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Original article

Novel, cost-effective spectrophotometric methods for the determination of Beta-carotene, Vitamin C, and Vitamin E in their ternary mixtures: Greenness and whiteness appraisal

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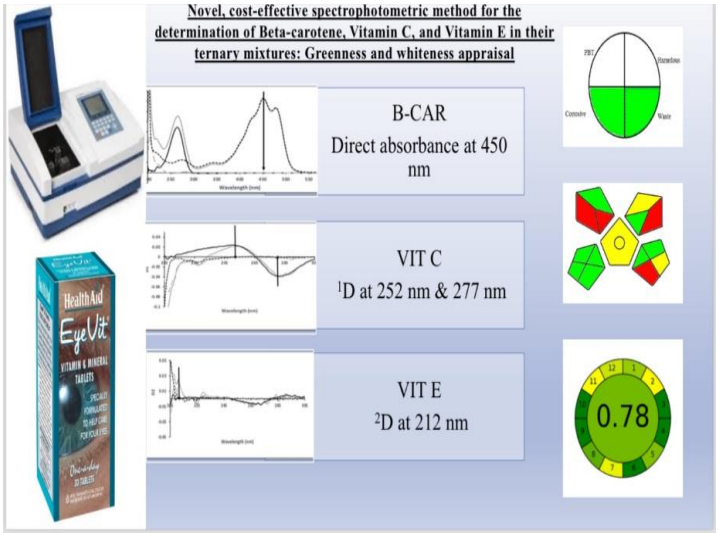
Abstract:



Oxidative mechanisms and excessive generation of reactive oxygen species (ROS) have been strongly associated with different pathological abnormalities. The use of antioxidants has gained attention in controlling body functions. Antioxidant vitamins including vitamin C (VIT C), vitamin E (VIT E), and beta-carotene (B-CAR) have been included in different health-managing formulations because of their ROS-scavenging effect. In this work, spectrophotometric methods are developed for the first time for the simultaneous determination of VIT C, VIT E, and B-CAR in tablet mixtures. B-CAR was determined by measuring direct absorbance at 450 nm. However, derivative spectrophotometry was applied to resolve the spectral overlap between VIT C/E by recording the absolute values of ^{1}D peak-to-peak amplitude at 252 and 277 nm for the determination of VIT C while the second derivative spectra were used for the determination of VIT E by recording the absolute values of ^{2}D amplitude at 212 nm. The validated methods permitted the determination of the antioxidant vitamins within the concentration ranges of 0.68 - 6.1, 3.35-53.6, and 3-24 µgmL^{-1} for B-CAR, VIT C, and VIT E, respectively. Good linearity was shown by high values of correlation coefficients >0.999. The National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI), and Analytical Greenness Calculator (AGREE) were used to provide a complementary approval of the degree of greenness

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of the proposed methods. Taking into consideration the low cost, greenness, and high quality of the proposed spectrophotometric methods, they can be regularly applied for routine quality control analysis of such vitamin combinations.

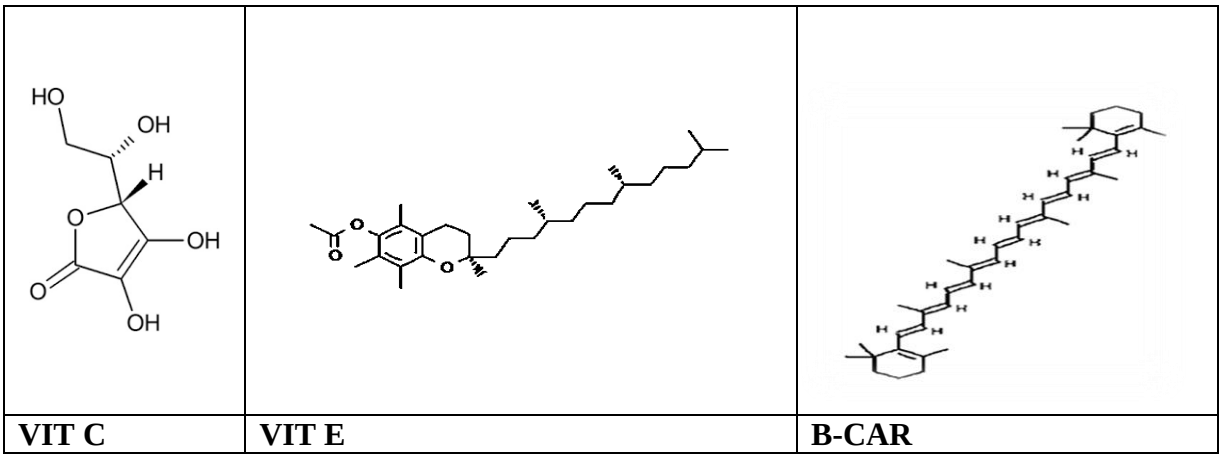
Keywords: Antioxidants, vitamins, derivative spectrophotometry, whiteness

1. Introduction

The role of free radicals in the development of different types of diseases, cancer progression, and hence the quality of life has been the main concern of recent research. This attracts attention to the use of antioxidants to control the release and oppose the effect of free radicals [(1)]. They act at three levels starting from prevention of the oxidation of oxidizable substrates, interception by scavenging free radicals e.g., ascorbic acid, known as vitamin C (VIT C), alpha-tocopherol, known as vitamin E (VIT E) and beta-carotene (B-CAR), and finally the level of repair by repairing damaged molecules e.g., glutathione. The free-radical scavenging effect of VIT C, VIT E, and B-CAR, makes them widely used as supplements to support the normal functions of many vital organs inside the body, e.g., liver, thyroid gland, cardiovascular system, nervous system, and the eye [(2)].

VIT E **(Fig. 1)** constitutes a group of lipophilic compounds with α-tocopherol being the most active form. It helps the body to maintain normal physiological processes targeting enhancement of the body's performance, immunological response, and skin quality [(3)]. B-CAR **(Fig. 1)** is a natural fat-soluble compound which is known as provitamin A. It is a potent antioxidant and oxygen scavenger that is found beneficial in protection against different types of illness including cancer, hepatic disorders, gastrointestinal, and cardiovascular diseases [(4)]. VIT C **(Fig. 1)** is a water-soluble vitamin that is well known for its antioxidant effect and helps in regenerating other antioxidants inside the human body including VIT E.

The effect of antioxidant vitamins in the control of visual impairment has been reported including cataract, glaucoma, eye dryness, and age-related macular degeneration (AMD). Different studies concerning the effect of VIT A, E, C in delaying the progression of AMD have been reported [(5, 6)]. VIT C is known to play a critical role in collagen formation, particularly in the cornea and sclera which is essential to maintain the structure of the eye.



VIT C	VIT E	B-CAR

Fig. 1: Chemical structures of VIT C, VIT E and B-CAR

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VIT A supports the corneal function, eye lubrication, and being a component of the eye protein rhodopsin, it aids in night vision. It was also reported that VIT A can improve conjunctival dysfunction and is thus beneficial in curing alkali-induced corneal burns in rats [7]. VIT E is essential in preventing lipofuscin accumulation and damage of photoreceptors and retinal dysfunction. The effect of VIT E in suppressing the increase in leptin levels in the retina associated with experimental uveitis has been reported [8]. A combination of VIT A, C, E, along with some minerals, has been formulated as antioxidant tablets marketed to maintain healthy life, improve CVS, maintain healthy eye functions, prevent eye dryness, and improve skin integrity.

Spectrophotometric determination of drug substances has gained much attention owing to its simplicity and cost-effectiveness compared with many other analytical techniques.

However, direct spectrophotometry usually suffers from spectral interference even for single-component analysis. Co-formulation excipients and matrix interference may also cause serious problems in the determination of active pharmaceutical ingredients (API). Spectral overlap is usually the main tackling problem in multi-component spectrophotometric analysis. Different mathematical treatments are hence required to resolve the spectral overlap and allow the determination of API with the elimination of interference. Among these is the derivative spectrophotometry which has been widely applied in pharmaceutical analysis [9, 10, 11, 12]. The first derivative treatment of absorption spectra is known to eliminate constant interference while the second derivative eliminates linear interference.

The literature was extensively reviewed for the spectrophotometric determination of the three antioxidant vitamins. Colorimetric determination of VIT C based on different types of redox reactions has been largely reported [13, 14]. The determination of VIT C in its pharmaceutical preparations was previously performed based on measuring the colored azo dye formed between VIT C and 2, 4-dichloroanailine [15]. Multivariate spectrophotometric methods [16] and mean centering of ratio kinetic profiles have also been reported for the determination of VIT C in its mixtures [17]. The reducing potential of VIT E was also previously applied in its colorimetric determination [13, 14, 18]. Also, colorimetric determination of VIT E was accomplished following the formation of gold complexes [19]. Being a colored compound, VIT E was determined by direct absorbance [20]. In addition, VIT E was determined in its binary mixtures with vinpocetine using absorbance difference and derivative ratio spectra [21]. Different spectrophotometric methods were also published for the determination of B-CAR including direct absorbance [22, 23] and derivative spectrophotometry [22]. B-CAR was determined in binary mixtures with anthocyanoside using the derivative spectrophotometric method whereas the third-derivative spectrophotometry method was used for their simultaneous determination using the zero-crossing technique [24].

Literature review reveals that HPLC-DAD has been proposed for the determination of the cited vitamins in their mixtures [25, 26, 27, 28]. Although HPLC methods were widely used for the analysis of vitamins mixtures, this technique is more complicated, time-consuming, requires personnel skills, and of much higher expenses compared with spectrophotometric techniques.

To our knowledge, no spectrophotometric method has been found in the literature so far for the simultaneous determination of the antioxidant vitamins, VIT C, VIT E, B-CAR. Thus, the present study aimed to develop spectrophotometric methods for the

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simultaneous determination of the three vitamins in their ternary mixtures and in the presence of co-formulated adjuvants in their pharmaceutical preparations without prior clean-up procedures. Although the direct spectrophotometric technique is suitable for the determination of B-CAR in the presence of VIT C and VIT E, it was not possible to determine the latter drugs using their absorption spectra due to spectral interference. Therefore, the first and second derivative spectra of the mixture were calculated.

The introduction of the dimension of methods' greenness and eco-friendliness in the assessment of analytical methodologies has gained much attention. Different metrics have been used to ensure the lack of environmental harm because of pharmaceutical analysis concerning chemicals and solvents, waste production, and energy consumption.

The most important are, the National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI), and Analytical Greenness Calculator (AGREE) [29-32]. Therefore, the greenness of the proposed spectrophotometric methods has been evaluated using these assessment metrics, giving a complementary overall picture of the proposed methods' eco-friendliness.

As an extension of Green Analytical Chemistry (GAC), the proposal of White Analytical Chemistry (WAC) has been proposed. WAC principles rely on the fact that the quality of analytical methods depends on three bases, analytical aspects, practical issues, and finally greenness evaluation [33]. Accordingly, the whiteness of the proposed spectrophotometric methods was also assessed in this study to give a wide holistic consideration of the methods' quality.

2. Experimental

2.1. Instrumentation

Spectrophotometric measurements were performed using a Shimadzu UV-1800 Double Beam UV/Visible Scanning Spectrophotometer, associated with LC solution software, Japan.

2.2. Materials and reagents

Authentic samples of VIT C, VIT E, and B-CAR were kindly provided by Pharco Pharmaceuticals, Egypt. Analytical grade Methanol (Scharlau) and HPLC grade tetrahydrofuran (THF) (Advent Chembio) were used all over the study.

2.3. Preparation of stock and working standard solutions

Stock solutions of VIT C and VIT E (500 μgmL^{-1}) were prepared in analytical grade methanol. The poor solubility of B-CAR in methanol forced us to prepare B-CAR stock solution (500 μgmL^{-1}) in HPLC grade tetrahydrofuran as no greener solvents were available to dissolve B-CAR. These stock solutions were separately diluted in methanol to obtain working standard solutions of concentrations 0.68 - 6.1, 3.35-53.6, and 3-24 μgmL^{-1} for B-CAR, VIT C and, VIT E, respectively.

2.4. General procedure and construction of calibration graphs

The absorption spectra of B-CAR, VIT C, and VIT E were separately measured in the wavelength range 200 – 600 nm against a blank that was prepared similarly to the working solutions with the same composition of solvents (methanol & THF). The absorbance data were recorded at 1 nm intervals. B-CAR was determined by measuring the direct absorbance at 450 nm. The first derivative spectra were calculated at 2 nm intervals and the absolute values of ^1D peak-to-peak amplitude at 252 and 277 nm were plotted against the corresponding concentration to construct the calibration graph of VIT C. The second derivative spectra were also calculated at 2 nm intervals,

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and the absolute values of ^{2}D amplitude at 212 nm were plotted against the corresponding concentration to construct the calibration graph of VIT E.

2.5. Application to pharmaceutical preparations

Commercial tablets of the vitamins mixture were not available in the Egyptian market, Eyevit® tablets (HealthAid, UK) labeled to contain VIT A 5000 IU, equivalent to 3000 µg B-CAR, VIT C 60 mg, and VIT E 30 IU, equivalent to 20.25 mg. Accordingly, lab-prepared tablets were prepared as follows: amounts of B-CAR, VIT C, and VIT E equivalent to those found in commercial tablets were mixed with tablets' excipients, microcrystalline cellulose, aerosil, and magnesium stearate. These excipients are most commonly used in tablet preparations and thus are approved in our proposal. Appropriate weights equivalent to one tablet were transferred into separate 100 mL volumetric flasks, extracted with 50 mL of a solvent mixture of methanol: THF (1:1), and completed to the mark with methanol. Aliquots of the resulting solutions, B-CAR (30 µgmL^{-1}), VIT C (600 µgmL^{-1}), and VIT E (204.5 µgmL^{-1}) were diluted with methanol to obtain a mixture of concentrations 0.68 µgmL^{-1} B-CAR, 13.5 µgmL^{-1} VIT C, and 4.62 µgmL^{-1} VIT E **(Table 4)**.

3. Results and discussion

3.1. Absorption characteristics

The absorption spectra of B-CAR, VIT C, and VIT E are presented in **Fig. 2**. B-CAR could be measured directly at 450 nm since at this wavelength both VIT C and VIT E showed zero absorption. Measurements at the former wavelength ensured the lack of THF interference as solvent since its cut-off point is 215 nm. However, the marked spectral overlap of VIT C and VIT E together with B-CAR in the UV region 200-350 nm, made their direct determination practically impossible. Accordingly, the first and second derivatives of the absorption spectra were generated, and the zero-crossing technique was applied in selecting the working points. The first derivative spectra of B-CAR, VIT C, and VIT E **(Fig. 3)** showed optima for the determination of VIT C by measuring the ^{1}D amplitudes at 252 nm and 277 nm (peak to peak measurement) with no interference from VIT E. On the other hand, the second derivative spectrum **(Fig. 4)** showed a signal for VIT E at 212 nm with zero contribution from VIT C. Therefore, the ^{2}D amplitude (peak to zero measurement) at 212 nm was found to be selective for the determination of VIT E. Derivative spectra calculation and peak-to-peak measurements eliminated any possible interference of solvents cut-off points. Thus, the three compounds could be determined by applying the proposed methods without any interference from one another.

The derivative spectra were calculated at different $\Delta\lambda$ values. $\Delta\lambda = 2$ nm was selected to be applied in the derivative spectrophotometric methods since it showed optimum sharp peaks with the highest sensitivity of the calculated amplitudes.

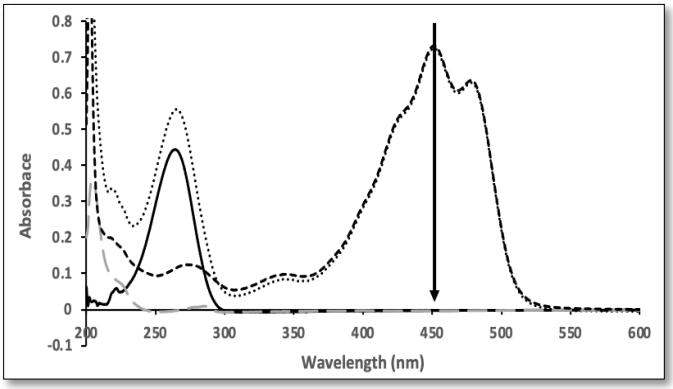

Fig. 2: Absorption spectra of 0.68 µgmL^{-1} B-CAR (---), 13.5 µgmL^{-1} VIT C (-), and 6.08 µgmL^{-1} VIT E (- - -) and their mixture (....), determination of B-CAR using direct absorbance at 450 nm

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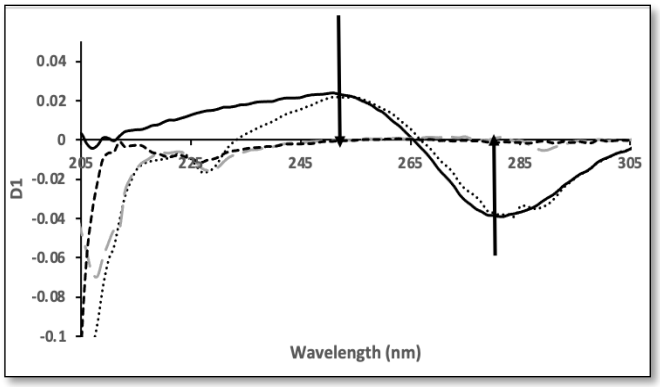

Fig. 3: First derivative ^{1}D spectra absorption spectra of 0.68 µgmL^{-1} B-CAR (---), 13.5 µgmL^{-1} VIT C (-), and 6.08 µgmL^{-1} VIT E (- - -) and their mixture (....), determination of VIT C using peak to peak ^{1}D at 252 and 277 nm

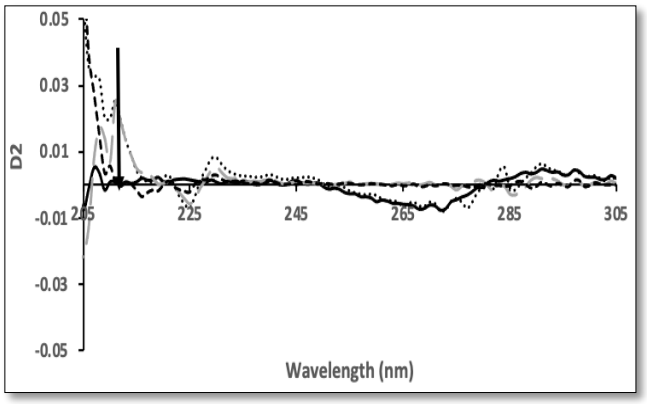

Fig. 4: Second derivative ^{2}D spectra of 0.68 µgmL^{-1} B-CAR (---), 13.5 µgmL^{-1} VIT C (-), and 6.08 µgmL^{-1} VIT E (- - -) and their mixture (....), determination of VIT E using peak to zero ^{2}D at 212 nm

3.2. Validation of the proposed methods

The developed spectrophotometric methods were fully validated as per ICH guidelines [34].

3.2.1. Linearity and range

The linearity of the proposed spectrophotometric methods was evaluated by analyzing a series of different concentrations of each component within the concentration ranges mentioned in **Table 1**. The proposed methods were applied following the established procedures. The absorbance or derivative values for each drug were recorded and plotted against the concentration to obtain the calibration curve of each compound with the corresponding regression equation. Regression and statistical parameters: including intercept, slope, variances around slopes, and intercepts are given in **Table 1**.

The good correlation coefficient values and small intercept values proved the methods' compliance with Beer's law and demonstrated outstanding linearity. Furthermore, the RSD percent of slope values was found to be minimal, showing that the proposed methods had acceptable linear correlations.

Table 1: Regression and statistical parameters of B-CAR, VIT C, and VIT E using the proposed spectrophotometric methods

	Method / Analyte		
	Direct Absorbance/ B-CAR	**^{1}D /VIT C**	**^{2}D /VIT E**
Wavelength λ(nm)	450	252, 277	212
Concentration range (µgmL^{-1})	0.68-6.1	3.35-53.6	3-24
Correlation Coefficient(r)	0.99949	0.99999	0.99987
Intercept(a)	0.023	0.002	-6.036×10^{-5}
Slope(b)	0.176	0.005	0.003
RSD% of slope	1.833	0.29	0.926
S_a	0.011	0.0003	0.0004
S_b	0.003	1.342×10^{-5}	3.231×10^{-5}
$S_{y/x}$	0.014	0.0005	0.0005
LOD (µgmL^{-1})	0.272	0.391	0.498
LOQ (µgmL^{-1})	0.823	1.186	1.51

S_a: Standard deviation of intercept
S_b: Standard deviation of slope
$S_{y/x}$: Standard error
LOD= 3 S_{blank}/ S
LOQ= 10 S_{blank}/ S

3.2.2. Accuracy and precision

Three replicate determinations of three different concentration levels of each

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compound were performed on the same day to assess the proposed methods' within-day precision, calculated as percentage relative standard deviation (RSD%), and within-day accuracy, calculated as percentage relative error (Er %). Three replicate determinations of the same concentration levels on three consecutive days were calculated in the same way to assess inter-day accuracy and precision. These values, being less than 2%, demonstrated the suggested spectrophotometric methods' great accuracy and precision (**Table 2).**

3.2.3. Limit of detection and limit of quantitation

According to ICH guidelines, the approach based on the standard deviation of the blank was used for determining the limits of detection and quantitation. To calculate LOD and LOQ, the general formulae were used: LOD= 3 S_{blank}/ S, LOQ= 10 S_{blank}/ S, where S_{blank} is the standard deviation of the blank and S is the slope of the calibration curve. The calculated LOD values were 0.272 μgmL^{-1}, 0.391 $\mu gmL^{-1,}$ and 0.498 μgmL^{-1} for B-CAR, VIT C, and VIT E, respectively while LOQ values were 0.823 $\mu g\ mL^{-1}$, 1.186 μgmL^{-1} and 1.51 μgmL^{-1} for B-CAR, VIT C, and VIT E, respectively.

Table 2: Accuracy and precision of the proposed methods for the determination of B-CAR using the proposed spectrophotometric methods

	Within-day (n = 3)			Between-day (n = 9)		
Nominal value (μgmL^{-1})	**Found ± SD (μgmL^{-1})**	**RSD (%)**	**|Er(%)|**	**Found ± SD (μgmL^{-1})**	**RSD (%)**	**|Er(%)|**
B-CAR						
0.7	0.698 ± 0.002	0.29	0.29	0.695 ± 0.004	0.58	0.71
1.8	1.82 ± 0.03	1.86	1.11	1.83 ± 0.02	1.09	1.83
5	4.98 ± 0.01	0.14	0.44	4.97 ± 0.03	0.6	0.56
VIT C						
6.7	6.79 ± 0.1	1.48	1.33	6.78 ± 0.05	0.8	1.25
13.4	13.63 ± 0.17	1.27	1.69	13.62 ± 0.21	1.55	1.66
40	39.87 ± 0.13	0.13	0.31	39.54 ± 0.14	0.34	1.14
VIT E						
4	3.98 ± 0.06	1.43	0.4	3.899 ± 0.10	2.64	2.53
8.1	8.17 ± 0.15	1.82	0.85	8.18 ± 0.12	1.52	0.93
20	19.78 ± 0.14	0.71	1.1	19.69 ± 0.18	0.91	1.55

SD: Standard deviation
RSD: Relative standard deviation
$|E_r\%|$: Absolute value of % Relative error

3.2.4. Selectivity

Within the linearity ranges, several mixtures of B-CAR, VIT C, and VIT E were prepared to test the methods' selectivity. The proposed methods were used to analyze the laboratory-prepared mixtures **(Table 3)** indicating that the absorbance, ^{1}D and ^{2}D values at the specified wavelengths, were only a function of the concentration of the corresponding component at the chosen wavelength and were unaffected by the concentration of the other components in the mixture. As a result,

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the tabulated results were satisfactory, showing that the proposed methods were selective.

Moreover, analysis of laboratory-prepared tablets including presumptive excipients such as microcrystalline cellulose, aerosil, and magnesium stearate showed no interference from these excipients. This proved the suitability of the proposed spectrophotometric methods for the selective analysis of VIT C, VIT E, and B-CAR in commercial tablets in the presence of possible excipients.

3.3. Stability of Solutions

The stability of VIT C, and VIT E stock solutions in methanol and B-CAR stock in THF was tested after storing at room temperature and in the refrigerator at 4°C for one week. Significant spectrophotometric variations were observed, and it was concluded that stock solutions were only stable within 5 days of preparation. Standard solutions were stable within 1 day of preparation when kept at room temperature. The stability of standard solutions was extended to 5 days when they were kept refrigerated.

Table 3: Determination of B-CAR, VIT C, and VIT E in laboratory-prepared mixtures using the proposed spectrophotometric methods (direct absorbance for B-CAR, ^{1}D for VIT C, and ^{2}D for VIT E)

Nominal Value (μgmL^{-1})			Found ± SD (μgmL^{-1})			RSD %			|Er%|		
B-CAR	**VIT C**	**VIT E**	**B-CAR**	**VIT C**	**VIT E**	**B-CAR**	**VIT C**	**VIT E**	**B-CAR**	**VIT C**	**VIT E**
8	8	8	8.01± 0.001	8.12± 0.05	7.95± 0.11	0.01	0.55	1.4	0.15	1.55	0.68
1.5	27	12.25	1.52± 0.02	27.003± 0.02	12.21± 0.06	1.52	0.17	0.46	1.13	0.01	0.31
1.35	6.75	5.52	1.35± 0.01	6.75± 0.07	5.51± 0.01	0.81	0.96	0.22	0.15	0.02	0.11
1.8	9	7.36	1.82± 0.03	9.13± 0.04	7.35± 0.03	1.86	0.46	0.44	1.33	1.49	0.08
2.7	13.5	11	2.69± 0.04	13.61± 0.04	11.21± 0.043	1.56	0.29	0.38	0.48	0.82	1.94

SD: Standard deviation
RSD: Relative standard deviation
|E_r%|: Absolute value of % Relative error

3.4. Analysis of laboratory-prepared mixtures

Five different laboratory-prepared mixtures were prepared containing B-CAR, VIT C, and VIT E within the linearity range mentioned in **Table 1.** The mixtures were analyzed according to the general procedure. The results of the analysis mentioned in **Table 3** showed that the accuracy and precision were within the accepted limit of 2% indicating that the methods were successfully applied for the determination of drugs' mixtures.

3.5. Application to pharmaceutical preparations

Since the pharmaceutical preparation of the mixture (Eyevit® tablets) was not available in the local market, the proposed methods were applied to determine B-CAR, VIT C, and VIT E in lab-made mixtures with

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concentrations equivalent to that in actual tablet formulation. The good recoveries with minimum deviations obtained in **Table 4** indicated that the proposed methods could be applied for the determination of the three vitamins in their multi-formulations with high accuracy and precision. The proposed methods were applied for the determination of B-CAR, VIT C, and VIT E, being the major constituents of the formulation. Other components of the tablet are minor, and it would be tedious and impractical to apply the proposed spectrophotometric methods for their quantitation. The proposed methods focused on the quantitation of the mainly active constituents.

The results obtained by the proposed methods were statistically compared with those of a reference method using the student's t-test and the variance ratio F-test. The calculated t- and F-values did not exceed the theoretical values which indicated a good agreement between the proposed and comparison reference methods. The reference method used involved analysis of the three drugs under investigation in addition to silymarin and N-acetylcysteine using the HPLC-DAD technique.

Table 4: Application of the proposed spectrophotometric methods for the determination of VIT C, VIT E, and B-CAR in their laboratory-prepared tablet solutions*

	Proposed methods Found (%) ± SD**	**Reference method [34] Found (%) ± SD****	**t-calculated**	**F-calculated**
B-CAR, Direct Absorbance	100.87± 0.88	100.71±0.46	0.62(2.3[d])	0.01(6.39[d])
VIT C, [1]D	99.86± 1.32	100.39±0.62	1.50(2.3[d])	0.001(6.39[d])
VIT E, [2]D	101.3± 0.95	101.57±0.09	1.26(2.3[d])	0.43(6.39[d])

*Concentration equivalent to 0.68 μgmL^{-1} B-CAR, 13.5 μgmL^{-1} VIT C and 4.62 μgmL^{-1} VIT E

**Mean % recovery ± SD for five determinations.

[d] Theoretical values for t and F at P =0.05 are 2.31 and 6.39, respectively.

3.6. Greenness and whiteness evaluation of the proposed spectrophotometric methods

Analytical chemistry constitutes an important tier of eco-conservation, and it has been widely linked to the exposure of biosystems to chemical pollution. Proper selection of analytical methodologies with obedience to 12 principles of Green Analytical Chemistry (GAC) can greatly contribute to preserving the environment with a positive impact on human health. In this respect, different metrics have been used to assess the degree of greenness of analytical methodologies, including NEMI, GAPI, and AGREE.

One of the oldest metrics to evaluate methods' greenness is NEMI which depends on using four fields to show the obedience of the method to four selected criteria. Green colored fields refer to: the reagents used that are not (persistent, bio-accumulative, and toxic), PBT, (field 1), not hazardous (field 2), noncorrosive (field 3), and the overall amount of waste generated does not exceed 50 g (field 4) [31]. **Fig. 5** shows the NEMI diagram of the proposed methods where the inclusion of THF as a solvent for B-CAR contributed to the non-obedience to fields 1 and 2. For comparison, selected reports describing the spectrophotometric determination of the selected vitamins in pharmaceutical preparations were used. The method selected for VIT C determination [15] was based on the reaction of VIT C with 2, 4-dichloroaniline forming a colored azodye with the use of sodium hydroxide and hydrochloric acid during the practical procedure rendering it corrosive, PBT, and

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hazardous with failure to compliance to the three corresponding fields. For VIT E, the reference method [18] depends on the colorimetric determination of the vitamin in its multivitamin capsules depending on the reduction potential of VIT E with tetrazolium blue following extracting the vitamin with petroleum ether from aqueous EDTA medium. The method also used sulphuric acid and sodium hydroxide with the application of heat failing compliance to the three fields, corrosive, PBT, and hazardous. However, the method selected for B-CAR was based on its determination in mixture tablets using third derivative spectrophotometry using methanolic hydrochloric acid as a solvent and diluent [24]. This imparts two failure fields, corrosive and PBT. As a result, the NEMI evaluation of our proposed spectrophotometric methods revealed that they had the same, or even superior degree of greenness, compared with the reference methods.

Although NEMI tool is considered simple and easy to apply and understand, it only provides a qualitative measure of the methods' greenness. More detailed tools were then developed including GAPI and AGREE. GAPI expresses the method's greenness in the form of five pentagons, each of which assesses the risk potential of a particular category, sample preservation (zones 1 to 4, pentagon 1), sample preparation (zones 6 to 8, pentagon 2), reagents used (zones 9 to 11, pentagon 3), and instrumentation (zones 12 to 14, pentagon 4), in addition to the central pentagon (zone 4) which gives a general picture of the whole method's greenness used for both qualitative and quantitative analysis. The degree of compliance of the methods under investigation is colored green, yellow, or red depending on the degree of fulfillment of the particular criterion [32]. Investigation of GAPI pictogram for the proposed spectrophotometric methods in comparison with the reference methods revealed a superior degree of methods' greenness, **Fig. 5**, since its polygon showed four red zones compared with the five zones for B-CAR [24], seven zones for VIT C [15], and eight zones including failure of the overall method's performance for VIT E [18]. The four red zones in the proposed methods referred to the use of a large amount of solvents for extraction, macro-extraction (zone 6), the use of THF in the solvent system which is considered nongreen solvent (zone 7) with safety hazard (zone 11) and lack of waste treatment (zone 15).

Lastly, the degree of the methods' greenness was evaluated using the AGREE tool which has been recently introduced to assess the compliance of analytical methodologies to the twelve principles of GAC. This tool represents the results of the evaluation in the form of a twelve-sectored polygraph using the green, yellow, and red colors with the green color being the best. The overall evaluation of the method's greenness was presented by a central value, the closest to unity, the greenest the method is [29, 30]. By analogy, comparing the proposed methods with the reference ones revealed the superiority of the former compared with the latter, 0.78 compared with 0.65 for B-CAR, 0.58 for VIT C, and 0.52 for VIT E, **Fig. 5**.

Finally, the use of the three metrics NEMI, GAPI, AGREE for assessing the method's greenness provided confirmatory proof of the superiority of the proposed spectrophotometric methods compared with the selected reference spectrophotometric methods for the vitamins analysis [15, 18, 24].

Recently, the concept of WAC has been introduced into the field of analytical methods evaluation. As per GAC, WAC considers 12 basic principles that describe the analytical performance, given a red color, practical issues, given a blue color, besides the method greenness, given a green color. The analytical efficiency of the developed

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methods is related to the validation parameters including accuracy, precision, linearity, limits of detection and quantitation, etc. Practical issues cover the applicability and economic factors. Finally, the method's greenness expresses the obedience of the method to the 12 GAC principles. Accordingly, this Red-Green-Blue (RGB) model uses colors to present the compliance of the investigated methods to the three pillars to be considered during the method's development. The saturation of these three primary colors results in the formation of the white color [33]. Recently, this algorithm was simply applied using Excel software which was used in our study to evaluate the whiteness of the proposed spectrophotometric methods in comparison with the selected reference methods [15, 18, 24]. **Fig. 6** illustrated that the whiteness of the proposed spectrophotometric methods was better than that of the reference methods for the three vitamins, whiteness % of 85.7% followed by the method for B-CAR determination [24] with a score of 82.4%, then VIT E determination [18] showing 78.2% whiteness score, and at last the method for VIT C determination [15] with 74.0% score. Concerning the single components of the methods' whiteness, analytical validation (%R) of the proposed methods was better than that of the reference methods [15, 18, 24], method greenness (%G) was the same as the third derivative method reported for B-CAR determination [24] while better than that described for VIT E (18) and VIT C [15] determination, and finally the degree of method's applicability (%B) which also had the same value as that for B-CAR determination [24] while higher than for VIT E [18] and VIT C [15] determination.

