPLC [11–14], and TLC [15] for the concurrent quantification of CIP and ORNI. Other studies also introduced two spectrophotometric approaches for determining ciprofloxacin alone in the existence of its degraded product in an acidic medium [16,17]; in order to determine CIPH concentration by these two well-established spectrophotometric methods, a long-time preparation of ciprofloxacin-induced degraded compound (*C*-DEG) is required to use its spectrum as a divisor in the process of CIPH quantification. No spectrophotometric approach for the synchronous estimation of CIPH and ORNI in the existence of ciprofloxacin-induced degradation compound is noticed in the prior studies.

Stability, indicating the drug sample and confirming that it is free from impurities or degradation compounds, is a significant point to reveal the alteration over time in the physical, chemical, or microbiological properties of drug compounds and drug products. In addition, defining the degradation products' structure is no less important as some drug compounds, when degraded, give inactive pharmacological products because of the loss of some functional groups in their molecular structure, which consequently causes the alteration in drug effectiveness that sometimes causes an undesirable effect on human health [18]. This gives rise to the need to conduct stability studies on the drug to detect any destruction in its formula.

Counting on the foregoing and due to the global trend toward developing green analytical methods [19–24], this manuscript focused on elaborating three green UV methods for the concurrent estimating of CIPH, ORNI, and *C*-DEG without the analyst needing to prepare and store the *C*-DEG spectrum in the spectra software. This manuscript also highlights the traits of these protocols and their potency to retrieve the component filtrated D^0 spectrum; in addition, the greenness of the instituted protocols was estimated and compared with the chromatographic reference protocol. The three new protocols are flexible in dealing with binary or ternary mixtures and do not demand specific programs or complicated calculations to handle, overcoming some constraints that may exist in some preceding approaches.

2. Theoretical background

2.1. UV spectrum filtration conception

The idea of whether the spectral approach is deemed a spectrum filtration approach depends on its ability to retrieve the drug substance zero-order spectrum, which represents the drug identity spectrum (ID spectrum) without any intervention from its accompanying components. The spectrophotometric approach can be made capable of spectral filtering by pairing it with some spectral tools that participate in the retrieval of the drug ID spectrum. These spectral tools are defined by the factorized and the

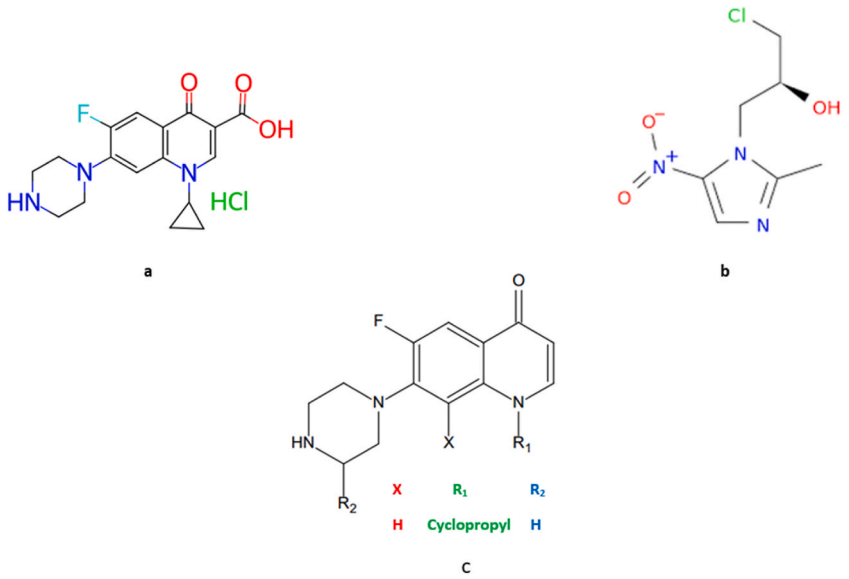

Fig. 1. The chemical structure of (a): ciprofloxacin HCl, (b): ornidazole and (C) acid degradation product of ciprofloxacin.

normalized spectrum, which works as spectral templates appointed in the spectral recovery pathway. The filtration process starts with producing a specific spectral template for each substance in the mix, relying on the phase at which the spectral processing is executed either in the zero-order phase, the ratio phase, or the derivation phase. These produced spectral templates are then stored in the spectra software to be ready later to be modified with the numeric spectral data of each substance upon performing the spectral filtration process as will be displayed next in the new protocols.

2.1.1. *The first spectral filtration protocol*

Ratio difference-isosbestic points (RD-ISO) coupled with the ratio difference-areas under the curve (RD-AUC)**;** this protocol involves two consecutive techniques

1st **technique**: ratio difference-isosbestic points (RD-ISO) is a developed technique [25], which handles the ternary mixture (A, B, C) by picking two isosbestic points between A and B (λ_{iso1}, λ_{iso2}), then dividing the D^0 spectrum of (A, B, C) mixture by the D^0 spectrum of A component, and subtracting the mixture amplitudes ($P_{\lambda iso1}$, $P_{\lambda iso2}$) to cancel the impact of A and B at them; therefore, the amplitudes difference at (λ_{iso1}, λ_{iso2}) will be linked to C concentration only as clarified in equations 1, 2 and 3.

$$P_{iso1} = [a_A C_A/A'] + [a_B C_B/A'] + [a_{C1} C_C/A'] \quad (1)$$

$$P_{iso2} = [a_A C_A/A'] + [a_B C_B/A'] + [a_{C2} C_C/A'] \quad (2)$$

$$P_{iso1} - P_{iso2} = (a_{C1} C_C - a_{C2} C_C)/A' \quad (3)$$

P_{iso1}, P_{iso2}: mixture amplitude at λ_{iso1}, and λ_{iso2}, respectively. C concentration is determined by establishing a linear equation between its concentrations and the analogous ($P_{\lambda iso1}$- $P_{\lambda iso2}$).

2nd **technique**: ratio difference-Areas under the curve (RD-AUC) is a technique that relies on the concept of dual amplitude difference [26]. It is utilized when no precision results are gained upon relying on the difference of only two points in the ratio stage to resolve the components of (A, B) mixture, where a larger number of spectral points are employed to cancel the error caused by the noise of the divisor or the error caused by subtracting two minor spectral points. To apply this technique, the component C ratio spectrum is recovered first as mentioned above, then canceled from the (A, B, C) mixture's ratio spectrum by a subtraction process to acquire the (A, B) ratio spectrum, which is handled then by (RD-AUC) technique by electing two areas under the curve ($AUC_{\lambda 1\text{-}\lambda 2}$, $AUC_{\lambda 2\text{-}\lambda 3}$) that carry an equal range of wavelengths (5 nm, 10 nm, …etc.) but with a variation in AUC value between them; then the AUCs difference ($AUC_{\lambda 1\text{-}\lambda 2}$- $AUC_{\lambda 2\text{-}\lambda 3}$) will be proportional to component B only since the constant difference is zero as in equation (4).

$$AUC_{(\lambda 1-\lambda 2)\ B} - AUC_{(\lambda 3-\lambda 4)\ B} = \text{slope} \times C_B \pm \text{intercept} \quad (4)$$

A linear equation between ($AUC_{(\lambda 1\text{-}\lambda 2)\ B}$- $AUC_{(\lambda 3\text{-}\lambda 4)\ B}$) and analogous $AUC_{(\lambda 1\text{-}\lambda 2)\ B}$ is constructed as equation (5), then component A concentration is determined by substituting the mixture ($AUC_{(\lambda 1\text{-}\lambda 2)}$ - $AUC_{(\lambda 3\text{-}\lambda 4)}$) value in the constructed equation (5) to attain $AUC_{(\lambda 1\text{-}\lambda 2)\ B}$, which is subtracted from $AUC_{(\lambda 1\text{-}\lambda 2)\ M}$ of (A, B) mixture to obtain $AUC_{(\lambda 1\text{-}\lambda 2)A}$, that is proportional to A concentration only as in equation (6).

$$AUC_{(\lambda 1-\lambda 2)B} - AUC_{(\lambda 3-\lambda 4)B} = \text{slope} \times AUC_{(\lambda 1-\lambda 2)B} \pm \text{intercept} \quad (5)$$

$$AUC_{(\lambda 1-\lambda 2)M} - AUC_{(\lambda 1-\lambda 2)B} = AUC_{(\lambda 1-\lambda 2)A} \quad (6)$$

$$AUC_{(\lambda 1-\lambda 2)\ A} = \text{slope} \times C_A \pm \text{intercept} \quad (7)$$

B component is quantified directly from its linear equation (4), while A concentration is detected from its linear equation (7) after estimating its $AUC_{(\lambda 1\text{-}\lambda 2)\ A}$ as mentioned above. This technique has the trait of getting rid of the fault that resulted from relying on only two minor amplitudes for the subtracting process, as sometimes the maximum peak cannot be involved in the subtraction process due to the high noise of the divisor. The condition for applying this technique is that both elected areas under the curve should possess the exact number of wavelengths to completely get rid of component A.

The spectral filtration pathways of components A, B, and C in the first protocol are as follows.

- Spectral filtration pathway of component A:

1 producing the factorized AUC spectrum of component A by choosing a ratio spectrum of one of its linear range concentrations and dividing it by its $AUC_{(\lambda 1\text{-}\lambda 2)}$.

$$[a_A\ C_A\ /a_A\ C'_A]/\ [\Delta a_{A\ AUC(\lambda 1\text{-}\lambda 2)}\ C_A/\ \Delta a_{A\ AUC(\lambda 1\text{-}\lambda 2)}\ C'_A] = [\text{constant}]' = [1]'$$

2 multiplying the recovered $AUC_{(\lambda 1\text{-}\lambda 2)}$ of component A by the prepared factorized spectrum to get the A component's ratio spectrum.

$$[1]'[\ [\Delta a_{A\ AUC(\lambda 1\text{-}\lambda 2)}\ C_A/\Delta\ a_{A\ AUC(\lambda 1\text{-}\lambda 2)}\ C'_A] = C_A\ /\ C'_A$$

3 multiplying the resulting ratio spectrum by the D^0 spectrum of divisor A to get the A component's D^0 spectrum.

$$(C_A\ /\ C'_A)\ .\ a_A\ C'_A = a_A\ C_A$$

-Spectral filtration pathway of component B.

1 -producing the factorized AUCs difference spectrum of component B by choosing a ratio spectrum of one of its linear range concentrations and dividing it by its AUC difference ($AUC_{(\lambda1-\lambda2)}$ - $AUC_{(\lambda3-\lambda4)}$).

$[a_B\ C_B\ /aA\ C'A]/\ [\Delta aB\ AUC(\lambda1-\lambda2) - AUC\ (\lambda3-\lambda4)CB/\ \Delta aA\ AUC(\lambda1-\lambda2) - AUC\ (\lambda3-\lambda4)\ C'A] = [(aB/aA).\ (\Delta a_{A\ AUC(\lambda1-\lambda2)-AUC\ (\lambda3-\lambda4)}/\ \Delta a_{B\ AUC(\lambda1-\lambda2)-AUC\ (\lambda3-\lambda4)})]'$

2 multiplying the mixture value of ($AUC_{(\lambda1-\lambda2)}$ - $AUC_{(\lambda3-\lambda4)}$) by the prepared factorized spectrum to get the B component's ratio spectrum.

$[(a_B/aA).\ (\Delta aA\ AUC(\lambda1-\lambda2) - AUC\ (\lambda3-\lambda4)/\ \Delta aB\ AUC(\lambda1-\lambda2) - AUC\ (\lambda3-\lambda4))]'[\ [\Delta aB\ AUC(\lambda1-\lambda2) - AUC\ (\lambda3-\lambda4)CB/\ \Delta aA\ AUC(\lambda1-\lambda2) - AUC\ (\lambda3-\lambda4)\ C'A] = aB\ CB\ /a_{\ A}.C'_A$

3 multiplying the resulting ratio spectrum by the D^0 spectrum of divisor A to get the B component's D^0 spectrum.

$(aB\ CB\ /a\ A.C'A).aA\ C'A = aB\ C_B$

-Spectral filtration pathway of component C.

1 -producing the factorized isosbestic difference spectrum of component C by choosing a ratio spectrum of a concentration of its linear range and dividing it by its amplitudes difference value (P_{iso1}- P_{iso2}).

$[a_C\ C_{C\ /}a_A\ C'A]/\ [\Delta aC\ (\lambda iso1-\ \lambda iso2)CC/\ \Delta aA(\lambda iso1-\ \lambda iso2)\ C'A] = [(aC/aA).(\Delta a_{A(\lambda iso1-\ \lambda iso2)}/\ \Delta a_{C\ (\lambda iso1-\ \lambda iso2)})]'$

2 multiplying the ($P_{\lambda iso1}$- $P_{\lambda iso2}$) value of the mixture at the two elected isosbestic points by the produced factorized spectrum to get the C component's ratio spectrum.

$[(a_C/aA).(\Delta aA(\lambda iso1-\ \lambda iso2)/\ \Delta aC\ (\lambda iso1-\ \lambda iso2))]'[\Delta aC\ (\lambda iso1-\ \lambda iso2)CC/\Delta aA(\lambda iso1-\ \lambda iso2)\ C'A] = aC\ CC\ /a_{\ A}.C'_A$

3 multiplying the C component's ratio spectrum by D^0 spectrum of divisor A to get the C component's D^0 spectrum.

$(aC\ CC\ /a\ A.C'A).aA\ C'A = aC\ C_C$

2.1.2. The second spectral filtration protocol

Ratio difference-isosbestic points (RD-ISO) coupled with dual-wavelength equation (DWE) consists of two consecutive techniques:

1st **technique**: Ratio difference-isosbestic points (RD-ISO) is utilized to recover the C component's D^0 spectrum in order to subtract it from (A, B, C) mixture's D^0 spectrum to gain (A, B) mixture's D^0 spectrum.

2nd **technique**: Dual-wavelength equation (DWE): This technique depends on the concept of Q absorbance ratio technique [27]. It handles (A, B) mixture in the D^0 stage by picking two wavelengths, which are λ_{iso} and λ_{Max} of A or B. In order to cancel the overlapping of B component at the λ_{Max} of A component, the next strategy is followed:

At $\lambda_{M}ax$: AM, Max = AB + AA (8)

$AM,Max = aB\ C_B + a_A\ C_A$ (9)

$$\frac{A_{M,Max}}{a_B} - \frac{a_A C_A}{a_B} = C_B \quad (10)$$

At λ_{iso}: C_M-C_A=C_B (11)

Where:

$C_M = A_{M,iso}/a_{iso}$ (12)

By substituting equation (12) in equation (11):

$(A_{M,iso}/a_{iso})$-C_A=C_B (13)

By substituting equation (13) in equation (10):

$$\frac{A_{M,Max} - a_A C_A}{a_B} = \frac{A_{M,iso}}{a_{iso}} - C_A \quad (14)$$

By rearrangement of equation (14):

$$C_A = \frac{\frac{A_{M,Max}}{a_B} - \frac{A_{M,iso}}{a_{iso}}}{\frac{a_A}{a_B} - 1} \quad (15)$$

a_A, a_B: absorptivity factor at 311 nm for component A and component B respectively, $a_{iso:}$ absorptivity factor of A or B at 320 nm. Equation (15) is utilized to detect A concentration (μg/ml) away from any overlapping from B, where B concentration is determined directly from equation (11) after computing A concentration. This novel technique is distinguished by its potential to correct component A absorbance at its λ_{Max} with no need for an expanded area of B spectrum; it also does not require additional processing steps such as division or derivative.

The spectral filtration pathways of components A, B and C in the second protocol are as follows:

Spectral filtration pathway of component A.

1 producing A component's normalized spectrum by summing some of the D^0 spectra of component A and dividing them by the total concentration.
2 multiplying the estimated concentration of component A by the produced normalized spectrum to get the A component's D^0 spectrum.

-Spectral filtration pathway of component B:

Subtracting A component's retrieved D^0 spectrum from (A, B (mixture's D^0 spectrum.

-Spectral filtration pathway of component C:

As mentioned in the first protocol.

2.1.3. The third spectral filtration protocol

This protocol involves signal retrieval by zero-crossing point technique (SRZ); this new technique relies on the concept of the amplitude factor technique [28]. It is able to detect component A and component B concentration in (A, B, C) mixture, using only one zero-crossing point in the derivative ratio stage through dividing the mixture's D^0 spectrum by C component's D^0 spectrum, and deriving the resulting ratio spectrum, then electing two wavelengths in derivative ratio stage, which are zero-crossing point of A (λ_{zero}) and the maximum peak of A (λ_{Max}). A linear equation between B component's signals at λ_{zero} ($P_{B,\ zero}$) and the analogous signals at λ_{Max} ($P_{B,\ Max}$) is constructed as in equation (17); hence, to quantify component A in the mixture (A, B, C), the mixture signal at λ_{zero} is substituted in equation (17) to recover B component's signal at λ_{Max} ($P_{B,\ Max}$). Then this recovered signal is subtracted from the mixture's signal at λ_{Max} ($P_{M,\ Max}$) to obtain A component's signal at λ_{Max} ($P_{A,Max}$) as in equation (18), thus determining its concentration through equation (19). B concentration is detected at λ_{zero} using equation (16):

$$\text{At}\lambda_{zero}: P_{B,zero} = \text{slope} \times C_B \pm \text{intercept} \quad (16)$$

$$P_{B,zero} = \text{slope} \times P_{B,Max} \pm \text{intercept} \quad (17)$$

$$\text{At } \lambda_{Max}: P_{M,\ Max} - P_{B,\ Max} = P_{A,\ Max} \quad (18)$$

$$P_{A,Max} = \text{slope} \times C_A \pm \text{intercept} \quad (19)$$

C concentration is detected in an identical way utilizing a convenient divisor of A or C. This technique is a proper solution when just one robust zero-crossing point exists in the derivative ratio stage or derivative stage.

The spectral filtration pathways of components A and B in the third protocol are as follows:

-Spectral filtration pathway of component A.

1 producing the A component's factorized amplitude spectrum by choosing a derivative ratio spectrum of one of its linear range concentrations and then dividing it by its signal at λ_{max}

$$[d/d_\lambda(a_A / ac)\ CA]/[d/d\lambda\ (aA/ac)\ \lambda Max\ CA] = [d/d\lambda\ (aA/ac)\ /d/d_\lambda(a_A/a_c)\ _{\lambda Max}]'$$

2 multiplying the A component's retrieved signal at λ_{Max} by the prepared factorized spectrum to recover A component's derivative ratio spectrum.

$$[d/d\lambda\ (aA/ac)\ /d/d\lambda(aA/ac)\ \lambda Max]'.\ [d/d\lambda(aA/aC)\ \lambda Max\ CA] = [d/d\lambda(a_A/a_C)\ C_A]$$

3 multiplying the resulting derivative ratio spectrum by the decoding spectrum to retrieve the A component's D^0 spectrum (the decoding spectrum was produced via dividing the A component's normalized spectrum by its derivative ratio spectrum).

$$[d/d_\lambda(aA/aC)\ CA].\ [aA/d/d\lambda(aA/aC)] = aA.C_A$$

-Spectral filtration pathway of component B.

1 producing the B component's factorized zero-crossing point spectrum by choosing a derivative ratio spectrum of one of its linear range concentrations and dividing it by its amplitude at λ_{zero}

$[d/d_{\lambda}(aB / ac)\ CB]/[d/d\lambda\ (aB /ac)\ \lambda zero\ CB] = [d/d\lambda\ (a_B/a_C)\ /d/d_{\lambda}(a_B\ /a_c)_{\lambda zero}]'$

2- multiplying the mixture's signal at λ_{zero} by the prepared factorized spectrum to recover the B component's derivative ratio spectra.

$[d/d\lambda\ (aB/aC)\ /d/d\lambda(aB\ /ac)\lambda zero]'.\ [d/d\lambda(aB/aC)\ \lambda zero\ CB] = [d/d\lambda(a_B\ /a_C)\ C_B]$

3 multiplying the resulting derivative ratio spectrum by the decoding spectrum to retrieve the B component's D^0 spectrum (the decoding spectrum was produced via dividing the B component's normalized spectrum by its derivative ratio spectrum)

$[d/d\lambda(aB\ /aC)\ CB]\ [aB\ /d/d\lambda(aB/aC)] = a_B.C_B$

3. Experimental

3.1. Equipment and software

JASCO V-650 spectrophotometer was employed for D^0 spectra scanning. Spectra manager® software, JASCO corporation, version 2, was employed to produce ratio, derivative ratio spectra, and spectral templates.

3.2. Substances and solvents

3.2.1. Pure samples

Ciprofloxacin HCl and Ornidazole were purchased from Chongqing Chemdad CO., Ltd., CHINA, having a purity of 99.35 ± 0.73 and 99.16 ± 0.62 according to British pharmacopeia [4] and Indian pharmacopeia [6] respectively.

3.2.2. Ciprofloxacin acidic degradation substance (C-DEG)

C-DEG was produced as stated in the protocol developed in Ref. [29] via dissolving 20 mg of pure CIPH in 20 ml of 2 N HCl, then heating the solution under reflux for 48 h; the resulting solution is cooled and neutralized with 2 N NaOH. Then it is evaporated until it dries under a vacuum. The residue was extracted into a 100-ml standard flask with methanol to acquire a methanolic stock solution with a 200 μg/ml concentration of *C*-DEG.

3.2.3. Pharmaceutical products

The commercial product Cifran-OZ® is labeled to consist of 500 mg of each ciprofloxacin and ornidazole in each film-coated tablet; it was manufactured by Sun Pharma Laboratories Ltd. INDIA, batch no. SXC0964A.

3.2.4. Solvents

Methanol solvent of the analytical grade was purchased from Panreac, Spain.

3.2.5. Standard solutions

- Stock standard methanolic solution of 1000 μg/ml of each CIPH, and ORNI.
- Working methanolic solution of 50 μg/ml of each CIPH, and ORNI

4. Procedure

4.1. Linearity ranges and calibration graphs

Six 5-ml standard flasks, which were set with a range of concentrations of 1.5–15 μg/ml for CIPH and 3–20 μg/ml for ORNI and *C*-DEG were produced separately in methanol, and then scanned and saved in spectra software. The saved spectra of the former standard solutions were processed to set the calibration diagram for the established protocols as follows.

4.1.1. The first protocol (RD-ISO/RD-AUC)

After dividing all the CIPH, ORNI, and *C*-DEG D^0 spectra by the ORNI 20 μg/ml D^0 spectrum, the following linear equations and factorized spectra were established.

- A linear equation was constructed between each of CIPH concentrations and the analogous amplitudes differences ($P_{277.5nm}$ -P_{320nm}), *C*-DEG concentrations and the analogous AUCs differences ($AUC_{310–320\ nm}$ -$AUC_{300–310\ nm}$), *C*-DEG AUCs differences ($AUC_{310–320\ nm}$ -$AUC_{300–310\ nm}$) and the analogous $AUC_{300–310\ nm}$, ORNI concentrations and the analogous $AUC_{300–310\ nm}$

- the factorized spectra were produced through a division process of the ratio spectrum of a chosen concentration from the linear range of each CIPH by its difference ($P_{277.5\ nm}$ -$P_{320\ nm}$), *C*-DEG by its AUCs difference ($AUC_{310–320\ nm}$ -$AUC_{300–310\ nm}$), and ORNI by its $AUC_{300–310\ nm}$

4.1.2. The second protocol (RD-ISO/DWE)

Using the D^0 spectra of ORNI and *C*-DEG, the following linear equations and spectral templates were established.

- A linear equation was constructed between each of ORNI concentrations and the analogous absorbances at 311 nm and 320 nm, *C*-DEG concentrations and the analogous absorbances at 311 nm and 320 nm.

-The absorptivity factor of each ORNI(a_{ORNI}) and *C*-DEG ($a_{C\text{-}DEG}$) was set through estimating the average of absorbance to concentration ratio at 311 nm and at 320 nm, where at 311 nm $a_{ORNI} = 0.0401$,$a_{c\text{-}DEG} = 0.0286$, and at 320 nm $a_{ORNI} = a_{c\text{-}DEG} = 0.0369$.

- The normalized spectrum of ORNI was prepared by summing a number of D^0 spectra and dividing them by the corresponding sum of concentrations.

4.2. The third protocol (SRZ)

After dividing each of ORNI and *C*-DEG D^0 spectra by CIPH 10 μg/ml spectrum, and dividing CIPH D^0 spectra by ORNI 20 μg/ml spectrum, all the resulting ratio spectra underwent derivation (1st order, 11 points, scale × 10), and the following linear equations and factorized spectra were established.

- A linear equation was constructed between each of the *C*-DEG concentrations and the analogous amplitudes at 271.7 nm, *C*-DEG amplitudes at 271.7 nm and the analogous amplitudes at 294.5 nm, ORNI concentrations and the analogous amplitudes at 294.5 nm, CIPH concentrations and the analogous amplitudes at 289.8 nm.
- The factorized spectra were produced through a division process of the derivative ratio spectrum of a chosen concentration from the linear range of each *C*-DEG by its amplitude at 271.7 nm, ORNI by its amplitude at 294.5 nm, and CIPH by its amplitude at 289.8 nm.
- Decoding spectra: dividing the normalized spectrum of each A and B by its analogous derivative ratio spectrum.

4.3. The analysis of in-lab-prepared mixes

Various mixes with varied portions of CIPH, ORNI, and *C*-DEG were produced and scanned in the region 200–400 nm, then saved in spectra software to apply the following established protocols.

4.3.1. The first protocol (RD-ISO/RD-AUC)

The stored D^0 spectrum of each produced ternary mixture (CIPH, ORNI, *C*-DEG) was subject to division by ORNI 20 μg/ml D^0 spectrum. Then ($P_{277.5nm}$-P_{320nm}) value of the resulting ratio spectrum was computed and multiplied by CIPH factorized isosbestic difference spectrum; the recovered CIPH ratio spectrum was then subtracted from (CIPH, ORNI, *C*-DEG) mixture's ratio spectrum to acquire (ORNI, *C*-DEG) mixture's ratio spectrum. To determine ORNI in the resulting binary mixture, the AUCs difference ($AUC_{310–320nm}$ -$AUC_{300–310nm}$) of the mixture's ratio spectrum was substituted in the corresponding equation between ($AUC_{310–320nm}$ -$AUC_{300–310nm}$ and $AUC_{310–320nm}$) to recover $AUC_{300–310nm}$ of *C*-DEG, which is subtracted from $AUC_{300–310nm}$ of the mixture ratio spectrum to get $AUC_{300–310nm}$ of ORNI. Each of the CIPH, ORNI, and *C*-DEG concentrations was detected using its constructed equation between signals and the analogous concentrations.

Spectral filtration pathway of CIPH, ORNI, and *C*-DEG.

1 multiply the factorized spectrum of each of CIPH by the mixture difference ($P_{277.5nm}$ -P_{320nm}), *C*-DEG by the mixture difference ($AUC_{310–320nm}$ -$AUC_{300–310nm}$), ORNI by the recovered $AUC_{300–310nm}$.
2 multiply each of the attained CIPH, ORNI, and *C*-DEG ratio spectra by ORNI 20 μg/ml D^0 spectrum.

4.3.2. The second protocol (RD-ISO/DWE)

CIPH's D^0 spectrum in each mixture (CIPH, ORNI, and *C*-DEG) was first recovered by (RD-ISO) technique, then subtracted from the corresponding ternary mixture's D^0 spectrum to get (ORNI, *C*-DEG) mixture's D^0 spectrum, the (ORNI, *C*-DEG) mixture's absorbances at 311 nm and 320 nm were substituted in the established equation of ORNI to detect its concentration; *C*-DEG was quantified through subtracting ORNI concentration from (ORNI, *C*-DEG) mixture concentration.

Spectral filtration pathway of ORNI and *C*-DEG.

1 multiply the determined concentration of ORNI by its stored normalized spectrum to attain the ORNI's D^0 spectrum.
2 subtract ORNI's D^0 spectrum from (ORNI, *C*-DEG) mixture's D^0 spectrum to acquire *C*-DEG's D^0 spectrum.

4.3.3. The third protocol (SRZ)

The stored D^0 spectrum of each ternary mixture (CIPH, ORNI, *C*-DEG) was divided by the D^0 spectrum of CIPH 10 μg/ml. The resulting ratio spectra were derived (1st order derivative, 11 points scale × 10), and the mixture signal at 271.7 nm was substituted in the constructed equation between $P_{271.7nm}$ and $P_{294.5nm}$ to recover the signal of *C*-DEG at 294.5 nm, which is subtracted from the mixture's signal at 294.5 nm to recover ORNI's signal at 294.5 nm. CIPH was determined in (CIPH, ORNI, *C*-DEG) mixture through a division process of the mixture's D^0 spectrum by ORNI's D^0 spectrum 20 μg/ml and the derivation of the resulting ratio spectrum (1st order derivative, 11 points, scale × 10). Each of the CIPH, ORNI, and *C*-DEG concentration was estimated using its constructed equation between signals and the analogous concentrations.

Spectral filtration pathway of CIPH, ORNI, and *C*-DEG.

1 multiply the factorized spectrum of each *C*-DEG by the mixture signal at 271.7 nm, ORNI by its retrieved signal at 294.5 nm, and CIPH by the mixture's signal at 289.8 nm.
2 multiply each component's resulting derivative ratio spectrum by its decoding spectrum.

4.4. The analysis of tablet formulations

Ten tablets of Cifran-oz® were weighed precisely and crushed well; then a specific weight equivalent to 10 mg of each CIPH and ORNI was taken into a 25 ml standard flask and dissolved with 15 ml methanol; the produced solution was sonicated for 10 min and filtered to 25 ml standard flask and completed with methanol to the line; a proper dilution was then made to produce the sample solution, which is then analyzed as mentioned in lab-prepared mixture section.

5. Results and discussion

To ensure the plausibility of replacing chromatographic methods with spectroscopic methods for conducting a green assay for ORNI

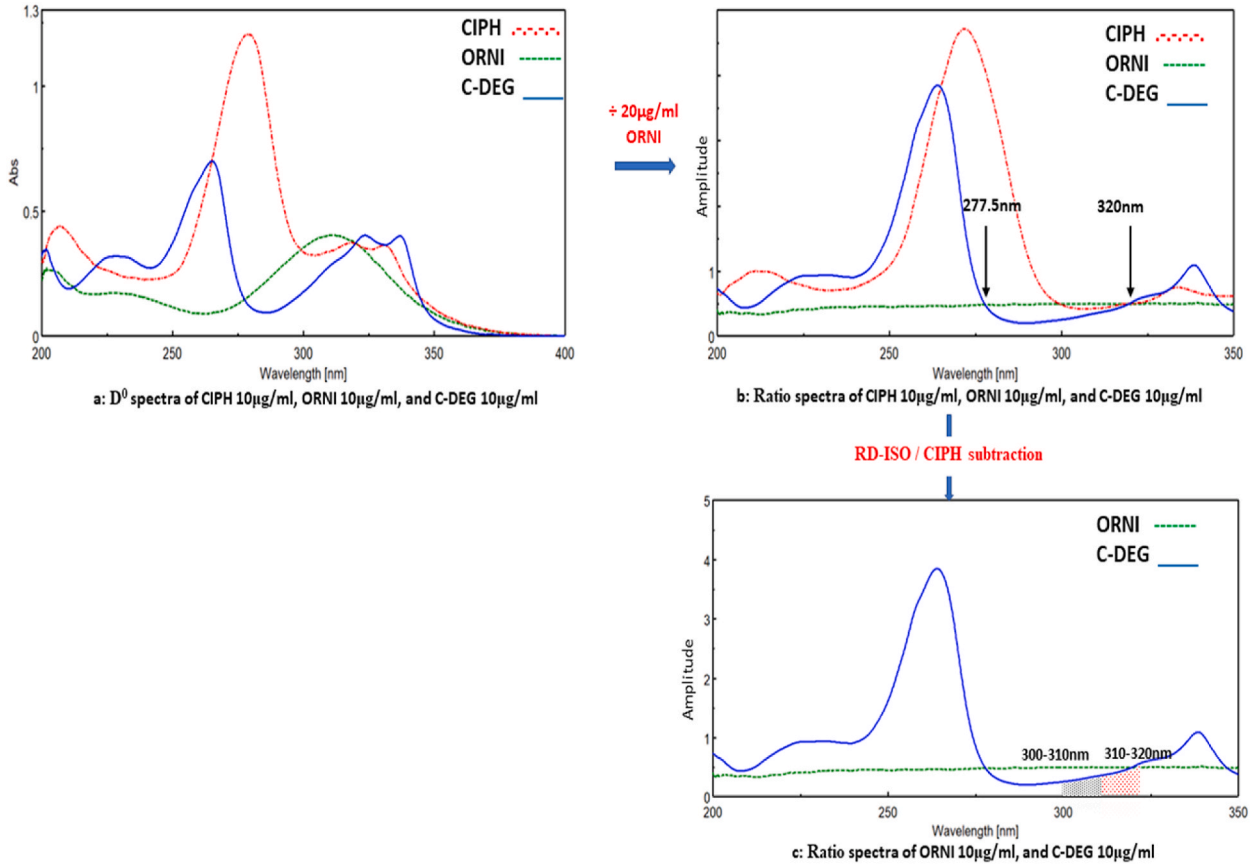

Fig. 2. Resolving the ternary mixture of CIPH 10 μg/ml, ORNI 10 μg/ml, and *C*-DEG10μg/ml using the first protocol (RD-ISO/RD-AUC). Where, a: the D^0 spectra of CIPH, ORNI, and *C*-DEG, b: the ratio spectra of CIPH, ORNI, and *C*-DEG after division by ORNI 20 μg/ml showing the two elected wavelengths, c: the ratio spectra of ORNI after applying RD-ISO and subtracting CIPH spectrum from the mixture spectrum showing the two elected AUCs.

and CIPH in the existence of *C*-DEG, a check of the preceding studies was achieved to detect the concerned drug's stability in an acidic medium and to estimate the related degradation compounds number in the condition of an acidic treatment. The achieved work in Ref. [29] showed that ciprofloxacin is degraded in an acidic medium to a single compound whose structure was recognized by infra-red, ^{1}H NMR, and mass spectra. Fig. 1 shows the loss of the carboxylic group from the *C*-DEG structure, which possesses a major function in bactericidal activity; hence it is essential to reveal the existence of the acidic degradation product of CIPH to ensure its actual antibacterial activity. Unlike CIPH, ORNI is more stable in acidic conditions as the accomplished HPLC study in Ref. [30] displays that, upon analyzing ornidazole by HPLC after acidic treatment, a small diminution in ornidazole peak was noticed without the manifestation of new peaks, which may be on account of the verity that the acidic degradation compound is non-chromophoric. Building on the aforesaid, the first UV study in an acidic medium was administered on CIPH and ORNI combination by developing three novel protocols that permit the synchronous estimation of CIPH and ORNI in an acidic medium away from any overlapping with the single degradation product of ciprofloxacin.

5.1. The first protocol (RD-ISO/RD-AUC)

It is obvious from Fig. 2 a and Fig. 2 b that ORNI and *C*-DEG intersect with two iso-points in the D^0 stage (277.5 nm, 320 nm); this intersection remains in the ratio stage at the exact two-wavelength. So, this protocol starts with the application of (RD-ISO) technique via dividing the produced triple mixture's D^0 spectrum by a suitable concentration of ORNI within its linearity range, for that, a divisor of 20 μg/ml ORNI was elected since it offered the finest recovery results for CIPH and *C*-DEG from their prepared mixtures with ORNI. The value ($P_{277.5nm}$-P_{320nm}) at the elected isosbestic wavelengths was computed to cancel the overlapping of ORNI and *C*-DEG at these chosen wavelengths, which consequently allowed estimating CIPH concentration via substituting the amplitude difference ($P_{277.5nm}$-P_{320nm}) in its related regression equation [$P_{277.5nm}$-P_{320nm} = 0.3627 × C_{CIPH}–0.0137]. CIPH's factorized isosbestic difference spectrum underwent multiplying by the computed $\Delta P_{277.5–320nm}$ value to regain its analogous ratio spectrum, and hence subtracting this regained ratio spectrum from the triple mixture's ratio spectrum to acquire the (ORNI, *C*-DEG) mixture's ratio spectrum.

To estimate *C*-DEG concentration in the attained binary mixture, the new ratio difference -Areas under the curve method was employed. Thus, many trials were done to elect the best region on the ratio spectrum to be reliable for detecting *C*-DEG and ORNI concentration. The range of wavelengths (300–320 nm) as shown in Fig. 2 c was the best region with the lowest noise of the divisor, where at the peaks of *C*-DEG ($P_{264nm,}$ and P_{339nm}) the noise was maximum, and no accurate results were achieved upon estimating *C*-DEG and ORNI using these regions.

The computed ($AUC_{310–320nm}$ -$AUC_{300–310nm}$), which is proportional to *C*-DEG concentration only, was substituted in the *C*-DEG's regression equation [$AUC_{(310–320nm)C\text{-}DEG}$ -$AUC_{(300–310nm)C\text{-}DEG}$ = 0.1175 × $C_{C\text{-}DEG}$ -0.019] to attain its concentration in the analyzed mixture; then the $AUC_{300–310nm}$ of *C*-DEG was recovered by substituting the computed ($AUC_{310–320nm}$ -$AUC_{300–310nm}$) in the linear equation [$AUC_{(310–320nm)C\text{-}DEG}$-$AUC_{(300–310nm)C\text{-}DEG}$ = 0.3959 × $AUC_{(300–310nm)C\text{-}DEG}$-0.0161]. A subtraction process between the $AUC_{300–310nm}$ of *C*-DEG and the $AUC_{300–310nm}$ of the mixture was done to attain the $AUC_{300–310nm}$ of ORNI, which consequently allowed estimating its concentration by substituting its attained $AUC_{300–310nm}$ in the constructed linear equation [$AUC_{(300–310nm)ORNI}$ = 0.4947 × C_{ORNI} +0.0093].

5.2. The second protocol (RD-ISO/DWE)

This protocol consists of the new dual-wavelength equation method preceded by (RD-ISO) technique, which is utilized first to recover the CIPH's D^0 spectrum and subtract it from the triple mixture's D^0 spectrum. The obtained binary mixture (ORNI, *C*-DEG) in

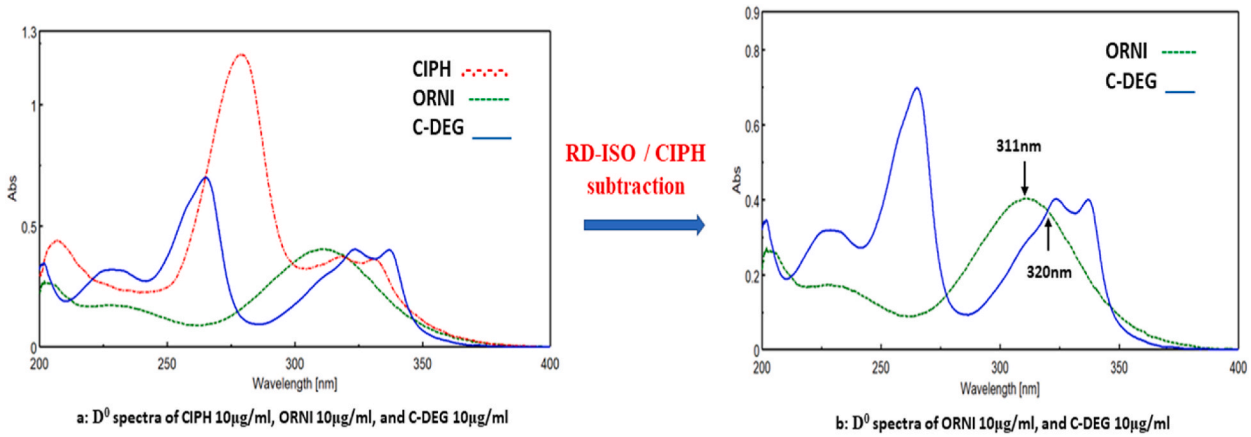

Fig. 3. Resolving the ternary mixture of CIPH 10 μg/ml, ORNI 10 μg/ml, and *C*-DEG10μg/ml using the second protocol (RD-ISO/DWE). Where, a: the D^0 spectra of CIPH, ORNI, and *C*-DEG, b: the D^0 spectra of ORNI, and *C*-DEG after applying RD-ISO and subtracting CIPH spectrum from the mixture spectrum showing the two elected wavelengths.

