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Development of an AQbD-Driven Ultra-Fast and Sustainable UPLC Method for Simultaneous Determination of Aztreonam and Avibactam: Validation and Multi-Metric Greenness Assessment

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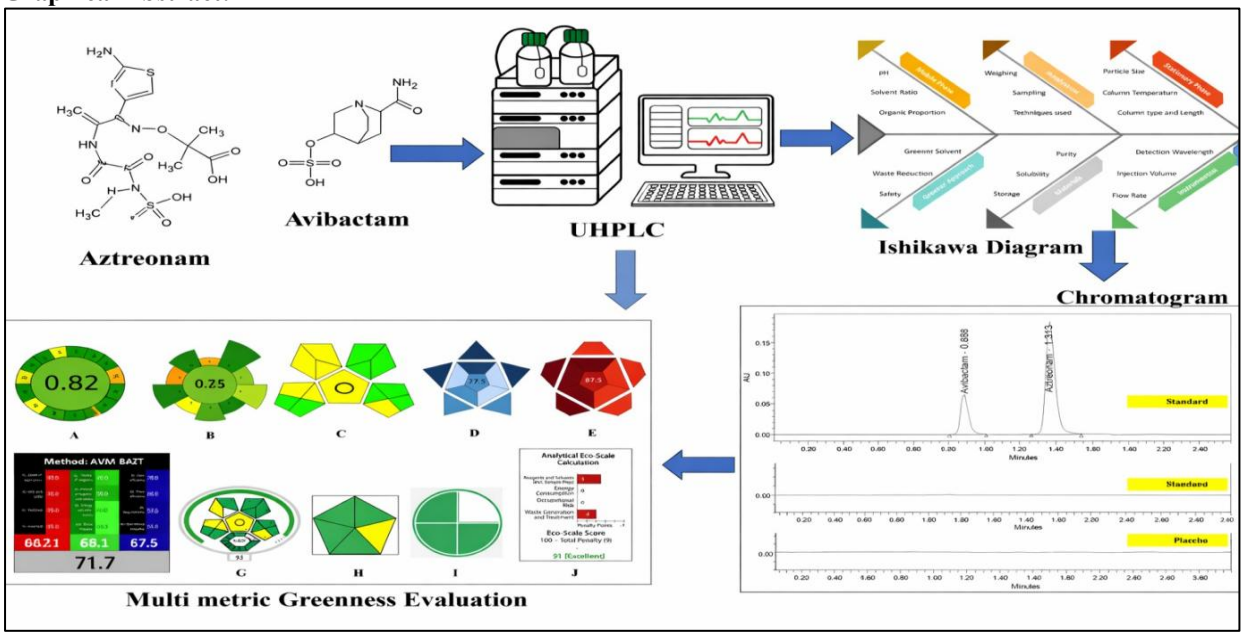
ABSTRACT
Managing multidrug-resistant Gram-negative infections requires combining Aztreonam (AZT) and Avibactam (AVB). However, simultaneous quantification is hindered by their divergent properties, often requiring high-waste chromatography or costly mass spectrometry. This study established a rapid, sustainable Ultra-Performance Liquid Chromatography (UPLC) method for their simultaneous estimation using an Analytical Quality by Design (AQbD) approach. Guided by ICH Q14, a Central Composite Design optimized chromatographic parameters to define a robust Method Operable Design Region following an initial Ishikawa risk assessment. The procedure was fully validated per ICH Q2(R2) standards. Environmental sustainability was quantified via the Analytical Eco-Scale, AGREE, GAPI, NEMI, and White Analytical Chemistry (WAC) frameworks. Sharp baseline separation was achieved within 3.0 minutes on a CHS C18 column (100 mm × 2.1 mm, 1.8 µm) using a methanol-phosphate buffer (40:60, v/v) mobile phase at a micro-flow rate of 0.3 mL/min. The method showed perfect desirability (1.000), linearity (R2 > 0.999), and quantitative accuracy. Generating just 0.9 mL of organic effluent, it scored 91 on the Eco-Scale, 0.82 on AGREE, and 71.7 on WAC. This validated UPLC method minimizes environmental impact while maintaining high analytical capability, offering an efficient, eco-friendly platform for routine quality control and therapeutic drug monitoring.