In conclusion, during the phase of development of analytical methods, not only method validation is important, but also methods' greenness and applicability/cost-effectiveness are of major concern, an important concept covered by calculating the method's whiteness.

4. Conclusions

To our knowledge, no report has been found in the literature so far dealing with the simultaneous determination of VIT C, VIT E, and B-CAR spectrophotometrically. This work introduced the first time-simple spectrophotometric procedures for the determination of VIT C, VIT E, and B-CAR in their ternary mixtures and mixture tablets providing no interference of one another. Direct absorbance at 450 nm was used for the determination of B-CAR while derivative absorption spectra were used for the determination of the other two drugs, ^{1}D signals (peak to peak) at 252 nm, 277 nm for VIT C and ^{2}D signals (peak to zero) at 212 nm for VIT E.

Methods' greenness was assessed using the following metrics, NEMI, GAPI, and AGREE giving a complementary picture of the superior degree of greenness of the proposed spectrophotometric methods compared with reference methods [15, 18, 24]. Moreover, the overall evaluation of the proposed methods was performed using (RGB) method for whiteness assessment showing a high degree of method validation, greenness, and applicability/cost. Therefore, the proposed spectrophotometric methods can be recommended as validated, eco-friendly, and cost-effective methods for routine analysis of the three drugs in their multivitamin preparations.

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

Data will be made available on request.

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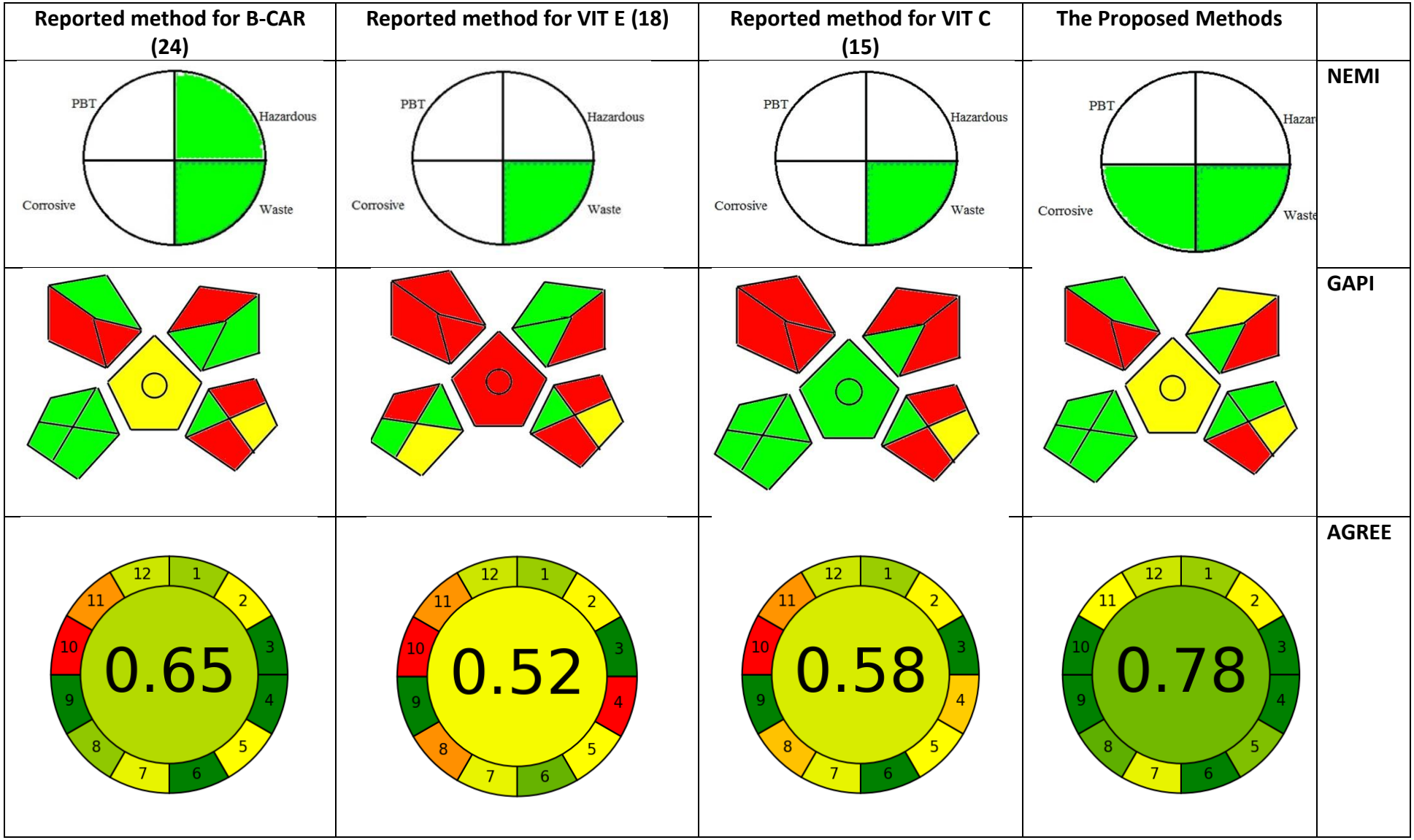