Fig. 3 a is then subjected to the dual-wavelength equation technique by electing two wavelengths comprising the iso-point of the overlapped components (ORNI, *C*-DEG) and the λ_{Max} of ORNI, or *C*-DEG, for that 320 nm and 311 nm were elected as the λ_{iso} and the λ_{Max} of ornidazole respectively Fig. 3 b; then the following developed equation was utilized to estimate ornidazole concentration:

$$C_{ORNI} = \frac{\frac{A_{311nm}}{0.0286} - \frac{A_{320nm}}{0.0369}}{\frac{0.0401}{0.0286} - 1}$$

The mixture's total concentration is estimated via a division process of the mixture's absorbance at the elected iso-point by the computed ORNI's absorptivity factor (absorptivity factor of ORNI and *C*-DEG is the same at λ_{iso}); then subtracting the determined concentration of ORNI from the determined concentration of the mixture to obtain *C*-DEG's concentration.

5.3. The third protocol (SRZ)

This new protocol processes data during the derivative ratio phase; thus, the produced ternary mixture as in Fig. 4 an underwent division by a proper concentration of CIPH, which gives the best recovery results for ORNI and *C*-DEG from their mixture with CIPH. The best concentration with this term was 10 μg/ml of CIPH. After the division step, a derivative process (1st order,11point. scale × 10) was implemented on the resulting ratio spectrum to acquire the analogous derivative ratio spectrum as perceived from Fig. 4 b. The overlapped ORNI and *C*-DG derivative ratio spectrum shows only one robust zero-crossing point of ORNI at 271.7 nm, whereas the zero-crossing point of *C*-DEG at 253.5 nm did not provide acceptable results respecting accuracy and repeatability when relying on it to quantify ORNI. Therefore, only the zero-crossing point of ORNI was inserted in the quantification process of both ORNI and *C*-DEG by substituting the mixture amplitude at 271.7 nm in the next established linear equation [$P_{271.7nm} = 0.0794 \times C_{C\text{-}DEG} + 0.0040$] to detect *C*-DEG's concentration. After that, the mixture signal at 271.7 nm was substituted in this linear equation [$P_{271.7nm} = -1.994 \times P_{294.5nm} - 0.005$] to retrieve *C*-DEG's signal at 294.5 nm, which is the ORNI λ_{Max}; this retrieved signal of *C*-DEG was subtracted from the mixture's signal at 294.5 nm to get ORNI's signal at its λ_{Max} (294.5 nm). ORNI's concentration is then determined easily by substituting its obtained signal at 294.5 nm in the linear equation [$P_{ORNI\ 294.5nm} = -0.0755 \times C_{ORNI} + 0.0049$]. To determine CIPH's concentration, a conventional zero-crossing point is applied by dividing the produced mixture's D^0 spectrum by ORNI's D^0 spectrum 20 μg/ml. Then we need to derive the resulting ratio spectrum (1st order,11point, scale × 10) and choose a proper zero crossing point of *C*-

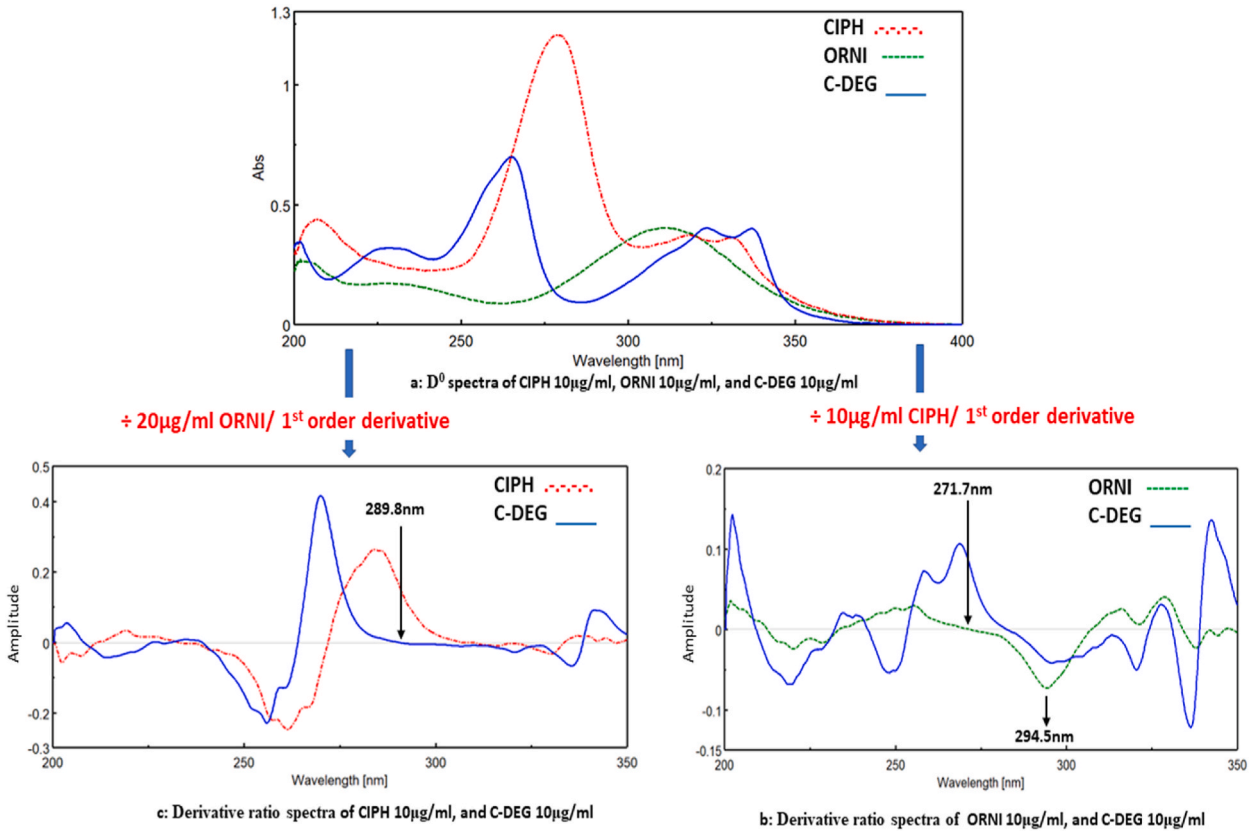

Fig. 4. Resolving the ternary mixture of CIPH 10 μg/ml, ORNI 10 μg/ml, and *C*-DEG10μg/ml using the third protocol (SRZ). Where, a: the D^0 spectra of CIPH, ORNI, and *C*-DEG, b: the derivative ratio spectra of ORNI, and *C*-DEG using CIPH 10 μg/ml as the divisor showing the two elected wavelengths, c: the derivative ratio spectra of CIPH and *C*-DEG using ORNI 20 μg/ml as the divisor showing the selected wavelength.

DEG to determine CIPH at it; the elected zero-crossing point of *C*-DEG was at 289.8 nm as shown in Fig. 4 c, which afforded the finest results in determining CIPH. So, by recording the signal at this wavelength and substituting it in the linear equation [$P_{CIPH\ 289.8nm} = 0.1768 \times C_{CIPH}$ -0.0019], the concentration of CIPH was obtained.

The application of the above protocols and their suitability for analytical purposes is governed by several criteria. Primary among these criteria is the need to obtain an acceptable recovery of the analyzed drugs and the presence of specific conditions in their spectra, such as sufficient iso points or robust zero-crossing points. Furthermore, simplicity and the number of steps in the protocol come into play after meeting the aforementioned criteria. Since all the proposed protocols demonstrated acceptable recovery results for CIPH and ORNI from synthetic mixtures and pharmaceutical formulations, it is possible to choose the preferred protocol based on the simplicity and the number of steps involved.

The RD-ISO/DWE protocol emerges as the preferable choice due to several reasons. It provides a straightforward equation for estimating ORNI and *C*-DEG in the zero-order stage without the need for additional division or derivative steps. Additionally, it offers a spectral filtration pathway with fewer steps compared to the first and third protocols. In contrast, the first and third protocols require two division processes to resolve the ternary mixture, and they also involve a multi-step spectral filtration pathway to recover the overlapped components.

5.4. Confirming the spectrum filtration process correctness

The Spectral Print Recognition Index (SRI) [31] was employed in this task to verify the effectiveness of the drug spectrum filtration process. This was accomplished by calculating the ratio between the absorbance at two selected wavelengths in the filtered drug spectrum and the original drug spectrum, using the following formula: [($A_{\lambda 1}$ filtrated spectrum/$A_{\lambda 2}$ filtrated spectrum)/($A_{\lambda 1}$ original spectrum/$A_{\lambda 2}$ original spectrum)]. For the estimation of SRI, the two selected wavelengths were as follows: 278.9nm/331 nm for CIPH, 311/343 nm for ORNI, and 265nm/323 nm for *C*-DEG. The computed SRI for each component in the prepared mixtures consistently showed a value of approximately 1, with a very slight deviation, as elucidated in Table 2. This underscores the efficacy of the three developed protocols in accurately extracting the filtered D^0 spectrum of each component.

5.5. Statistical comparison

A statistical comparison was conducted between the developed approaches and the reference HPLC approach [13], as outlined in Table 4. The f-test and *t*-test values indicated no significant differences among them.

5.6. Greenness evaluation

A comparison of greenness was conducted between the introduced protocols and the reference HPLC [12] protocol to assess which of them exhibits a greener trait through the utilization of NEMI [32], GAPI [33], AGREE [34], and CALIFICAMET [35] tools.

The NEMI metric relies on four criteria to estimate the procedure's greenness: PBT (the reagent is listed in EPA's TRI chemical list) [36], Hazardous (the reagent is listed in F, K, P, and U lists for environmentally hazardous waste) [37], Corrosive (The analysis is performed at pH < 2 or >12), and waste (The waste quantity generated by the analytical procedure is more than 50 g). If any of the above criteria is met, its quadrant will be colored white; the more white-colored quadrants count, the less green the method is.

GAPI is a quantitative tool that assesses the method's greenness by evaluating 15 different criteria during three stages of the

Table 1
Validation data and parameters of the elaborated protocols for CIPH, ORNI, and *C*-DEG estimation.

Protocol	RD-ISO/RD-AUC			RD-ISO/DWE		SRZ		
Drug Substance	ORNI	*C*-DEG	CIPH	ORNI	*C*-DEG	CIPH	ORNI	*C*-DEG
Linearity range	3–20 μg/ml	3–20 μg/ml	3.5–15 μg/ml	3–15 μg/ml	3–15 μg/ml	1.5–15 μg/ml	3–20 μg/ml	3–20 μg/ml
Linear equation	Y = 0.4947 × −0.0093	Y = 0.1175x-0.019	Y = 0.3627x-0.0137	$C_{ORNI} = \frac{\frac{A_{311nm}}{0.0286} - \frac{A_{320nm}}{0.0369}}{\frac{0.0401}{0.0286} - 1}$	Y = 0.0364x+0.003	Y = 0.1768X-0.0019	Y = −0.0755X+0.0049	Y = 0.0794X+0.0040
Correlation coefficient	0.9998	0.9997	0.9999	0.9998	0.9998	0.9999	0.9998	0.9999
Mean%±SD*	101.48 ± 0.85	100.26 ± 1.35	100.52 ± 1.14	100.89 ± 0.93	100.84 ± 1.39	100.16 ± 0.93	99.67 ± 0.84	100.80 ± 0.93
Repeatability**	1.19	1.18	0.97	0.91	1.00	1.16	1.02	1.41
Intermediate Precision**	1.28	1.48	1.38	1.22	1.28	1.41	1.18	1.54
DL μg/ml	0.077	0.242	0.104	0.134	0.114	0.076	0.130	0.107
QL μg/ml	0.232	0.733	0.315	0.407	0.345	0.229	0.393	0.325

* Expresses the accuracy of three concentrations (4,6,12)μg/ml of CIPH, ORNI, and *C*-DEG.
** Intermediate Precision and Repeatability are computed as the RSD% of three drug concentrations (5,7,13)μg/ml of CIPH, ORNI, and *C*-DEG.

Table 2
Quantification and SRI results of CIPH, ORNI, and *C*-DEG in the prepared mixes by the suggested protocols.

Drugs substances ratio (μg/ml) CIPH: ORNI: *C*-DEG	RD-ISO/RD-AUC			RD-ISO/DWE		SRZ		
	CIPH	ORNI	*C*-DEG	ORNI	*C*-DEG	CIPH	ORNI	*C*-DEG
10:10:10*	100.50	99.58	100.35	98.80	99.41	100.03	98.92	100.59
5:12:10**	99.33	101.50	100.77	100.45	101.03	98.22	99.29	100.95
12:6:6	99.76	99.47	100.43	100.18	100.27	99.86	98.78	100.23
12:8:4	100.54	100.80	101.13	100.70	99.94	100.53	99.32	99.45
average±sd	100.03 ± 0.59	100.41 ± 0.90	100.47 ± 0.49	100.03 ± 0.85	100.16 ± 0.83	99.66 ± 1.00	99.04 ± 0.23	100.31 ± 0.64
SRI±SD***	0.9997 ± 0.001	0.9992 ± 0.001	0.9993 ± 0.003	0.9996 ± 0.001	0.9988 ± 0.002	1.0005 ± 0.002	0.9997 ± 0.001	1.0000 ± 0.001

* Cifran-OZ® tablets announced to contain 500 mg of each CIPH and ORNI.
** Average of three repetitions.
*** Average of SRI±SD for the filtrated spectra of each component.

Table 3
Quantification results of CIPH, and ORNI in Cifran-OZ® tablets utilizing the suggested protocols.

Cifran-OZ®	CIPH		ORNI		
	RD-ISO/RD-AUC	SRZ	RD-ISO/RD-AUC	RD-ISO/DWE	SRZ
Assay±SD[a]	100.18 ± 0.86	99.95 ± 1.83	101.23 ± 0.97	99.09 ± 1.55	100.19 ± 1.62
SRI	0.998	0.997	0.997	0.999	0.996
Standard addition [b,c]	99.29 ± 0.38	99.05 ± 0.68	101.44 ± 0.41	100.91 ± 0.62	99.76 ± 1.34

[a] Average of three analyses ±standard deviation.
[b] The standard addition is (4,5, 6 μg/ml) for each CIPH and ORNI.
[C] The calculated value is the average ± SD for the three concentrations of the standard added with three repetition.

Table 4
Statical comparison between the introduced UV protocols and the reference HPLC method for CIPH and ORNI quantification in commercial forms.

Cifran-OZ®	CIPH		ORNI			Reported method* [13]	
	RD-ISO/RD-AUC	SRZ	RD-ISO/RD-AUC	RD-ISO/DWE	SRZ	CIPH	ORNI
Average**	100.18	100.08	101.31	99.07	101.51	100.43	100.26
SD	0.96	1.41	0.69	1.21	0.68	0.92	1.42
Variance	0.9139	1.9821	0.4779	1.4568	0.4603	0.8518	2.0222
t-value***	0.431	0.463	1.482	1.421	1.772	–	–
f-value***	1.073	2.327	4.232	1.388	4.392	–	–

* HPLC using a C-18 column utilizing a mobile phase of acetonitrile: water in (45:55v/v) ratio, pH adjusts to 3.0 with *O*-phosphoric acid, flow rate 1 ml/min, detection at 299 nm.
** n = 5.
*** f (0.05)6.388, t (0.05)2.306.

analytical procedure (sample preparation, reagents, and solvents, instrumentation). Green, yellow, and red colors indicate low, moderate, and high greenness impact of the analytical procedure. The more red-colored sections in the generated pictogram, the lower the greenness impact.

The AGREE calculator comprises the twelve basics of green analytical chemistry (sampling procedure, sample size, apparatus location, sample setting steps, automation and miniaturization, derivatization, waste amount, analysis output, energy consumption, reagent source, toxicity, operator's safety), where the higher the computed score in the pictogram center, the greater the procedure's greenness.

CALIFICAMET is a new assessment tool that evaluates the sustainability of the established analytical procedure by summarizing various analytical information in a hexagon. The hexagon is divided into 6 equilateral triangles, each representing the assessment of a different aspect of the analytical procedure, such as sample processing, approach characteristics, calibration step, accuracy, quality control, toxicity, safety, waste, carbon footprint, and cost, as clarified in Fig. 5. Each criterion in each triangle is evaluated within a range from 0 to 4, relying on the penalty score calculated for each criterion [35]. The higher the penalty points accumulated for each criterion, the more negatively the method contributes to that criterion.

CAPI, CALIFICAMET, and AGREE tools are superior to NEMI as they deal with more criteria for estimating the procedure's greenness. Therefore, they express the greenness of the procedure more comprehensively and accurately. It is not accurate to rely solely on the NEMI tool for estimating the procedure's greenness. Otherwise, the CALIFICAMET tool handles more criteria than GAPI, AGREE, and NEMI, as it evaluates the sufficiency of analytical parameters in the sample, quality control of the established procedure, as well as the hazard, safety, ecological impact, and the cost of the analytical procedure. As shown in Fig. 5, both the established

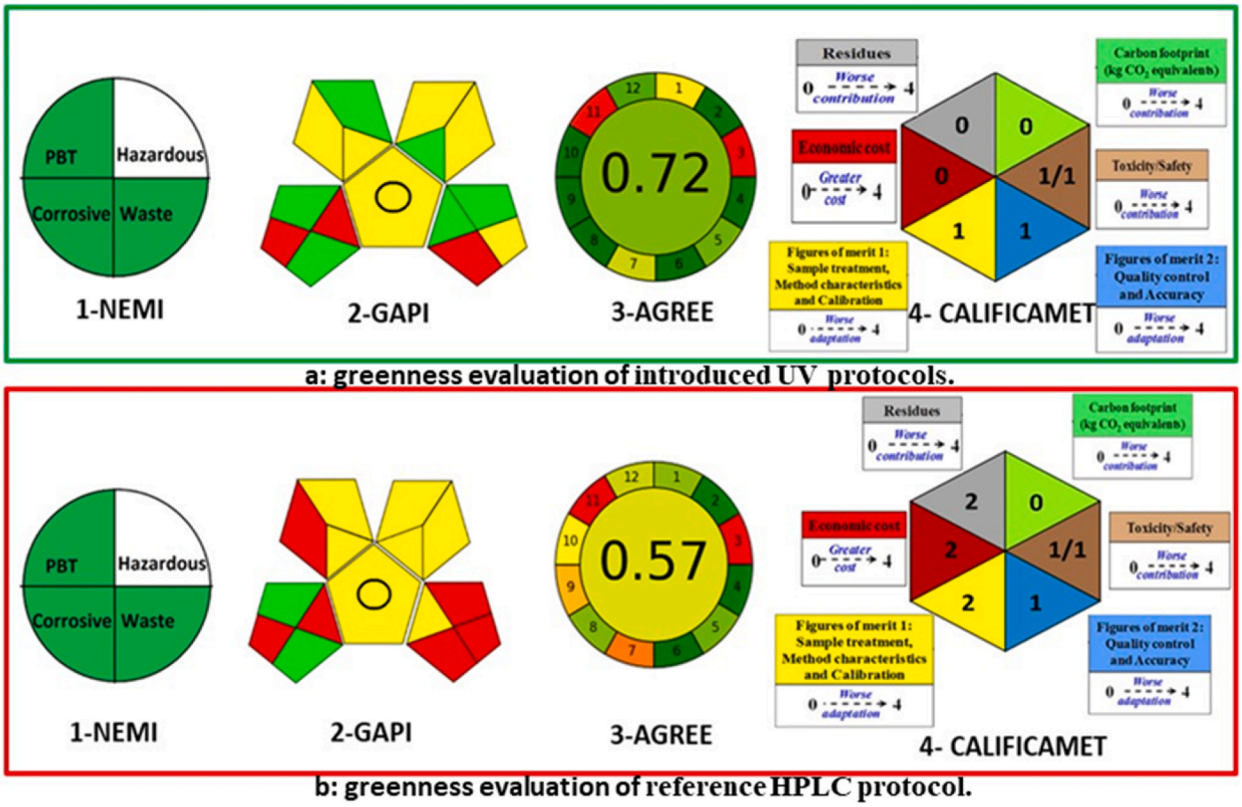

Fig. 5. Greenness comparison between the introduced UV protocols(a) for CIPH and ORNI, and the reference HPLC protocol (b).

approaches and the HPLC approach show the same greenness evaluation in the NEMI tool, as both methanol (used in the newly established protocols) and acetonitrile (used in the HPLC protocol) are listed as Hazardous wastes. AGREE, GAPI and CALIFICAMET tools express a higher greenness impact of the newly established UV protocols than the HPLC protocol, as stated in Fig. 5, because the HPLC approach is considered an energy and reagent consumer and also generates more waste during the analysis operation. Therefore, the newly announced spectroscopic approaches are considered greener substitutes for the chromatographic approach.

6. Validation

Validating the newly announced approaches was done following the directives of ICH [38].

- linearity:

Using the experimental circumstances explained above, linearity was maintained by producing the analyzed components' calibration graphs in the concentration ranges as in Table 1.

- Detection limit (DL) and quantitation limit (QL):

DL and QL were computed as described in ICH by utilizing the calibration curve slope and SD of the response. Excellent value of LD and LQ emphasize the approach's sensitivity.

- Accuracy:

Accuracy was demonstrated by applying the proposed protocols to both pure components and commercial formulations, utilizing the standard additions technique as outlined in Table 3. The calculated recovery percentages yielded satisfactory results, with a relative standard deviation (RSD) of less than 2.

- Precision:

Precision was confirmed by obtaining an acceptable RSD value when analyzing three different concentrations of the pure drugs on

the same day and over three days using the described methods, as detailed in Table 1.

- Specificity:

Specificity was established by applying the proposed protocols to in-lab-prepared mixtures and tablet preparations, as described in Table 2 and Table 3, respectively. The calculated mean percentages, along with their standard deviations, were found to be satisfactory, highlighting the absence of any overlap with other substances or excipients. It's important to note that the developed protocols are capable of quantifying drug mixtures with various ratios, with the exception of mixtures containing the maximum concentrations of CIPH, ORNI, and *C*-DEG simultaneously (15 μg/ml CIP + 20 μg/ml ORN + 20 μg/ml *C*-DEG). This exception is due to the recording of a spectrum with an absorbance value exceeding 2, which is considered analytically unreliable.

7. Conclusion

This manuscript introduces a green spectroscopic study for the estimation of CIP and ORN in the presence of ciprofloxacin-induced degradation compounds, serving as an alternative to chromatographic methods. This is achieved through the development of three new protocols, each incorporating novel mathematical techniques for processing overlapping spectra. These protocols operate in the ratio stage, D^0 stage, and derivative ratio stage, converting spectral data into valuable information for quantification and filtration purposes. All the developed protocols can successfully extract the ID spectrum of drug substances from synthetic mixtures or commercial formulations by linking them to a convenient spectral template, simplifying the quantification and purity verification process. This highlights the significance of these developed protocols in analytical procedures, eliminating the need for specialized software or complex mathematical operations. It is recommended to implement these protocols as part of routine analytical procedures in drug control laboratories due to their environmentally friendly impact, as confirmed by NEMI, AGREE, GAPI, and CALIFICAMET tools.

Consent for publication

Not Applicable.

Ethics approval and consent to participate

Not Applicable.

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Data availability

- Data associated with this study have not been deposited into a publicly available repository.
- Data will be made available on request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Amir Alhaj Sakur: Writing – review & editing, Supervision, Methodology. **Duaa AL. Zakri:** Writing – original draft, Validation, Investigation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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