Talanta Open 7 (2023) 100226

Contents lists available at ScienceDirect

Talanta Open

journal homepage: www.sciencedirect.com/journal/talanta-open

Environmentally sustainable computationally spectrophotometric resolution strategy for analysis single-tablet regimens of antihypertension with overlapped spectra

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ARTICLE INFO

Keywords:
Parent spectrum restoration technique
Fingerprint
Derivative ratio Transformation
Dual Amplitude Difference
Green assessment
White analytical chemistry

ABSTRACT

Single tablet regimens (STRs) for hypertension suppression improve patient satisfaction, quality of life, medication and adherence compared to multi-tablet regimens (MTRs). Development of simple cost effective and at the same time green an ecofriendly method for analysis and resolution of multicomponent and complex mixtures is an increasingly important issue in the field of pharmaceutical industry in general and analytical chemistry in specific. Thus, the presented research deals with two main goals, the first one is concerned with developing and applying a fully validated resolution plan using numerical data of signals on manipulated spectra for successive extraction of parent spectra of drugs in single-tablet Regimens (STRs) used in antihypertensive treatment. The STRs contains three drugs with deconvoluted spectra, Atenolol (ATL), Amiloride (AMD) and Chlortalidone (CLN). The resolution plan encompasses two simple and affordable spectrophotometric methods employing resolving spectra either normalized spectrum (NS) or factorized spectrum (FS) which acts as a master key for unlocking the overlapped spectra of ternary mixture with no need for any preliminary physical separation, sample pretreatment or pH adjustment. These two main methods namely, dual amplitude difference (DAD) and derivative ratio transformation (DDT). The presented approaches required a simple spectrophotometer with its built-in program using primitive mathematical operations to restore the parent spectrum for individually drug cited in the mixture understudy. The proposed methods validity was checked and evaluated through the ICH guidelines and displayed linearity within concentration range 4.0-40.0μg/mL for ATL, 3.0-20.0μg/mL for both AMD and CLN. While the specificity evaluation was performed via assaying the three drugs accurately and precisely in their synthetic mixtures and in their STRs. Generally, these methods could be recruited for the fast and effective analysis and determination of the purity index for AMD, CLN and ATL in bulk materials and available market formulation, Teklo ® tablets.

The second main goal of the research was the evaluation of the methods greenness and whiteness where the applied UV-methods and the reported HPLC one had significantly different greenness scores when compared using three metrics: the Green Certificate Classification (GCC), the Complex Green Analytical Procedure Index (Complex-GAPI), and the Analytical Greenness metric approach (AGREE).

Also, the whiteness scores, which reflected the degree of the achieved sustainability for the HPLC reported technique and the produced UV ones were done by using the ideologies of the white analytical chemistry (WAC) tool. All the differences between the four aforementioned metrics are discussed in this study.

1. Introduction

Some dosage forms were formulated to increase the patient compliance specially those suffering chronic diseases such as cardiovascular and diabetes type II which necessitate the usage of more than one or two drugs to control the diseases and prevent their complications on the long run. Different factors may contribute to elevated blood pressure; thus, a single therapy approach may not be sufficient to

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https://doi.org/10.1016/j.talo.2023.100226

Available online 26 April 2023

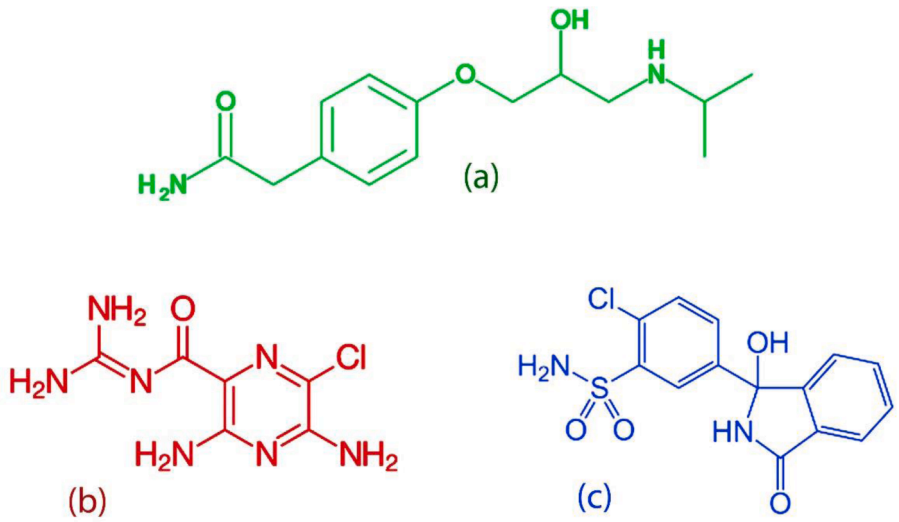

Fig. 1. Illustration of ATL (a), AMD (b) and CLN (c) chemical structures.

control it. It is usually preferred to combine multiple therapy acting through synergistic mechanisms to achieve maximum therapeutic effect with least side effects. The therapeutic regimen is better administered as a combined fixed dose in one tablet or capsule to enhance patient adherence and compliance especially for geriatrics [1].

Pharmaceutical analysis plays a very important role and has a great significance in the pharmaceutical field. It includes quantification of the active pharmaceutical compounds and the used additives in their bulk powders or finished products. Recently a tremendous evolution occurred in spectrophotometric methods where different research articles were reviewed and published explaining how some smart innovative approaches based on the basic mathematical calculations could solve hardest and complex overlapped mixture [2–9].

After literature review, the choice was settled on a ternary mixture widely used in combination treatment of hypertension many years ago and present in the market under the name of Teklo®. The selected mixture included Atenolol (ATL), Amiloride (AMD) and Chlorthalidone (CLN).

ATL one of the selective beta blockers that could be administrated alone or combined with other drugs, into treat hypertension and manage angina [10]. It is reported that the use of ATL among children is increasing where it couldn't cross the blood brain barrier [11].

CLN is a thiazide diuretic that acts by blocking Na^+, K^+, and water reabsorption by the kidney leading to diuresis and elevating high blood pressure through decreasing the plasma extracellular fluid volume [12]. While AMD is a diuretic with potassium sparing properties [13] used in combination with thiazide diuretics to increase the natriuretic effect without causing hypokalemia [14]. The chemical structure of the three drugs were drawn and displayed in Fig. 1.

Although the ternary mixture under investigation had been launched in the market years ago, few number of methods were mentioned in the literature for their simultaneous estimation including chemometric methods [15,16]which require the purchase and the use of sophisticated programs. In addition to chromatographic methods; densitometry [17–19], HPLC [20] and electrophoresis [21].

Combining two or three dugs in one dosage form proved to have health benefits by increasing patient adherence and convenience by decreasing the pill burden. Therefore, analysts need develop reliable methods for drug analysis in such combined formulations to assure their safety, freedom from impurities and adulteration before reaching the end users. To preserve the cost effectiveness, spectrophotometric techniques were found to be a good choice where it doesn't require any expensive solvents, reagents, programs, or instrumentation. Multicomponent analysis using spectrophotometry became a promising tool for analysts since microprocessor-based spectrophotometers linked to computers are widely used. The multi-component spectrophotometric analysis steps can be utilized for dismissal of intersecting spectra with any degree of spectral overlap even in the presence of two or more spectra with the almost the same spectral features could be smartly manipulated, and others could be induced using simple mathematical approaches [22–23] Fig. 2, clarifying the steps in resolution plane.

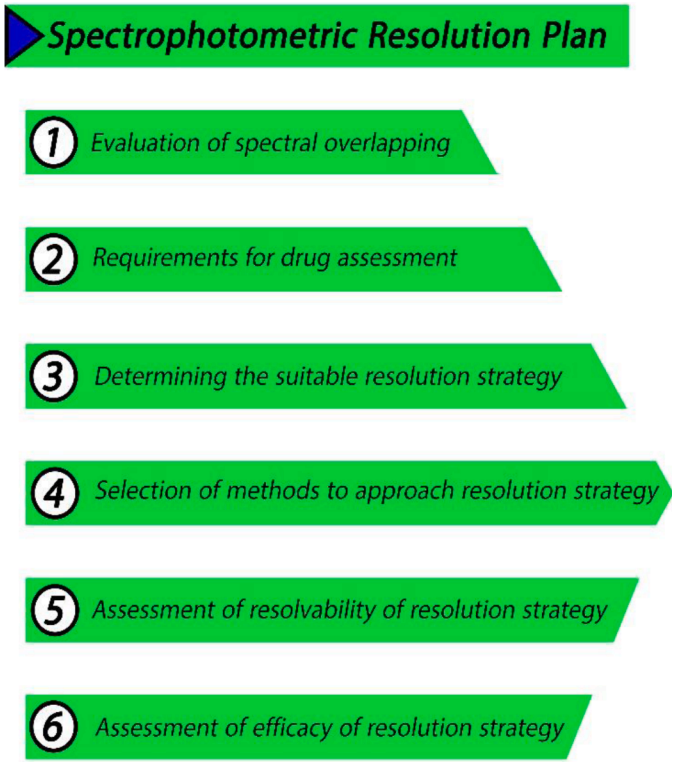

Fig. 2. The main spectral analytical steps of a resolution plan.

Upon searching the literature, it was found that there were no reported **univariate** spectrophotometric methods based on the intrinsic features of ATL, AMD and CLN absorption spectra for their simultaneous quantification in their single-tablet regimens. The adopted restoring technique was developed and validated successfully for estimating ATL, AMD and CLN successively despite of the severe overlap exhibited

between their absorption spectra hindering their direct determination by classical spectrophotometric methods.

Analyzing the three drugs in Teklo® tablets combination was challenging for two main reasons, their overlapped spectra as well as their **critical ratio (20: 1: 5) ATL, AMD and CLN**, respectively which hinder the analysis of the mixture using traditional spectrophotometric methods.

The Parent Spectrum Restoration technique (PSRT) is an innovative signal restoration technique from complicated data samples that is used in the present work. The current technique is an advanced approach for recovering the parent spectrum of the proposed drugs and highlighted the basic concepts, privileges, and applications of this technique. This recovered parent spectrum (D^0) acts as fingerprint of the studied drugs and confirmed their purities as well as allows quantitative determination of their concentrations at their maxima with maximum accuracy and precision [24,25].

In this study, beside quantification of the therapeutic drugs, the aim was also to assess the greenness of the adopted procedures. Over the past two decades in the pharmaceutical industry, the usage of solvents, reagents, and waste produced have been increased which motivated the researcher in chemistry generally [26,27] and analytical chemistry specifically [28,29] to study the effect of such practices on the environment or the ecosystem. Many metrics have been devised and recently reported to assess the greenness of the analytical procedures [30–36] depending on the 12 Green Analytical Chemistry (GAC) principles. At the present time, many quantitative tools like GCC [37] and AGREE [38] have been established, the considered criteria for evaluation included: the type of the solvent, amount of waste, and energy consumption. The main purpose of developing the Complex-GAPI tool is to cover the wide range of parameters which define the analysis procedure and the pre-analysis ones such as reagents, preparation steps and techniques [39].

The sustainability of the developed analytical methods in another words the "whiteness" is measured by merging three main criteria: the validity of the analytical method represented by red color, the impact on the environment that takes on the colour green and the economic aspect that represents by the colour blue. Results for the previous colored connection produced the white color of the method, which can be assessed by the WAC tool [40–42].

In the present study, three separate green evaluation tools and one whiteness metric were applied to evaluate the developed UV-methods and the reported HPLC one, as well as a comparative study for the practical green and white tools were illustrated to clarify the advantages and disadvantages of the applied evaluation tools.

1.1. Spectrophotometric resolution plan

- Evaluation of spectral overlapping:

Strategic spectral analysis, including an assessment of spectral resolution in their critical ratio ATL, AMD and CLN successively despite of the severe overlap exhibited between their absorption spectra hindering their direct determination by classical spectrophotometric methods.

- Requirements for drug assessment.

Recovery of the parent spectra (zero order) of the studied drugs to permit their assessment. Thus, the requirements are regression equations which computed using absorbance at their maxima as well as these recovered D^0 spectra confirmed their purity.

- **Determining the suitable resolution strategy**

Based on the extent of spectral overlapping and the available resolution tools such as normalized spectrum and factorized spectrum of one or more of the studied drugs

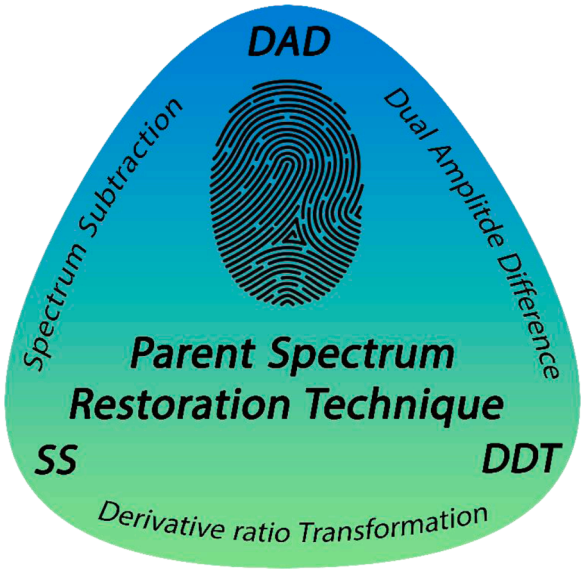

Fig. 3. Resolution strategies for the extraction of parent spectra from ternary mixtures.

- **Selection of methods to approach resolution strategy.**

Survey of the requirements and limitations of the spectrophotometric methods and to develop a novel method to reach optimum resolution with minimum manipulation steps so use recently developed DAD a well as a novel one namely derivative transformation in addition to well-known spectrum subtraction method.

- **Assessment of resolvability of resolution strategy**

Analysis of different laboratory prepared blends comprising different drugs ratios and the recovery % of the added drugs are calculated.

- **Assessment of efficacy of resolution strategy**

By analyzing studied drugs in its dosage form with excipients present and using the method of standard addition for proving the accuracy of the results.

1.2. Theoretical framework

Parent Spectrum Restoration Technique (PSRT) is an innovative process employed for the quantization of ternary or multicomponent mixtures with high selectivity and sensitivity beside its ability to disengage the overlapped spectra. The proposed PSRT had the power to recover their parent spectra of which is zero order spectra that act as spectral profiles or fingerprints for drugs. The component concentrations in mixture were simply estimated via three separate regression equations relating the maximum absorbance λ_{max} to the relevant concentrations.

This technique included three computational methods as illustrated in Fig. 3, includes dual amplitude difference (DAD) via factorized spectra, derivative ratio transformation (DDT)using normalized spectra or any other divisor using the built-in spectrophotometer software, and the famous smart method spectrum subtraction (SS).

1.2.1. Dual amplitude difference (DAD)

The recent approach, *DAD,* can beusedtoanalyze3 drugs in a triple combination (X, Y, Z) where the spectrum of one drug (Z) is prolonged over the other 2 drugs (X and Y). DAD could be applied using the built-in spectrophotometer programmer employing the factorized ratio

spectrum (FS) of the extended drug (Z) [43].

The previously FS of Z in its pure form was easily obtained with the aid of the spectrophotometer built-in software in three successive steps:

Step 1- Z ratio spectrum was obtained through the division of D^0Z in its pure form in any concentration inside the linearity range by D^0Y' in its pure form (as a divisor).

Step 2-Calculation of the amplitude difference (ΔP) between two carefully selected wavelengths (λ_1 and λ_2), where X has no contribution or equal amplitudes difference at the selected wavelengths.

Step 3-The ratio spectrum obtained in (Step 1) was then divided by the numerical amplitude difference value calculated in (Step 2) to get the FS of Z, as follows:

$$\text{Factorized ratio spectrum of Z} = \frac{Z/Y'}{\Delta P} \quad (1)$$

The mixture gross spectrum was divided by D^0Y'. The (ΔP) between (λ_1 and λ_2), where X exhibited no contribution, was calculated as summarized in the following equations.

$$P_{m1} = \left(\frac{Y}{Y'}\right)1 + \left(\frac{Z}{Y'}\right)1 \quad (2)$$

$$P_{m2} = \left(\frac{Y}{Y'}\right)2 + \left(\frac{Z}{Y'}\right)2 \quad (3)$$

$$\Delta P = \left\{\left(\frac{Y}{Y'}\right)1 + \left(\frac{Z}{Y'}\right)1\right\} - \left\{\left(\frac{Y}{Y'}\right)2 + \left(\frac{Z}{Y'}\right)2\right\} \quad (4)$$

$$= \frac{Z}{Y'}1 - \frac{Z}{Y'}2$$

Where the mixture amplitudes at (λ_1 and λ_2) are denoted by P_{m1}, P_{m2}, respectively. The amplitude difference between (λ_1 and λ_2) is denoted by ΔP.

The product of multiplication between the stored FS of Z and the numerical value of (ΔP) was found to be the original ratio spectrum ($\frac{Z}{Y'}$) which could be recruited for resolvingD^0 Z.

$$\Delta P \frac{\frac{Z}{Y'}'}{\Delta P} = \frac{Z}{Y'} \quad (5)$$

$$\frac{Z}{Y'} x Y' = z \quad (6)$$

The ratio spectrum ($\frac{Z}{Y'}$) also served another function, where it aided in regaining X and Y as binary mixture upon its subtraction from the mixture gross ratio spectrum as summarized in the following equations:

$$P_m - \frac{Z}{Y'} = \left\{\frac{X}{Y'} + \frac{Y}{Y'} + \frac{Z}{Y'}\right\} - \frac{Z}{Y'} \quad (7)$$

$$[P_r] = \frac{X}{Y'} + \frac{Y}{Y'} \quad (8)$$

DAD employing the factorized spectrum as a resolving tool offered the privilege of minimizing the data analysis of the restored spectra signal output, assuring high sensitivity and accuracy for determination of cited drugs.

1.2.2. Derivative ratio transformation (DDT)

For estimating the resolved binary mixture (X, Y) shown in Eq. 8, the novel method, DDT, could be used. Pure X factorized zero order absorption spectrum is prepared using spectrophotometer software where recorded X zero order absorption spectrum within linearity range is divided by the calculated numerical absorbance value at $\lambda_{max.}$

Numerical response factor is calculated, this factor is the average of the division products (A/P) for different concentrations of pure X within linearity range of X which calculated for each concentration of X separately relating absorbance at its maxima of zero order spectrum (A) relative to its corresponding amplitude (P) of derivative ratio spectrum of X using Y as a divisor.

Upon applying derivative ratio method to the resolved ratio spectrum of binary mixture of X and Y (Eq. 8), $\frac{X}{Y'}$would have spectrum with amplitude relating to X only. This amplitude value is multiplied by the calculated response factor to get the numerical absorbance value at λ_{max}. The parent D^0 of X spectrum in each mixture could be easily restored by multiplying this numerical absorbance value at λ_{max} by the factorized spectrum of X.

DDT method superior over the derivative ratio of ternary mixtures such as zero crossing derivative ratio and double divisor derivative ratio since it has minimum manipulation steps with less requirement and no need to search to zero crossing or coincidence point as well as it transforms derivative ratio spectra to their corresponding parent original spectra of each component separately.

1.2.3. Spectrum subtraction (SS)

To restore the D^0Y spectrum originally present in the mixture, the recovered parentD^0 Z spectra (using DAD) and D^0X (using DDT) in to each mix was subtracted from the respective ternary mix gross spectrum adopting the facile spectrum subtraction method [44]

Thus, quantification of the three overlapping components under study could be simply accomplished via construction of three divergent regression equations relating the absorbance at λ_{max} against their corresponding concentrations.

Upon applying the suggested methods, the concentration of the three drugs in their complex mixture could be simply determined at its λ_{max} against the corresponding concentrations with optimum accuracy and precision as well as allowing the assessment of content uniformity of tablet dosage form. In addition, using the factorized spectrum as a manipulating tool diminishes random errors that may result due to standard preparation as a divisor since it is prepared using response and not concentration.

1.3. Greenness and whiteness evaluation methods

There are variety of strategies for assessing how environmentally friendly an analytical process is; three announced greenness evaluation tools and one whiteness assessment method were recruited in study.

1.3.1. Analysis of greenness using metric (GCC)

The Green Certificate Classification is considered as a modified tool of Eco-Scale, its criteria take into account the typical analytical procedure parameters like reagent harmful effects, instrument energy usage, the quantity of hazardous compounds discharged into the environment, including the volumes of reagents and solvents used and the quantity of waste produced, all this previously mentioned parameters are scored using a penalty-point theory.

The "Green Certificate" scale, which has been proposed, is based on the use of a color code with letter, A class with dark green color is considered as the most environmentally friendly class, analytical techniques in the A class have fewer than 10 undesirable penalty points, whereas in the G class with red color, methods have gained over 81 undesirable penalty points and considered as a non-green method [45].

1.3.2. Analysis of greenness using metric (AGREE)

The 12 rules of GAC are the basis in the Analytical Greenness approach (AGREE). These rules are turned into scores between 0 and 1 and the average of them giving the final result in the middle of the AGREE pictogram. As well as, in this tool we can giving more scores by giving more weight for the selected criteria according to its importance [30].

The results are shown as a simple read pictogram that resembles a clock. Additionally, colour is highlighted from deep red for zero result and deep green for one.

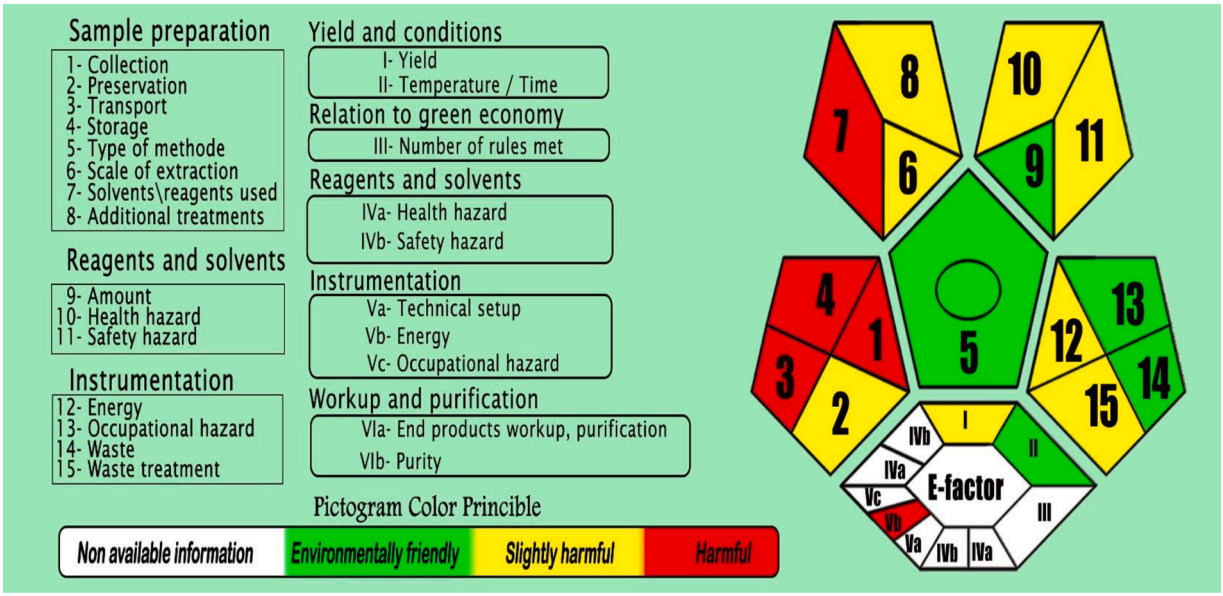

Fig. 4. Six-block regular pictogram produced by Complex-GAPI tool for greenness assessment. The explanation for each block, and the color scale principle in pictogram.

The circular pictogram's central portion shows the overall outcome of the analytical approach. Dark green will be used to indicate how "green" the analytical technique is if the result is close to the value one. For evaluation the analytical approaches, the energy consumption, automation, use of safe or hazardous chemicals, waste generation and management, sample size, sample processing, measurement site, and the analyst's safety were all calculated using the easy and simple AGREE software. The primary benefit of AGREE is that it covers all of the GAC rules. Each GAC rule is evaluated using a color, and a score, the final result is given in both a color and a numerical format so that, the reader immediately has an idea of how environmentally friendly the analytical process was overall.

1.3.3. Analysis of greenness using metric (Complex-GAPI)

Complex-GAPI is the new updated version of GAPI tool, it is the result of combining two tools, the first is the GAPI tool that is responsible for evaluating the analytical process in terms of sample preparation [31], evaluation of the solvents used, and the nature of the analytical tool used, as shown in the close numbers (1 through 15). The second tool added in Complex-GAPI is concerned with the procedures carried out before applying the final analysis, and it is illustrated in the Fig. 4 in the numbers (I to VIb) reflecting the pre-analysis processes' green nature.

The quantity of solvents, reagents, and generated waste product are itemized to be the source of the E-factors value, so the low value indicated good for environment while the higher values referred to the more waste produced, and that unfavorable environmental effects. The ring in the middle of hexagonal appears when the applied method is qualification and quantification, no circle is shown if the applied method is simply used to qualify.

Complex-GAPI uses two or three assessment-colored levels for each step. The created color pictogram allows the evaluation and quantification of the results. The red, yellow, and green colors represent the low, medium, and high environmental consequences related to each phase of the pre-analysis procedure and the analytical approach. (Color scale was clarified in Fig. 4). Each field represents a distinct aspect of related to the sample processes and analytical procedure, and if certain green conditions are satisfied, a green fill is applied to that field. Any non-available information is colored by white. More information about Complex-GAPI can be found in this article [32].

1.3.4. Analysis of whiteness using (WAC) tool

The validity of the analytical procedure and all 12 GAC criteria are covered by WAC principles. Additionally, WAC illustrates the analytical method's whiteness by merging the color effects of the three groups: analytical efficiency group (Red), adherence to green chemistry principles group (Green), and economic growth effectiveness group (Blue). The method's final colour, which symbolized the whiteness of the analytical method, is produced by adding the three main colors. [33,34]

2. Experimental

2.1. Apparatus

UV-VIS double-beam spectrophotometer (JASCO V-630) with serial number (C367961148), using quartz cells (1.00 cm) was used. All scans were performed using 1.00 cm quartz cuvettes in wavelength range from200 nm -400 nm. Manipulation of the spectra was performed using built-in software Spectra Manager II.

2.2. Chemicals

Pure samples: ATL, CLN and AMD were offered from Sigma Pharmaceutical Company with purity of 99.89±0.22, 100.45±0.12 and 99.78±24, respectively in accordance to the reported method [14].

***Dosage form*:** Teklo® tablets, labelled to contain 100 mg ATL,5 mg AMD and 25 mg CLN.

Solvents: Spectroscopic grade methanol was used as a solvent (Fischer scientific, UK).

2.3. Standard solutions

Stock solutions of concentration (1000 *μ*g/mL): ATL, CLN and AMD were prepared into three separate calibrated volumetric flasks. Methanol was utilized for both dissolving and topping off the volume to the proper level.

Working solutions of concentration 50.0*μ*g/mL: were prepared for ATL, CLN and AMD by accurately transferring 0.5 mL of their respective stock solution to three separate calibrated volumetric flasks (10-mL) and methanol was used to adjust the final volume.

2.4. Synthetic laboratory blends

For the creation of five synthetic lab mixes of ATL, AMD, and CLN in various ratios, precise aliquots from their respective working solutions were properly transferred to five different volumetric flasks (5-mL), and methanol was employed to adjust the final volumes. For blind mixes which containing AMD under its linearity, stander spectrum addition with 4.0μg/mL of pure AMD was applied mathematically by spectra manager two, then the added spectrum was subtracted to get the real concentration of the minor AMD.

2.5. Pharmaceutical dosage form extraction

The accurate weight of ten Teklo® tablets was recorded. The weighed tablets were then grinded into fine powder and uniformly mixed. A portion claimed to contain (2.0 mg ATL,0.5 mg CLN and 0.1 mg AMD) was accurately weighed and transferred into a small beaker in which10 mL of methanol was added and sonicated for 5 minutes to guarantee the complete dissolution of the active drugs. For getting rid of interfering tablet additives as the diluents, filtration was performed for the resulted suspension. One millilitre from filtrate solution was received in a calibrated volumetric flask (10-mL) and the final volume was adjusted using the same solvent. From this prepared solution, with claimed concentration 20.0μg/mL ATL, 1.0μg/mL of AMD and 5.0μg/mL CLN.The procedures described analysis of Synthetic laboratory blends were followed. For calculating the minor AMD, the added spectrum 4.0 μg/mL of AMD was subtracted to calculate the stated concentration of AMD that presented in the tablet.

2.6. Procedure

2.6.1. Resolution plan

2.6.1.1. Evaluation of spectral overlapping. The ATL, AMD and CLN standard solution, 10 μg/mL, each were scanned in the wavelength region of 200–400 nm. The $\lambda_{max,}$ was found to be 226 nm ($\lambda_{max,}$ of ALT), 362nm ($\lambda_{max,}$ of AMD) and 230 nm ($\lambda_{max,}$ of CLN), respectively utilizing methanol as blank and stored using the built-in software.

2.6.1.2. Requirements for drug assessment. Construction of three separate calibration graphs were achieved correlating absorbance values in D^0 spectra ATL, AMD, and CLN at their λ_{max}226 nm, 362 nm, and 230 nm, respectively versus the corresponding concentrations and computation of three separate regression equations was performed.

2.6.2. Determining the suitable resolution strategy

If D^0 of the proposed drugs are not allowed so the decision is application of ratio or derivative strategy or both.

2.6.3. Selection of the methods to approach resolution strategy

The resolution tools used for the selected methods

- ***Factorized D^0spectrum of CLN:*** The stored D°CLNwas divided by its corresponding absorbance at 230 nm.
- ***Factorized ratio spectrum of AMD:*** Any concentration of AMD (within linearity range) could be divided by ATL (4.0 µg/mL), as a divisor to get a ratio spectrum. The resulted ratio spectrum was divided by the amplitude difference value calculated between 325 nm and 350 nm.
- ***Numerical response factor for CLN***: The average of the division product of different concentrations of CLN within its linearity range (A_{230nm} /P_{217nm}) relating the absorbance at λ_{max} 230 nm(A_{230nm}) relative to amplitude of the first derivative of ratio spectrum of CLN using 4.0 µg/mL ATL, as a divisor at 217 nm (P_{217nm}) using $\Delta\lambda$ of 4 and scaling factor 10.

2.6.4. Assessment of resolvability of the proposed methods (Lab-prepared blends s analysis)

Step 1 (Application of DAD): The D^0mixture spectrum was divided by D^0 ATL' spectrum (4µg/mL) and the amplitude difference value for the resulted ratio spectrum, between 325 nm and 350 nm was calculated. This calculated amplitude difference was multiplied by the stored AMD factorized spectrum, where the ratio spectrum of (AMD/ ATL') was obtained. This obtained ratio spectrum (AMD/ ATL') was multiplied by the ATL' divisor to restore parent spectrum (D^0) of AMD. The ratio spectrum of (AMD/ ATL') was subtracted from ratio spectrum of the mixture to recover the ratio spectra of ATL and CLN. The concentrations of AMD in each mixture were calculated using the corresponding regression equations constructed at 362 nm.

Step 2 (Application DDT): Derivatization of the resulted ratio spectrum of ATL and CLN in step 1 using first derivative mode at $\Delta\lambda$ of 4 and scaling factor 10. The amplitude value at 217 nm was recorded then multiply by response factor. The resulted numerical absorbance value was multiplied by factorized spectrum of CLN to restore its parent spectrum (D^0) in the mixture. The concentrations of CLN in each mixture is calculated using the corresponding regression equations constructed at 230 nm.

Step 3 (Application of SS): To restore the parent spectrum of ATL in the mixture, recovered parent spectra (D^0) of both AMD (Step 1) and CLN (Step 2) were subtracted from the corresponding gross D^0 spectrum of ternary mixture. The concentrations of ATL in each mixture is calculated using the corresponding regression equations constructed at 226 nm.

The concentrations of ATL, AMD and CLN are calculated using the corresponding regression equations constructed at their maxima.

2.6.5. Assessment of resolvability and recovery percentages of the proposed plan (Dosage form analysis)

The previously prepared extract of the tablet dosage form was scanned, and the recorded spectrum was manipulated as detailed for mixture analysis, section (2.5.4.).

2.6.6. Assessment of efficacy of the selected methods

Dosage form accuracy was further accredited using standard addition technique as summarized in Table 3 after analysis of specific and known different concentrations of AMD, ATL and CLN standard authentic employing the previously detailed procedures and the attained results were acceptable.

3. Results and discussion

Years ago, binary mixtures was simply resolved using derivative ratio spectrophotometry [44]. Then ternary mixtures was successfully determined by Nevada et al. [46] using derivative ratio spectra zero-crossing [47–50], while Dinc et al. [49,51] used double divisor method. Lofty et al. [52–54] developed the derivative subtraction method in conjugation with constant multiplication to get the derivative spectrum binary mixture components separately.

Nowadays, basics of these methods together with other smart techniques based on mathematical manipulation of the tangled spectra, depending on either intrinsic properties present in the drug spectra such as extensions [55], isosbestic points and dual wavelengths [56] or obtaining some resolving spectra such as normalized [57,58] and factorized spectra [59] opened the door for the analysis of multicomponent mixtures in their dosage forms with minimum cost and effort but yet accurate and precise, sparing the hyphenated or expensive techniques to critical cases and complex matrices including different body fluids or food samples.

The common and the main concept of the two presented spectrophotometric methods was to unlock the tangles between the spectra step by step where the parent spectrum of each component in the mixture.

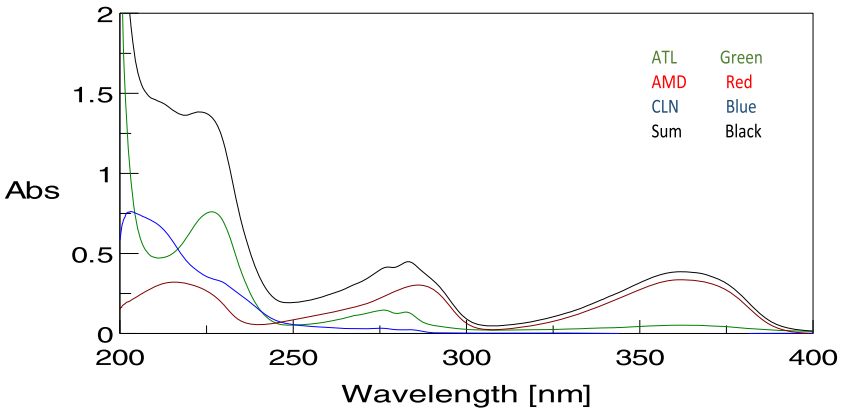

Fig. 5. Zero order spectrum of ATL (20 μg/mL), AMD (5 μg/mL), CLN (5 μg/mL) and the sum of them which represent the ratio spectra of the dosage form after spectral enrichment technique (20 ATL: 5 AMD: 5 CLN).

3.1. Spectral features for the studied dugs

Zero order spectrum of the three drugs, each was separately scanned and then overlaid over each other, it was observed that the three spectra were severely overlapped and the spectra of AMD and ATL were more extended than that of CLN. Since the mixture of drugs under investigation needed to be analysed in simple matrices such as tablet dosage form and laboratory prepared mixtures prepared in methanol just to guarantee that the right amount of the drugs would be delivered to the patients to cause its required therapeutic effect, spectrophotometric technique being the most available and affordable one, was the best choice for estimating of ATL, AMD and CLN simultaneously in their combined fixed dosage form.

Upon overlaying the stored spectra of the three drugs in their zero order over each other, a sever overlap was revealed that hindered their direct determination and necessitated the use of smart new resolution techniques. In addition to, the existing of AMD in the ternary mix with minimum concentration and absorptivity was obstructed its analysis, to determine this minor concentration, standard spectrum 4.0 μg/mL of pure AMD was added mathematically to the spectrum of the pharmaceutical preparation and to each blinded mix that containing AMD under its linearity, then to obtain the real concentration of AMD the pure added spectrum was simply subtracted by spectrum manager software. In Fig. 5 zero order spectrum of the three drugs in ratio (20 μg/mL ATL: 5.0 μg/mL CLD: 4.0 +1.0 μg/mL AMD and the Sum of them) giving a clear imagination about the spectral features of the studied ternary mix.

3.2. PSRS for resolving the ternary mixture of AMD, CLN, and ATL

3.2.1. Via DAD method for restoring AMD

Dual amplitude difference method using factorized spectrum, started by getting the factorized spectrum of AMD using spectrophotometer software through dividing any concentration of pure AMD by selected ATL concentration,4.0μg/mL. Then, the obtained ratio spectrum was divided by the calculated amplitude difference between two specific wavelengths selected in the region (325 nm and 350 nm) where ATL and AMD are overlapped while spectrum of CLN shows zero amplitude difference at these selected wavelengths. This factorized spectrum was stored to be recalled when necessary, during the mixture analysis.

Each spectrum in Fig. 6(a) of lab-prepared blends of ATL, AMD and CLN was divided separately by the previously selected ATL divisor as presented in Fig. 6(b). The numerical amplitude difference between 325 nm and 350 nm was calculated then multiplied by the stored factorized spectrum to obtain the ratio spectrum $(\frac{AMD}{ATL})$ originally present in the mixture, Fig. 6(c) followed by multiplication by ATL divisor to restore the parent spectrum of AMD where its corresponding concentration could be easily calculated from the respective regression equation using its maxima at 362 nm, Fig. 6(d).

3.2.2. Via DDT method for restoring CLN

The obtained ratio spectrum of $(\frac{AMD}{ATL})$ for each lab-prepared blends was subtracted from the previously manipulated ratio spectrum of ternary mixture to get the ratio spectrum $(\frac{ATL}{ATL})$ and$(\frac{CLN}{ATL})$ as a binary mixture, Fig. 6(e).First derivatization of the obtained ratio spectrum was applied using Δλ of 4 and scaling factor 10, Fig. 6(f). The peak amplitude value at 217 nm was recorded then multiplied by calculated numerical response factor which is the average of the division product of different concentrations of CLN within its linearity range (A_{230nm} /P_{217nm}) relating the absorbance at λ_{max}230 nm(A_{230nm}) relative to amplitude of the first derivative of ratio spectrum of CLN using 4.0 μg/mL ATL, as a divisor at 217 nm(P_{217nm}) using Δλ of 4 and scaling factor 10.

Upon multiplying the calculated numerical absorbance value of CLN at 230 nm by its factorized spectrum of CLN, its parent D^0spectrumin the mixture was restored as shown in Fig. 6(g). The concentrations of CLN could be estimated utilizing the absorbance values at 230 nm.

3.2.3. Via SS method for restoring ATL

Finally, D^0 of ATL as illustrated in Fig. 6(h), was solely resolved after subtracting the restored zero order spectra (D^0) of AMD and CLN from their respective ternary mixture of ATL, AMD and CLN. The absorbance values at 226 nm, was recorded to calculate ATL concentration from the respective regression equation.

3.3. Greenness and whiteness assessment

The results acquired from evaluating the created methods' greenness and whiteness were accomplished and compared to those from the previously published chromatographic method [15]. Firstly, the methods' Greenness were evaluated using three different methodologies; the Green Certificate Classification (GCC), the Analysis of Greenness (AGREE), and the Complimentary Green Analytical Procedure (Complex-GAPI).

For the GCC method, the direct spectroscopic analysis received a high score 89 and the green classification in the class B, as a result of low instrument energy consuming, the little amount of the used solvent and the wastes produced. According to the GCC, HPLC reported method is 84 green score. Due to the extensive use of solvents, the quantity of waste they produce, the failure to miniaturize analytical method, as well as the high energy consumption. However, GCC tool still catalogs this procedure as having a good green impact in the class B [45], so after getting these results, another greenness tools should be applied to clarify the previously confusing score. The results of the GCC were illustrated in Fig. 7(a).

By applying the second tool AGREE, it was demonstrated that the

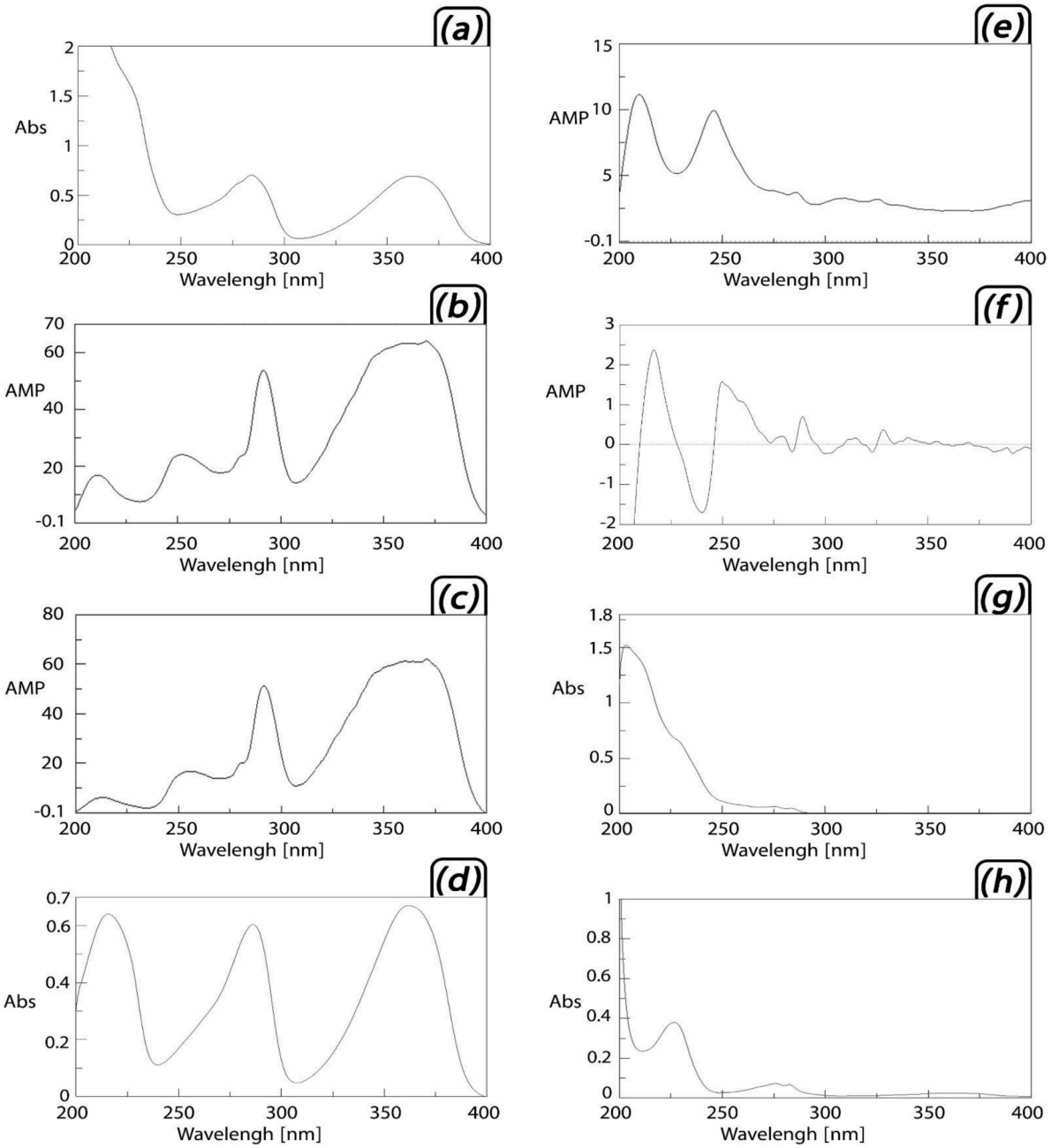

Fig. 6. (a) a lab-prepared blend mix containing AMD, ATL and CLN (10μg/mL, each)
(b)Ratio spectrum of the previously mix using ATL' (4 μg/mL) as a divisor.
(c) Ratio spectrum of AMD 10μg/mL using ATL' (4 μg/mL) as a divisor.
(d) Recovered parent spectrum AMD (10μg/mL).
(e) Regained ratio spectra of ATL and CLN binary mixture, (10 μg/mL) each.
(f) First derivative of ratio spectrum of CLN and ATL (10μg/mL, each) using ATL' (4 μg/mL) as divisor.
(g) Restored parent spectrum of CLN (10μg/mL).
(h) Restored parent spectrum of ATL (10μg/mL) after subtracting parent spectrum of CLN (10μg/mL) from their resolved binary mixture.

reported HPLC and the proposed UV-methods capture different greenness scores. The HPLC reported technique used to determine AMD, CLN, and ATL had a moderate score for greenness (0.58) and on the other hand the UV-methods received a higher score for green performance (0.76), mainly because they were used less energy and solvent [60].

As illustrated in Fig. 7(b), both techniques were categorized as red due to their off-line status and as yellow according to the off-line testing samples. The HPLC mobile phase's big volume production incorporates orange color categorization in the section five of the HPLC AGREE pictogram, while the UV-methods scores can be discovered in a middle situation, which is colored yellow. Additionally, AGREE program gave full information about the analytical process connected to each of the green chemistry rules in a form of pdf files, indicating the analytical methods' weakest points that need further greenness correction. Comprehensive reports with vibrant pictograms and highlighted reports were acquired for the preceding chromatographic and UV-methods, and they were saved as supplementary pdf files (S1,S2-Supplementary materials).However, AGREE metric does not take into account the chemicals, energy, and waste generated during the sample preparation. So, the Complex-GAPI metric was used in this study to cover this point. The

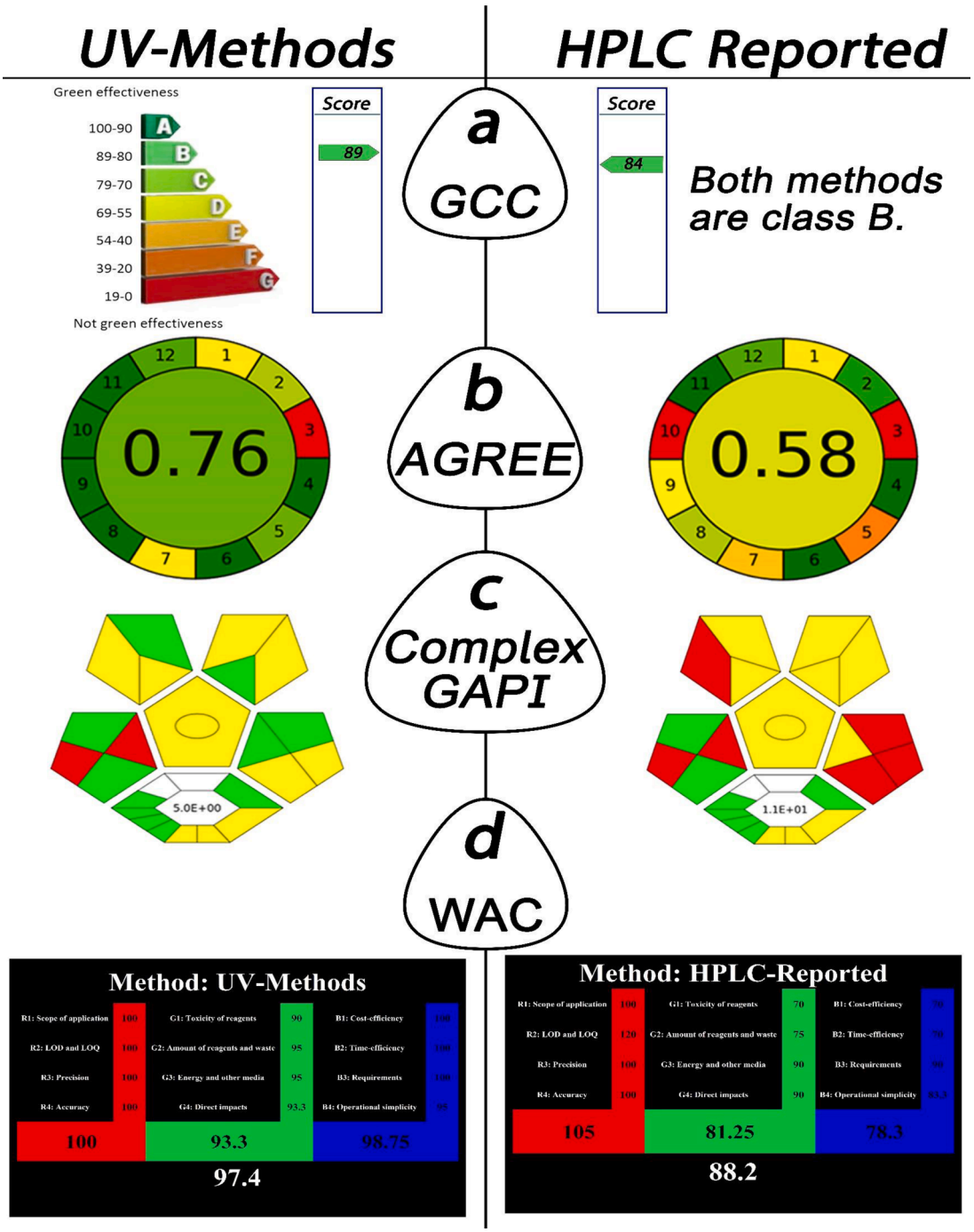

Fig. 7. Greenness and whiteness evaluation results of UV -methods and Reported HPLC method by (a) GCC, (b) AGREE, (c) Complex-GAPI, and(d) WAC tools.

greenness of the analytical methods used in this study is evaluated from more than one side, and each side complements the other to give complete evaluation results [61].

Furthermore, The Complex-GAPI evaluation for the two examined analytical approaches UV-methods and HPLC reported was completed. Regarding sample treatment evaluation, both approaches required an off-line collection of samples, hence the red evaluation was given. Due to sample transportation, both techniques received a red evaluation. The two analytical methodologies considered enables the quantification of the AMD, CLN, and ATL in pharmaceutical samples, displaying a ring in the center pentagram to notice this point. Simple sample treatments were required for both methods, so a yellow color is present in the middle of the complex-GAPI pictograph. Some parameters displayed yellow color in the Complex-GAPI pictogram (Fig. 7(c)) created for the UV-methods. Since the procedure is not solvent-free, the waste has been produced and there is no waste treatment. As well as a green solvent methanol was only utilized and less than 10 milliliters of biodegradable waste were produced. The utilization of spectroscopy equipment requires an energy consumption around 0.1 kWh1, and it is notable by a green color Additionally, the E-factor has a low value due to the less amount of waste is produced through this method.

In the reported HPLC (acetonitrile: heptane sulphonic acid sodium salt) were used as a mobile phase. The flow rate was adjusted to 1.0 mL per minutes, the time required to complete the analysis is about 9 minutes, and the required amount of solvent is more than 10 milliliters for completing just one run. The previously data about HPLC clarified the

red and yellow colors results, as well as, the high value for the E-factor produced in the Complex-GAPI pictogram created for evaluation the HPLC procedure. Energy use, waste production and occupational hazard are all considered in Complex-GAPI evaluation. As shown in Fig. 7(c), the employment of HPLC instrumentation results in an energy consumption that is more than 0.1 kWh per hour; as a result, a yellow color assignment was made for this method. However, the utilization of spectroscopy equipment requires an energy consumption around 0.1 kWh1, and in this situation is notable by a green color. HPLC is classed as a red occupational hazard, while spectroscopy equipment is classed as a green due to the preventing vapor emissions to the atmosphere. The type of the method for HPLC& UV considered as a simple sample preparation, so the differences between the two methods were just in the used solvent. In HPLC method, both methanol and acetonitrile solvents were used, while in UV-methods just methanol was responsible for the sample preparation results.

The analytical method's capacity to produce accurate results and its efficiency in detecting low concentrations of the samples are the important keys to the researcher in developing the analytical methods. However, the GCC, AGREE and Complex-GAPI metrics weren't considered these points into their criteria. To avoid this limitation, the WAC tool was applied.

The WAC tool was employed and the results revealed that the suggested UV-methods were accurate, precise, environmentally benign since they utilized methanol, a safer solvent, and produced less waste that decomposes naturally without harming the environment. Fig. 7(d)

When compared the previously results to the reported HPLC, the spectroscopic approach is less complicated, quicker, and more affordable, earning it a score of 97.4% overall.

Reported HPLC is considered as an expensive, time and solvent consuming method which could be reflected on less score in green and blue group, but it is recognized for its accurate procedures which was reflected in high score in red group, its total whitening score was 88.2% as demonstrated in Fig. 7(d).

3.4. Comparative study between the applied greenness and whiteness tool

Different results on the greenest strategy were obtained using the three metrics for judging the greenness of both the proposed spectrophotometric method and chromatographic one. Primarily due to the HPLC method which used the acetonitrile as a solvent, all the tools classified it as the least environmentally friendly. while rendering the UV-process more user-friendly by utilizing methanol solvent. The four metrics were compared, and it became clear that GCC's capacity to assess how environmentally friendly the analytical techniques was constrained. Given that the criteria used during the green evaluation could not sufficiently distinguish between differences among the adapted methods, it was the least effective instrument for assessing and comparing the greenness, as the green evaluation results for different analytical method are very close to each other (89 for UV-methods vs. 84 for HPLC) and both methods were classified as a green in the class B.

The free AGREE tool is quantitative, supports qualitative, visual analysis, and incorporates all 12 GAC ideologies into its evaluation criteria to provide accurate results when comparing various analytical approaches.

The free AGREE program is quantitative and also enables qualitative and visual analysis, in addition to taking all 12 GAC principles into its evaluation criteria to offer satisfied and truth results between different analytical methods.

The green results according to AGREE tool ranging between 0.76 score for UV-methods to 0.58 score for HPLC. The high variances in the values were observed between the compared methods due to the differences related to the type of the used solvent and the amount of the generated waste.

The AGREE methodology's total greenness is yet uncompleted because it does not take into account the sample preparation prior to the final sample analysis. In comparison to the GAPI-complex and WAC tools, using AGREE tool is a little bit simpler since it has an added benefit of allowing each assessment criterion to be weighted in accordance with the analyst's objectives.

Table 1
Parameters of assay and validation data sheet for the proposed PSRT to estimate ATL, AMD and CLN.

Parameter	AMD *DAD*	CLN *DDT*	ATL *SS*
λ_{max} nm	362	230	226
Linearity (μg/mL)	3.0-20.0	3.0 -20.0	4.0-40.0
Slope	0.0670	0.0634	0.0378
Intercept	0.0003	0.0024	0.0012
Correlation Coefficient	0.9998	0.9999	0.9999
Accuracy[a] Mean ± SD	99.61±0.13	99.92±0.37	99.82±0.21
Repeatability (RSD%[b])	0.34	0.12	0.12
Intermediate precision (RSD%[c])	0.45	0.48	0.48
Robustness[d]	0.281	0.323	0.245

[a] average of 3 experiments.
[b] (n = 9) average of three different concentration repeated 3 times in the same day.
[c] (n = 9) average of three different concentration performed three times each on three separate days.
[d] all results is less than 2%

The Complex -GAPI is a free tool that is simple to use and may provide you with more details about how sample preparation affects the environment. It is semi-quantitative, facilitates visual evaluation, and offers a complete perspective of all phases of an analytical process from beginning to conclusion. The Complex -GAPI green evaluation includes more factors amended than the GCC and AGREE tools, particularly those pertaining to pre-sample handling and treatment. A quick check at the Complex -GAPI pictograms can show the analytical procedure's green index and the areas that still need to be improved in terms of greenness. However, a few restrictions are also clearly discernible. Both the volume of waste and the wide variety of reagent/solvent quantities are not taken into consideration of this tool. For instance, whether the used volume is 10.1 mL or 400 mL, it will receive the same label (red) and the same score. From this point, using more than one tool to obtained reliable green information for the applied analytical methods is required. [42, 45,60]

The WAC tool, which takes into account the sustainability principles and contains standards that incorporates protection of the environment, worker safety, the quality and the cost of the analytical techniques, can be seen as the most comprehensive of the several metrics for evaluating various analytical methods.

The WAC is considered as a reasonable, logical, and well-judged tool for evaluating different analytical methods, as it takes into account the high efficiency and sensitivity of the chromatography method into consideration for the final result, and this were already noticed through the high red color value compared to the spectral method 105% vs.100%.

In order to make the best decision when choosing greener approaches, this tool must be used by a skilled analyst who is capable of understanding the full analytical process. This is a result of the greater amount of data that must be considered during the evaluation and the requirement to give weight to various factors. The free excel programmed datasheet making WAC tool available for many analyzers.

According to the evaluator's goals, all the tools described here, ranging in complication, were judged to be helpful for assessing and comparison the greenness of analytical procedures and were able to incorporate the key GAC ideologies. The tools vary from one another because they provide varying levels of detail for each area that is

Table 2
Estimation of AMD, CLN and ATL concentrations in lab-prepared blends s after application of the resolution plan.

lab-prepared blends				Recovery% [a]		
No	Concentration (μg/mL)			AMD	CLN	ATL
	AMD	CLN	ATL	*DAD*	*DDT*	*SS*
[b]1	5.0	5.0	20.0	[c]99.35	99.98	100.31
2	10.0	10.0	10.0	99.40	99.32	100.27
3	10.0	5.0	5.0	99.13	100.32	100.59
4	5.0	20.0	2.0	98.54	100.11	99.82
5	3.0	4.0	40.0	98.52	99.97	99.87
Mean recovery ±SD				98.99±0.43	99.94±0.37	100.17±0.32

[a] Average of three determinations.
[b] Mixture no. 1 represent the ratio of drugs present in the dosage form after mathematically spectrum adding of 4.0 μg/mL AMD.
[c] Results after mathematically spectrum subtraction of 4.0 μg/mL AMD.

assessed. Thus, the analyst must decide which metric will work best given the circumstances.

Several metrics are available for assessing the greenness and whiteness of approaches, engaging a number of characteristics for developing sustainable analytical methods. The described HPLC method was evaluated by all the tools as being the least environmentally friendly than UV-methods, mostly because it used acetonitrile as the mobile phase and this instrument consumes a lot of energy.

There are gaps in the metrics, even though all of those given here can be used to evaluate how an analytical approach would affect the environment. The optimal statistic to employ will depend on the goal of the evaluation and the practical needs.

4. Spectrophotometric methods validation

The proposed resolution techniques were developed and validated in accordance to the guidelines of ICH. The data were summarized and displayed in Table 1. It was proved through results that within the specified range of linearity, the applied methods were specific, accurate and precise.

4.1. Analytical performance parameters

Adherence to ICH (Q2B) validation was performed in adherence to ICH (Q2B) guidelines [62] the results as summarized in Tables 1 and 2 revealed accuracy, precision, selectivity and robustness of the presented methods

4.1.1. Range and linearity

Linearity was assessed through constructing different calibration graphs on three successive days. Each concentration was analyzed in triplicates. The assays were performed following the previously detailed procedures. Setup of the calibrationrange was achieved considering the practical range adherence to Beer's law as well as the cited drugs concentrations in their single tablets regimen for providing linear, accurate and precise results. Evaluation of the method linearity was accomplished through analysis of each drug in different concentrations ranging between 4.0-40.0μg/mL for ATL, 3.0-20.0μg/mL for both AMD and CLN. The concentration ranges and calibration formulas are illustrated in Tables 1 and 2.

4.1.2. Accuracy

4.1.2.1. Active ingredients. Accuracy evaluation was established along the specified ranges of the analytical procedures using the suggested consecutive steps to determine different samples of ATL. AMD and CLN. The selected concentrations for AMD and CLN were (5.0,7.0,12.0,15.0,18.0μg/mL) while those for ATL were (5.0,15.0,20.0,25.0,30.0and 35.0μg/mL). the respective regression equations previously computed were employed to calculate the concentrations and the obtained percentage recoveries assured satisfactory accuracy as presented in Tables 1 and 2.

Table 3
Assessment of AMD, CLN and ATL concentrations in their STRs and the results of standard addition technique

Pharmaceutical dosage form.		Found % [a]		
		AMD[c]	**CLN**	**ATL**
		DAD	***DDT***	***SS***
Teklo® tablet	Mean	100.11	99.61	100.07
100mg (ATL)/ 25 mg(CLN)/5 mg AMD	±SD	±0.21	±0.59	±0.25
Standard addition	Mean	Recovery % [b]		
	±SD	**AMD**	**CLN**	**ATL**
		DAD	***DDT***	***SS***
		99.32	99.49	99.72
		±0.47	±0.42	±0.18

[a] Average of three determinations.
[b] Average of three determinations (authentic added equivalent to 4.0, 8.0,16.0 μg/mL of ATN, 4.0,6.0,8.0 μg/mL of AMD and CLN).
[c] After mathematically spectrum adding of 4.0 μg/mL AMD.

4.1.2.2. Pharmaceutical products. The suggested analytical methods were adopted significantly for 4.0-40.0μg/mL for ATL,3.0-20.0μg/mL for AMD and CLN in their combined pharmaceutical product Teklo® Tablets. actually, the pharmaceutical product accuracy could be assessed in accordance to the ICH guidelines via the application of either standard addition technique or comparing the proposed technique results versus those of reported method or spiked placebo.

In the presented technique, the validation was assured usingstandard addition technique through determining three fortification levels. The values of percentage recoveries were calculated, and the results affirmed method suitability for determination of the analytes under investigation in their pharmaceutical dosage form without any impact from the added excipients as stated in Table 3.

4.1.3. Precision

4.1.3.1. Repeatability and intermediate precision

Three different ATL, AMD and CLN concentrations were determined in triplicates. The concentrations used were (4.0, 10.0, 16.0μg/mL) for both AMD and CLN, (20.0, 30.0, 40.0 μg/mL) for ATL. The standard solutions were analyzed separately intra-daily and inter-daily on three consecutive days adopting the suggested methods. Relative standard deviations were estimated as shown in Tables 1 and 2.

4.1.4. Specificity

The method specificity was assessed through preparing and analyzing some lab-prepared blendsof ATL, AMD and CLN within their linearity ranges. The data were processed, and the results were satisfactory as shown in Table 3.

4.1.5. Robustness

Studied analytes standard solutions were kept in the fridge where the temperature was retained at (0 ± 5 °C) for nearly seven days. The methods robustness was assessed via estimating the standard solutions with different concentration levels (6.0, 8.0, 10.0 μg/mL) to verify that the applies methods are valid without any critical variations. Along this period, the obtained results didn't go below the minimum percentage in accordance with the literature about the stability of the cited drugs. The purpose behind this practice was to prevent the occurrence of errors that may result from the loss of solvent loss owing to alcohol (methanol) volatility that could happen even if stored in the fridge resulting in inaccurate results. Virtue of procedures were followed to examine the stability of solutions and mentioned for each presented method. Analysis credibility concerning international variations in the parameters of

Table 4
The purity index for ATL, AMD and CLN in Teklo® tablets.

Number of tablets	DDT AMD[#] Purity index	DAD CLN Purity index	SS ATL Purity index
Tablet 1	0.991	0.990	0.995
Tablet 2	0.992	0.994	0.995
Tablet 3	0.990	0.993	0.991
Tablet 4	0.995	0.997	0.993
Tablet 5	0.990	0.996	0.994
Tablet 6	0.994	0.999	0.998
Tablet 7	0.988	0.999	0.991
Tablet 8	0.989	0.994	0.996
Tablet 9	0.996	0.999	0.991
Tablet 10	0.992	0.999	0.997
Mean	0.992	0.996	0.994
SD	0.003	0.003	0.003

[#] After mathematically spectrum adding of 4.0 μg/mL AMD.

assay was revealed through obtaining the Mean % recoveries and % RSD as declared in Table 1

4.5. Assessment of drugs in Teklo® tablets

The presented resolution UV techniques were also successfully applied for simultaneous estimation of the AMD, CLN and ATL in their Teklo® tablets. The obtained results showed percentage recoveries with acceptable values confirming the methods suitability for routine and fast estimation of the cited drugs without any interference from the other tablet components. The results obtained were displayed in Table 3.

5. Assessment of average content of Teklo® tablets

To assure the therapeutic potency of dosage forms lie within the limits of specified acceptance range, the uniformity of content was investigated for ATL, AMD and CLN, the content uniformity of each dosage unit in Teklo® tablets was assessed according to the British pharmacopeia (BP) guidelines [13]. The detailed results were acceptable and within the endorsed limits of BP as displayed in Table 4.

Evaluating the purity index [63,64] was also performed ATL, AMD and CLN using ten Teklo® tablets. The calculation was achieved through the extracted parent spectra of 20.0 μg/mL ATL,5.0 μg/mL CLN and (1.0+4.0) μg/mL of AMD from their combined tablet dosage form via applying the procedures of the three proposed methods and the equivalent parent spectra of raw ATL, CLN and AMD. The values of purity index were found to be satisfactory indicating of ATL, CLN and AMD purity in the tablets and the obtained results were displayed in Table 4.

6. Statistical analysis

The results of the newly proposed spectrophotometric methods including in resolution plan were statistically compared to those of the HPLC reported method [15] as shown in Table 5. The t and F calculated values were found to be less than the theoretical values indicating that there were no significant differences between the presented UV-methods and the reported HPLC one regarding accuracy and precision.

7. Conclusion

Eco-friendly sustainable parent spectrum restoration technique proved to be simple, sensitive, specific and reliable technique including three successive methods dual amplitude difference, derivative ratio transformation and spectrum subtraction for the estimation of AMD, CLN and ATL in different matrices including bulk powder, lab-prepared blends and tablet formulations. It could also be utilized for determination of other multicomponent mixtures where this technique restores the parent spectrum of each drug in the combination which is the fingerprint of the drug and could be used to confirm its purity as well as the analysis of the cited drugs at their maxima offers maximum accuracy and precision. These methods offer the advantages of minimum mathematical intervention and no need for complementary methods. It is possible to use the methods described in this article to regularly determine the presence of the cited drugs in their pharmaceutical dosage forms in quality control laboratories because they are simple, accurate, rational, and valid and don't require any expensive equipment or special software for data analysis and signal processing. The employability of the technique could also be extended to be applied in serum or different dissolution media. The methods could also be recruited for analysis of different ternary mixtures in any quality control laboratories to check the purity and concentration of the drugs in any step of production or even in the finished product to assure that shipment and storage didn't have any deliberate effects to guarantee that the right dose is delivered to the patients.

Several metrics are available for evaluating the greenness and whiteness of methods, employing a number of characteristics, and play a significant role in the creation of more sustainable analytical procedures.

While the GCC tool supplied a quantitative simple classification of examined methods for the energy usage, consuming chemical, hazard exposures, and waste quantity produced, the AGREE tool assesses the many steps of the overall technique with help from the application of the online accessible AGREE calculator. The Complex-GAPI metric gave a complete visual representation of the green scenario in accordance with the GAC principles while taking into account all the procedures steps taken before to and throughout the analytical processes. Because of this, each one has a different focus, but regardless of the overall rating, they

Table 5
Statistical comparison between the results ATL, AMD and CLN obtained upon applying PSRT and the reported HPLC method [15].

Values	**AMD** *DAD*	Reported Method [15][a]	**CLN** *DDT*	Reported Method [15][a]	**ATL** *SS*	Reported Method [15][a]
Mean	99.29	99.43	99.92	99.87	99.82	99.76
SD	0.14	0.19	0.37	0.29	0.22	0.31
RSD%	0.14	0.19	0.37	0.29	0.22	0.31
Variance	0.0196	0.0361	0.1369	0.0841	0.0484	0.0961
Student's t-test[b] (2.23)	0.1265		0.8538		0.7333	
F –value[b] (5.05)	1.84		1.63		1.99	

[a] ODS-80 TM column, mobile phase 5mM sodium heptane-sulphonic acid and acetonitrile in a 20:80, pH was 4.4. The detector's setting was 274 nm.
[b] The figures in the parenthesis are the corresponding theoretical values of t and F at p =0.05 n=6
AMD in DAD procedure always be calculated after mathematically spectrum adding of 4.0 μg/mL AMD

have all offered a similar scale of method greenness.

However, the WAC tool appears to be a quantitative and online-accessible assessment metric, looking for incorporate green principles and method effectiveness on evaluating various methods to make more sustainable analytical determinations.

The described HPLC method was evaluated by all the tools as being the least environmentally friendly than UV-methods.

Applying different greenness and whiteness tools were provided a complete greenness profile for the applied analytical methods which given the analysers more idea about the steps that really were needed to improve.

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.

Data availability

The data that has been used is confidential.

Funding

No specific grant for this research was provided by funding organizations in the public, private, or non-profit sectors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2023.100226.

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