Graphical Abstract:



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1. INTRODUCTION:

The alarming proliferation of multidrug-resistant (MDR) Gram-negative bacterial pathogens constitutes one of the most formidable challenges to modern global public health [1]. Strains producing metallo-β-lactamases (MBLs) are particularly catastrophic, as they possess the enzymatic capacity to hydrolyze nearly all commercially available β-lactam antibiotics, resulting in high clinical treatment failure rates[2]. To combat these aggressive infections, the concurrent clinical administration of Aztreonam (AZT), a potent monobactam antibiotic resistant to MBL hydrolysis, and Avibactam (AVB), a highly innovative non-β-lactam β-lactamase inhibitor, has emerged as a paramount therapeutic strategy[2,3]. The distinct chemical architectures of these active ingredients, which dictate their divergent physicochemical behaviors, are illustrated in Fig. (1).

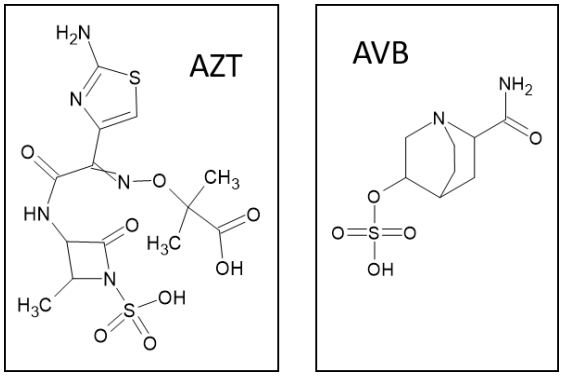

Figure 1 Chemical Structures of AZT and AVB respectively

Despite undeniable clinical success, the routine quantitative analysis and therapeutic drug monitoring (TDM) of the AZT/AVB combination presents a profound analytical hurdle [4]. AZT and AVB possess fundamentally contrasting polarities, solubilities, and chromatographic retention dynamics. AVB is an extremely polar molecule lacking a strong ultraviolet chromophore, making robust column retention difficult, whereas AZT exhibits entirely distinct elution requirements [4]. Consequently, legacy analytical methods in the public domain are heavily fragmented. Existing literature relies either on highly complex, expensive ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) platforms that require multiple sequential analytical runs [4], or conventional reverse-phase HPLC procedures [5–7]. These conventional HPLC methods suffer from severe operational limitations, including prolonged run times (>10 to 20 minutes) and the massive consumption of highly toxic, hazardous organic solvents like acetonitrile [5,6].

Rationale, Motivation, and Regulatory Need:

The primary motivation for this research stems from the urgent practical need to democratize the analysis of the AZT/AVB combination. High-resolution LC-MS/MS instrumentation is economically prohibitive and technically complex for standard pharmaceutical quality control laboratories and regional hospital settings[4]. Furthermore, the continued heavy usage of toxic acetonitrile in routine HPLC contradicts global environmental, social, and governance (ESG) mandates [11]. Concurrently, the international regulatory landscape is undergoing a massive conceptual evolution. The recently finalized ICH Q14 guideline for Analytical Procedure Development explicitly discourages empirical, unoptimized trial-and-error method development[8]. Instead, it strongly mandates an "Enhanced Approach" rooted in Analytical Quality by Design (AQbD) principles [8,9]. By utilizing systematic risk assessment and Design of Experiments (DoE), analytical scientists can mathematically model multivariate interactions, define Critical Method Parameters (CMPs), and establish a Method Operable Design Region (MODR) [9]. When coupled with the updated ICH Q2(R2) guideline for method validation [10], this enhanced paradigm guarantees built-in lifecycle robustness, sharply reducing out-of-specification (OOS) investigations during routine commercial use.

Opportunities and Challenges in AQbD Method Development:

Developing an ultra-fast, green UPLC method for AZT and AVB presents distinct scientific opportunities and operational challenges. The primary **opportunity** lies in leveraging chromatographic chemistry (1.8µ particle size) to achieve massive gains in theoretical plate efficiency, allowing for micro-flow rates (**0.3 mL/min**) that drastically curtail chemical waste. Conversely, the major **challenge** involves identifying an isocratic mobile phase that balances the divergent polarities of AZT and AVB without utilizing harsh ion-pairing agents or toxic acetonitrile [4,7]. Furthermore, optimizing the procedure requires precisely balancing organic modifier concentrations to ensure rapid elution while avoiding the ecological penalties associated with solvent flammability and occupational hazard [11, 15].

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Contributions and Objectives of the Present Work:
To bridge these critical gaps, the present investigation establishes the first AQbD-driven, simultaneous UPLC method for the concomitant quantification of AZT and AVB. Moving completely away from hazardous reagents, this study replaces acetonitrile with an ecologically benign methanol-buffer system. Systematic optimization was executed via a Central Composite Design (CCD) under the ICH Q14 framework, followed by comprehensive validation adhering to ICH Q2(R2) criteria. Finally, to demonstrate absolute superiority over existing academic literature, the environmental sustainability and functional utility of the optimized method were rigorously quantified using advanced green and white analytical chemistry frameworks, including NEMI, GAPI, AGREE, the Analytical Eco-Scale, and WAC [11–15].

2. MATERIALS AND METHODS:

2.1 Instrumentation:

All chromatographic assessments were done on a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. The instrument was furnished with quaternary pump, an in-built vacuum degasser, a temperature-controlled column oven, and a TUV detector. The complete system was regulated by Empower 3 software. Analytical separation was achieved using a CHS C18 column (100 mm × 2.1 mm, 1.8 µm particle size). For preliminary spectrophotometric scanning, a Shimadzu UV-1800 double-beam UV-visible spectrophotometer interfaced with UV-Probe software was employed, along with standard 1.0 cm path-length quartz cuvettes. Buffer pH adjustments were monitored using a Sartorius BSA2745-CW pH meter fitted with a sensitive glass electrode. Highly purified water, essential for all analytical preparations, was freshly obtained from a Milli-Q water purification system (Millipore, USA).

2.2. Chemicals and Reagents:

Pure reference standards of aztreonam (AZT) and avibactam (AVB) were used throughout the study. All solvents and chemical reagents, including methanol and ammonium acetate, were of analytical or HPLC grade. Phosphate buffer was prepared according to standard laboratory protocols. All mobile phase components and analytical solutions were prepared with HPLC-grade ultrapure water. Prior to use, the final mobile phase was vacuum-filtered through a 0.45 µm membrane filter and subjected to ultrasonication. This pretreatment confirms complete solubilisation and removal of any dissolved gases.

2.3. Preparation of Solutions:

2.3.1. Preparation of 0.01 M Potassium Dihydrogen Orthophosphate Buffer:

An accurately weighed quantity of 1.36 g of potassium dihydrogen orthophosphate was taken and transferred into a 1000 mL volumetric flask which contains approximately 900 mL of water. The mixture was exposed to ultrasonication to confirm complete solubilisation and degassing, after which the volume was made up to the mark with water. Subsequently, 1.0 mL of triethylamine was added to mixture, and the final pH of the solution was adjusted to 4.0 using a dilute orthophosphoric acid solution.

2.3.2. Preparation of Standard Solutions:

For preparation of the primary standard stock solution, accurately weighed quantities of Aztreonam (AZT) 60 mg and Avibactam (AVB) 60 mg from reference standards were transferred into a specific 50 mL volumetric flask. Starting with, approximately 30ml of the was filled with diluent. The contents were proceeded for ultra-sonication for 10 minutes to complete dissolution, after this the volume was adjusted to the 50 mL mark with the diluent. This procedure produces a combined standard stock solution having concentrations of 1200 µg mL-1 for AZT and 400 µg mL-1 for AVB. To prepare the standard working solution which mimic the 100% target assay concentration, a 1.0 mL of the stock solution was pipetted into a 10 mL volumetric flask and make up to the mark with diluent. This attained final concentrations of 120 µg mL-1 for AZT and 40 µg mL-1 for AVB.

2.3.3. