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ARTICLE

A Green and Lean Method Certified by NEMI, ESA, AGREE, GAPI and BAGI for the Analysis of Ivermectin in Injection Solution for Veterinary Use

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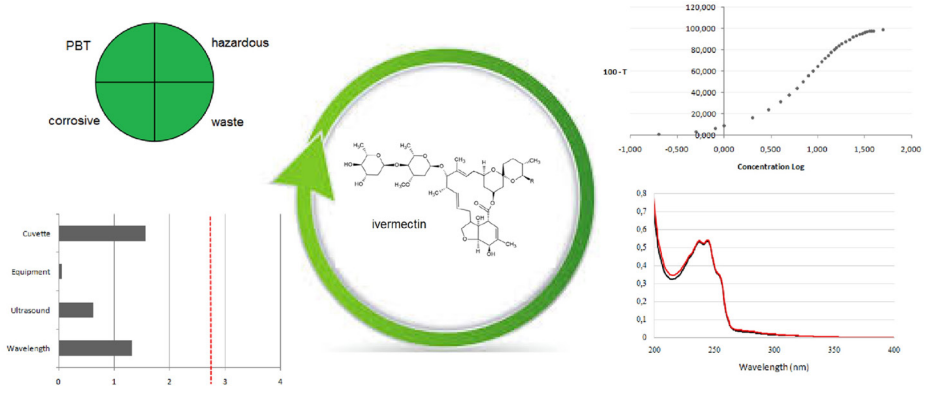

The use of antiparasitics, such as ivermectin (IVE), is extremely important to public health and the economy. Quality control and analytical development are necessary to guarantee the efficacy, safety, and quality of medicines. This work covered the development and validation of a green and lean method by UV to quantify IVE in injection solution for veterinary use. UV methodology using ethanol as a diluent, a quartz cuvette and a spectrophotometer at 245 nm were used. In order to bring objectivity in relation to the greenness of the proposed method, 5 tools were used: National Environmental Method Index (NEMI), Eco-Scale Assessment (ESA), Analytical GREEnness Metric (AGREE), Green Analytical Procedure Index (GAPI), and Blue Applicability Grade Index (BAGI). The proposed method was linear in the range of 6-16 μg mL^{-1}, precise (RSD < 5%), selective and indicative of stability by forced degradation, exact (100.07%) and robust against small and deliberate modifications. NEMI showed the 4 green quadrants, GAPI showed predominantly green and yellow quadrants, ESA, AGREE and BAGI showed scores of 93, 0.82 and 65, respectively. The method is an excellent and lean green option for evaluating final IVE product. It has an environmentally friendly footprint, which can be advantageously employed by pharmaceutical chemical laboratories worldwide.

Keywords: National Environmental Method Index, Eco-Scale Assessment, Analytical GREEnness Metric, Green Analytical Procedure Index, Blue Applicability Grade Index

INTRODUCTION

Ivermectin (IVE) is a macrocyclic lactone used as an anthelmintic in human and animal health. It is a semi-synthetic compound of > 80% IVE B1a ($C_{48}H_{74}O_{14}$) and < 20% IVE B1b ($C_{47}H_{72}O_{14}$) and is sold in pharmaceutical dosage forms such as tablets, pastes, and injectable solutions.[1-3] The fact that parasites cause harm to public health and the economy makes the development of analytical methods fundamental.

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They are indispensable for guaranteeing the effectiveness, safety, and quality of medicines, avoiding cases of resistance, residues in food, allergies, and any negative impacts on human, animal, and ecosystem health.[4-5]

However, just the existence of the analytical method is not enough; currently, the need is for the method to also be clean, green and optimized, based on the fundamentals of Green Analytical Chemistry (GAC).[6-16] This context brings objectivity regarding to the greenness of the methods, tools such as National Environmental Method Index (NEMI), Eco-Scale Assessment (ESA), Analytical GREEnness Metric (AGREE), Green Analytical Procedure Index (GAPI), Blue Applicability Grade Index (BAGI) are used.

NEMI is a graphical representation of a circle divided into four parts, which are categorized. The first corresponds to no products belonging to the list of persistent, bioaccumulative and toxic chemicals (PBT). The second corresponds to no product being on the hazardous waste list. The third corresponds to the pH of the sample being between 2 and 12. The fourth corresponds to the amount of waste generated being less than 50 g. The ESA is a penalizing tool. Thus, penalty points (PP) are reduced from a base of 100. Consequently, the closer the final score is to 100, the more sustainable the analytical method is considered. Scores >75 indicate excellent green analyses, > 50 points mean an acceptable green analysis, and <50 points are considered an inappropriate green analysis. The AGREE tool considers the 12 principles of GAC. Each principle has a score that ranges from orange to green. The result ranges from 0 to 1, and 1 represents the dark green color, i.e. high environmental performance. GAPI takes into account the entire analytical procedure, from sample collection to waste disposal. This tool uses a color scheme, in which there are two or three levels for evaluating the steps. There is an evaluation of reagents, procedures and instrumentation. Five staves are divided into 15 parts, which each part corresponds to a specific parameter. Additionally, a color-coding system, green (low), yellow (medium) and red (high), is used to represent the impact of the analyses. In addition, the central staves indicate the type of method, quantitative (circle present in the center of the pentagon) or qualitative (no circle in the center of the pentagon). BAGI assesses the practicality of the method. It belongs to White Analytical Chemistry (WAC) and is regarded as a complement to sustainable tools. It is represented by an asteroid, which the bluer it is, the more practical and easier the method is. The score can be between 25 and 100, where 25 corresponds to a worse performance of the method in relation to practicality and 100 represents excellent performance. Thus, the method is considered practical when a minimum value of 60 points is assigned.[17-23]

This study aims to develop and validate an ecological and lean method by UV for quantifying IVE in injection solution for veterinary application. Moreover, the NEMI, ESA, AGREE, GAPI, and BAGI tools will be used to evaluate the method's greenness.

MATERIALS AND METHODS

IVE standard with content of 99.8% for H2B1a/(H2B1a+H2B1b) and 96.1% for H2B1a + H2B1b. The sample used was an injection solution (50 mL) with a declared content of 1%. The raw material and sample were donated by Noxon®. Absolute ethyl alcohol (Sciavicco®) and purified water (Gehaka®) were used to prepare the solutions.

Equipment

Spectrophotometer model Genesys 10S UV-Vis (Thermo Scientific®), ultrasound (Unique®), water ultrapurifier (Gehaka®), analytical balance model AUW22OD (Shimadzu®), 10 mm quartz cuvettes with 4 mL capacity (Qualividros®), heating bath and UV light chamber were used.

Stock and work solution preparation

Stock solutions were prepared with the equivalent of 2.5 mg of IVE standard, which was transferred to a 50 mL volumetric flask. Then, a small amount of ethanol was added and taken to ultrasound for 15 minutes and then the volumes were completed with ethanol, obtaining a concentration of 50 μg mL^{-1}.

The working solutions were prepared from the stock solution, so that 6 concentrations, namely 6, 8, 10, 12, 14, 16 μg mL^{-1}, were achieved. The aliquots were transferred to a 5 mL volumetric flask and the volumes were completed with purified water.

Ringbom curve

The Ringbom curve was determined at 245 nm using 33 standard IVE concentrations ranging from 0.2 to 50 μg mL^{-1}, in order to define the linear region and thus proceed with the validation of the method. The analyses were performed in triplicate.

Validation parameters

The international guidelines were followed to validate the proposed analytical method, considering parameters such as linearity, precision, selectivity, accuracy and robustness.[24-31]

Linearity

The linearity of the proposed method was proven through statistical analyzes (straight line equation, least squares, correlation coefficient, analysis of variance – ANOVA and residual graph) of the results from concentrations 6, 8, 10, 12, 14 and 16 μg mL^{-1}.

Precision

Precision was evaluated at a concentration of 12 μg mL^{-1} regarding the proximity of the results at three different levels: intraday, interday, and interanalyst.

Intraday precision evaluated the proximity of results obtained on the same day and with the same analyst. Interday precision evaluated results acquired by the same analyst on different days. Finally, inter-analyst precision evaluated the proximity of results obtained by different analysts on different days.

The precision of the proposed method was established through the dispersion of results, based on the relative standard deviation (DPR %).

Selectivity

The selectivity of the proposed method was proven through comparison of the spectra of the sample and standard solutions at a concentration of 12 μg mL^{-1}, and also by the forced degradation method.

The forced degradation study or stress test for IVE in injection solution occurred at a concentration of 15 μg mL^{-1} under stress conditions: acidic (HCl 0.1 M for 2 hours at 60 °C), basic (NaOH 0.01 M for 1 hour at 60 °C), neutral (diluent for 1 hour at 60 °C) and photolytic (UV light for 24 hours at room temperature).

Accuracy

The accuracy of the proposed method was evaluated by standard recovery test, in which standard solutions are added to the sample solution (6 μg mL^{-1}) and then analyzed. Three levels were used: recovery 1 to 80% (8 μg mL^{-1}), recovery 2 to 100% (10 μg mL^{-1}) and recovery 3 to 120% (12 μg mL^{-1}). Accuracy was assessed in triplicate on three distinct days.

Robustness

The robustness of the proposed method was verified by modifications in the reading wavelength (245 nm - normal x 243 nm - modified), ultrasound time (15 minutes - normal x 10 minutes - modified), equipment (Thermo Scientific®, Genesys 10S UV-Vis - normal x Biospectro® SP-220 - modified) and cuvette capacity (4 mL - normal x 1 mL - modified).

The working solutions were prepared at a concentration of 12 μg mL^{-1} in triplicate. Variations were evaluated using the F test and *t* test.

Content analysis

Standard and sample solutions were prepared at a concentration of 12 μg mL^{-1}. Readings were taken at a wavelength of 245 nm through 6 replicates and over three days. The analysis result must comply with official compendia for IVE in injection solution.

National Environmental Method Index (NEMI)

NEMI issues (a) persistent, bio-accumulative and toxic (PBT); (b) hazardous; (c) corrosive and (d) waste were evaluated. The result was presented through the presence or absence of the color green in 4 quadrants, each representing the parameters mentioned above.

Eco-Scale Assessment (ESA)

The penalty points (PP) were calculated according to the Equation 1.

$$ESA = 100 - \left[\begin{matrix}(\text{chemical reagents pictogram x quantity of reagents x signal words}) + \\ \text{energy} + \text{ocupacional hazard} + (\text{waste amount x waste characteristic})\end{matrix}\right] \quad \text{Equation 1}$$

Analytical GREEnness Metric (AGREE)

The analytical conditions of the proposed method were evaluated against the 12 GAC principles and the data was included in the AGREE calculator.

Green Analytical Procedure Index (GAPI)

In addition to the analytical choices, the processes involved and the instrumentation were evaluated and measured by GAPI.

Blue Applicability Grade Index (BAGI)

BAGI evaluated the 10 characteristics of the proposed method, namely: type of analysis, number of analytes evaluated simultaneously, instrumentation and analytical technique, number of samples which can be analyzed simultaneously, sample preparation, number of samples analyzed per hour, type of reagent and materials, preconcentration requirement, degree of automation and sample quantity.

RESULTS AND DISCUSSION

Ringbom curve

The Ringbom curve presents the compatible linear region for the validation stage (Figure 1A). In this case, the points chosen were 6, 8, 10, 12, 14 and 16 μg mL^{-1}.

Linearity

The correlation coefficient was 0.9999, therefore, it is a value higher than 0.99 as recommended in the guides.[26,28] The linear regression was significant, while the linearity deviation was not, which corroborates the linearity of the method (Table I). Furthermore, the residual graph shows that the points are distributed randomly without a trend (Figure 1B).

Table I. ANOVA results to evaluate the linearity of the method

Parameters	Value
Wavelength (nm)	245
Linearity range (μg mL^{-1})	6-16
Slope	0.0503
Intercept	0.0557
Correlation coefficient (r)	0.9999
Regression	1994.09* (4.75)
Lack of fit	0.30 (3.26)

*Value p< 0.05

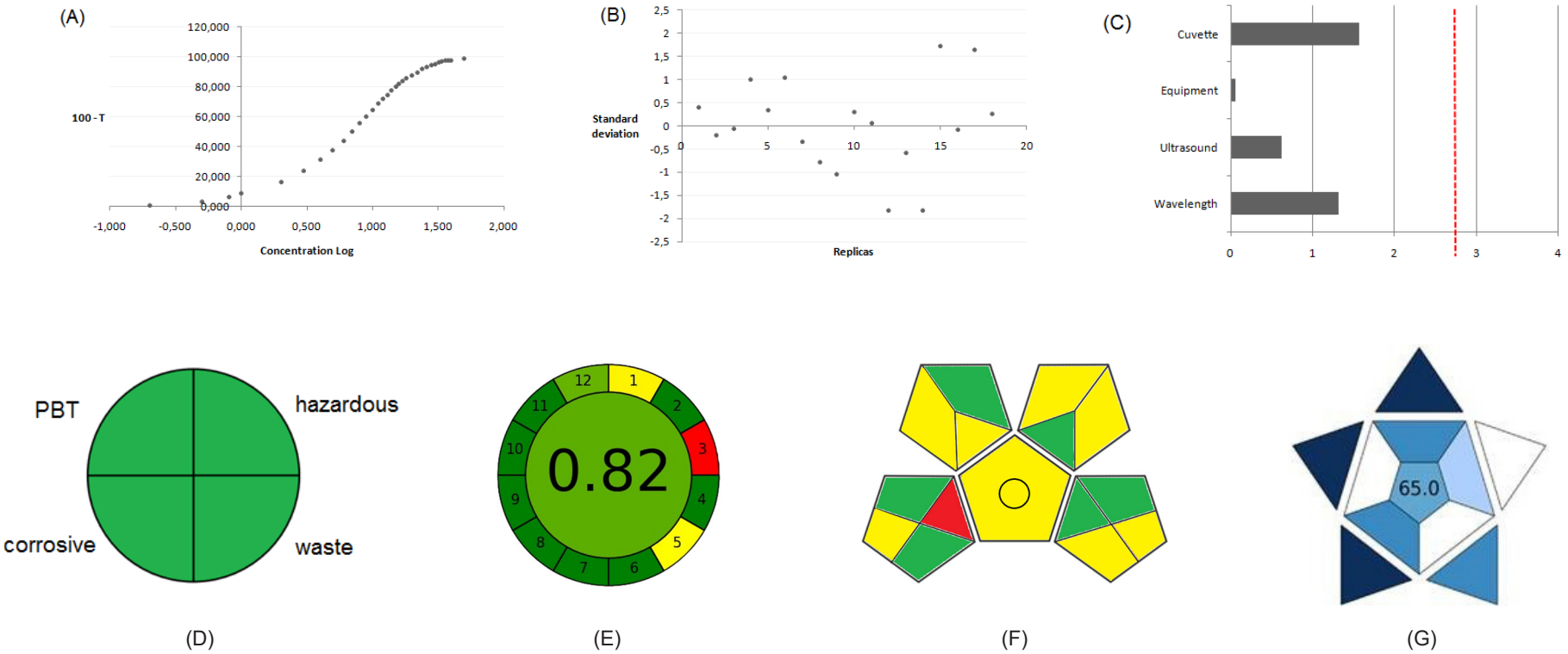


Figure 1. (A) Ringbom curve, (B) residue graphical, (C) effect of robustness modifications, (D) NEMI, (E) AGREE, (F) GAPI, and (G) BAGI results.

Precision

The RSD (%) of the evaluated precision levels were smaller than 5%, thus demonstrating the proposed method´s precision (Table II).

Table II. Absorbance results to evaluate the precision of the method

Wavelength	Level	Absorbance						RDS (%)
		1	2	3	4	5	6	
245 nm	Intraday	0.529	0.525	0.534	0.536	0.529	0.527	0.79
	Interday	0.545	0.554	0.547	0.538	0.555	0.545	1.34
		0.538	0.537	0.531	0.536	0.542	0.539	
	Interanalyst	0.538	0.537	0.531	0.536	0.542	0.539	0.99
		0.529	0.525	0.534	0.536	0.529	0.527	

Selectivity

The overlap of standard and sample IVE spectra (Figure 2) shows the method's ability to identify IVE in the injection solution, since the presence of adjuvants did not reveal interference.

Furthermore, selectivity was proven through forced degradation. In the development and validation of methods, the stress test is an indication of stability for the method. It is important to highlight that each formulation presents different stress rates and conditions. Therefore, comparison of stress test results should be cautious; the way the pharmaceutical product was exposed (powder or solution), for how long and at what temperature are decisive for the comparison. The conditions chosen were strategically designed so that there was neither excessive nor insufficient degradation (Table III). Exacerbated stress can result in inappropriate conditions and endpoints, which increases the chances of generating degradation products of no interest for study, that is, degradation products that do not correspond to reality. On the other hand, ineffective stress generates insufficient results, providing false methods indicative of stability.[27,29-30] The objective of the present work was to show that the proposed method is indicative of stability, as in all stress conditions tested the method was able to attest to the degradation of IVE due to the change in the absorption profile (Figure 2).

Table III. Degradation values obtained in the stress test for IVE in injection solution

Degradation condition	Absorbance (time 0)	Absorbance (1*, 2**, 24*** hours)	Degradation (%)
Acidic (HCl 0.1 M at 80 °C)	0.615	0.577**	6.18
Neutral (ethanol at 60 °C)	0.860	0.747**	13.14
Basic (0.01 M NaOH at 60 °C)	0.586	0.520*	11.26
Photolytic (UV at 254 nm)	0.783	0.699***	10.73

Accuracy

The average recovery obtained on different days and in triplicate was 100.07% (Table IV). The value obtained in the recovery test is within the specification range for pharmaceutical analysis, which is 98 to 102%, therefore, the proposed method is accurate.

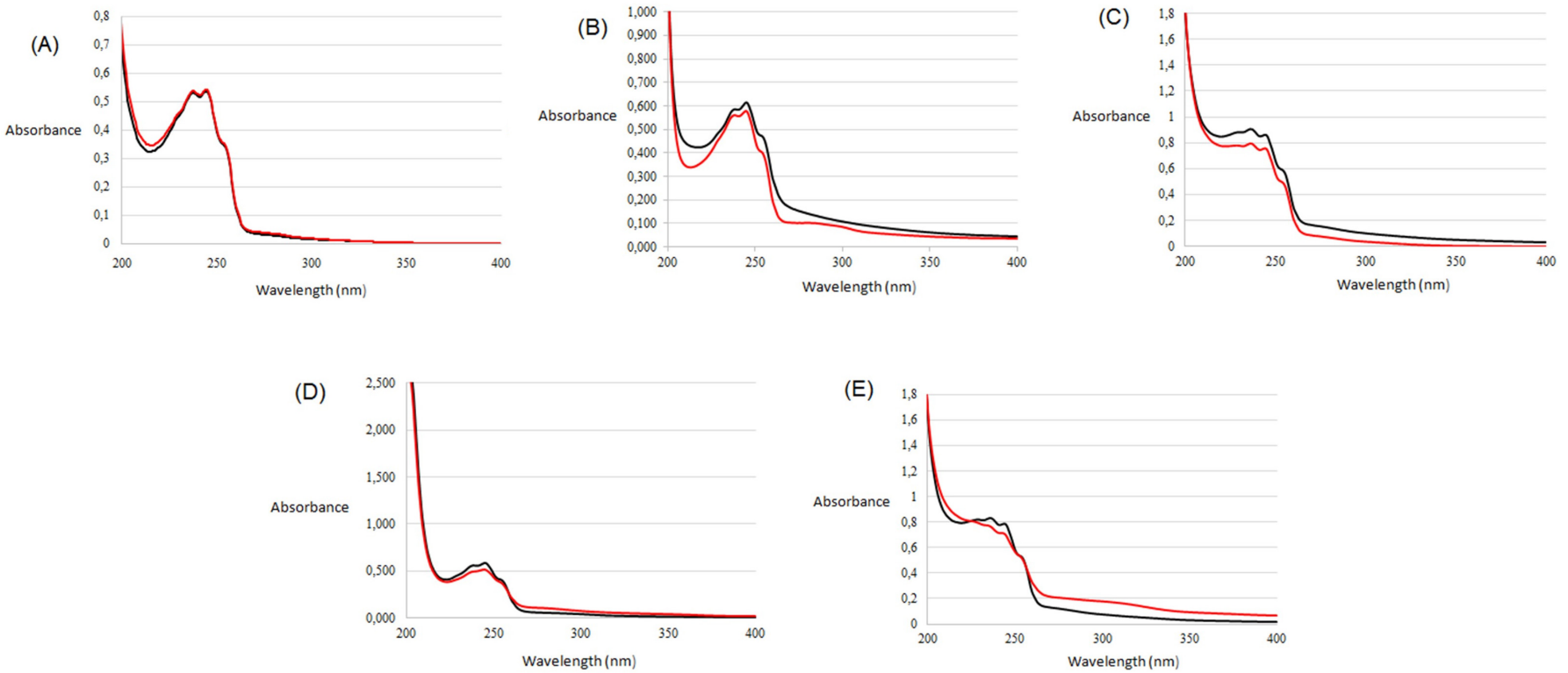


Figure 2. (A) Overlay of the spectra of the standard (black) and sample (red) IVE solutions at 12 μg mL^{-1} and 245 nm. From (B) to (E): absorption spectra profiles from the forced degradation test under the following conditions – (B) acidic, at 0 h (black) and 2 h (red); (C) neutral, at 0 h (black) and 1 h (red); (D) basic, at 0 h (black) and 1 h (red); (E) photolytic, at 0 h (black) and 24 h (red). All (B) to (E) spectra were recorded at 15 μg mL^{-1} and 245 nm.

Table IV. Accuracy results of the proposed method based on the recovery test

	IVE standard added (μg mL^{-1})	IVE standard recovered (μg mL^{-1})	Recovery* (%)	Mean recovery (%)	RSD (%)
R1	2.0	1.98	98.78		
R2	4.0	4.00	100.07	100.07	0.06
R3	6.0	6.08	101.35		

*Average of 3 determinations in triplicate

Robustness

There were no statistically significant differences for changes in wavelength, equipment, ultrasound time and cuvette capacity (Figure 1C). Thus, the proposed method is robust to such deliberate changes, that is, the $t_{calculated}$ was smaller than the $t_{tabulated}$ (2.78), which reveals that such changes do not impact the proposed method.

Content analysis

According to the American Pharmacopoeia,[29] the IVE content in the final product (injection solution) must present a lower value of 95% and an upper value of 105%, thus the value found (101.30%) using the proposed method meets specifications (Table V).

Table V. Results of the content assessment of IVE in injection solution by the proposed method

Day	Average content* (%)	Final content (%)	RSD (%)
1	101.73		
2	100.56	101.30	0.63
3	101.60		

*Average of 3 determinations in triplicate

National Environmental Method Index (NEMI)

NEMI evaluates four different parameters. The proposed method uses only ethanol, which is non-persistent, bioaccumulative, toxic, and dangerous. Therefore, it is not on the TRI and EPA list. In relation to the corrosive quadrant, the pH of the solution was 7, thus, the pH is in the range of 2 to 12. Furthermore, the residue per sample corresponds to 5 mL, being less than 50 mL. Therefore, the proposed method presents the 4 quadrants in green, as shown in Figure 1D.

Eco-Scale Assessment (ESA)

Assessment by ESA is based on PP, as shown in Equation 1. Therefore, in relation to the reagents, the quantity used is less than 10 mL (1 PP), the ethanol label contains two pictograms and the signal word 'Danger' (4 PP). Regarding the instrument, the spectrophotometer consumes energy <0.1 kWh per sample (0 PP). In relation to occupational risk, the analytical process is hermetized. Waste generation was 5 mL per sample (3 PP) and treatment is by degradation (1 PP).

Point count using Equation 1: ESA = 100 - [(1x4) + (0) + (0) + (3x1)] = 100 - 7 = 93

The analysis presented an ESA value of 93, therefore, the proposed method is indicated as an excellent green analysis.

Analytical GREEnness Metric (AGREE)

AGREE showed a score of 0.81 and a green color (Figure 1E), so with a value close to 1, the proposed method is considered green.

Green Analytical Procedure Index (GAPI)

GAPI is a semi-quantitative tool that evaluates processes from start to finish, so the pentagrams for the proposed method were filled mostly in green and yellow, that is, the proposed method has a low-medium environmental impact, as shown in Figure 1F.

Blue Applicability Grade Index (BAGI)

BAGI demonstrated that the proposed method is practical, since the score was 65 with the asteroid predominantly blue, as shown in Figure 1G.

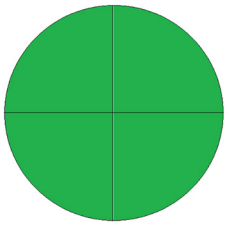
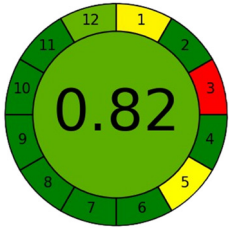
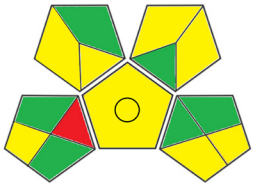
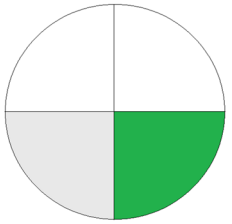
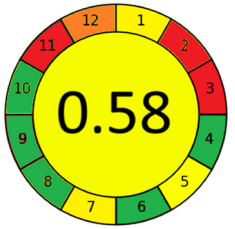
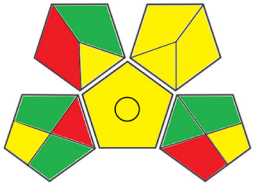
Environmental impact assessment of the proposed method

Considering the objective of routine analysis in the quality control of final IVE products, spectrophotometry is a fast, simple, easy-to-use, and economical technique that requires less solvent and generates less waste than high-performance liquid chromatography, for example. Furthermore, it offers greater sensitivity.[32-33]

Table VI compares the proposed method with a previously reported method for analyzing injectable IVE solutions. The proposed method employs ethanol as a diluent. Ethanol is both less toxic and more economical, not only in terms of purchase cost, but also in disposal. In contrast, methanol is metabolized into formaldehyde and formic acid, which are causing serious poisoning. Similarly, acetonitrile can yield cyanide upon metabolism, which causes respiratory toxicity.[10,35] The traditional method for evaluating IVE in injectable solution, as described in the USP, is HPLC. The mobile phase consists of acetonitrile, methanol, and purified water (106:55:39, v/v/v), using a 250 x 4.6 mm column with a flow rate of 1.5 mL min^{-1} and injection volume of 20 µL.[29]

Therefore, the proposed method offers advantages over both the literature method and the conventional method, the solvent used is considered green, it generates less waste, and it requires a lower concentration of the stock solution. Furthermore, compared to the traditional method, it is faster and economical. Overall, based on the tools employed, the method can be considered more eco-efficient than existing methods and innovative for evaluating IVE in injectable solution.

Table VI. Comparison of the proposed method with another method from the literature

Method	Diluent	Stock solution (concentration used)	Work solution (volume used)	Greeneess profile				
				NEMI	ESA	AGREE	GAPI	BAGI
UV*	Ethanol	50 μg mL^{-1}	5 mL		93			
UV[34]	Methanol or acetonitrile	100 mg L^{-1}	10 mL		79			

*Proposed method

CONCLUSIONS

Current scientific literature and chemical-pharmaceutical laboratories advance with the proposed green and lean method. It is linear (6-16 μg mL^{-1}), selective, precise (RSD < 5%), exact (100.07%) and robust. In addition to being indicative of stability and an excellent green option by ESA, NEMI, AGREE, GAPI and BAGI to evaluate IVE in injection solution for veterinary use.

Conflicts of interest

The authors declare that they have no conflict of interest.

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