Fig. 5: Schematic presentation of the proposed spectrophotometric methods greenness in comparison with the reference method for VIT C (15), VIT E (18), and B-CAR (24), using NEMI, GAPI, and AGREE metrics

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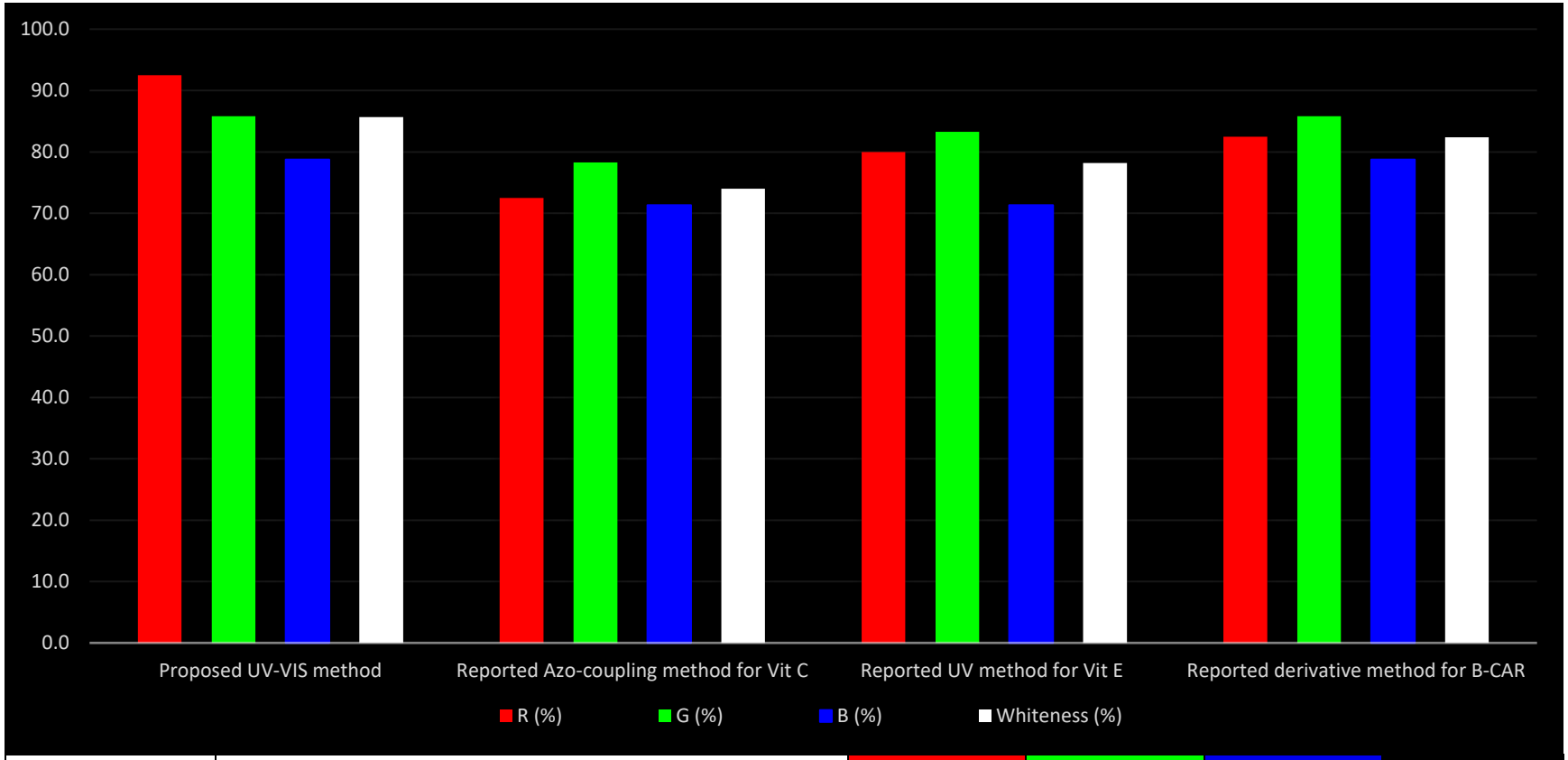

Method number	Method name	R (%)	G (%)	B (%)	Whiteness (%)
1	Proposed UV-VIS methods	92.5	85.8	78.8	85.7
2	Reported Azo-coupling method for Vit C (15)	72.5	78.3	71.3	74.0
3	Reported Spectrophotometric method for Vit E (18)	80.0	83.3	71.3	78.2
4	Reported derivative method for B-CAR (24)	82.5	85.8	78.8	82.4

Fig. 6: Presentation of the proposed spectrophotometric methods whiteness in comparison with the reference method for VIT C (15), VIT E (18), and B-CAR (24), using (RGB) model, along with the underlying detailed table

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Authorship Contribution Statement

Heba K. Ashour: Conceptualization, Methodology, Writing - review and editing, Supervision.

Hadir M. Maher: Conceptualization, Methodology, Data curation, Formal analysis, Validation, Writing – original draft, Supervision.

Shaza M. Kamel: Conceptualization, Methodology, Data analysis – writing – original draft.

Fawzy El-Yazbi: Conceptualization – Writing – review and editing, Supervision.

Conflict of Interest

Declared none.

Highlights

- Direct absorbance and derivative spectrophotometric methods are developed to quantify vitamin C, Vitamin E, and β Carotene, simultaneously.
- The methods have been successfully applied to laboratory-prepared mixtures.
- The Greenness and whiteness of the spectrophotometric methods have been confirmed using NEMI, GAPI, and AGREE metrics.

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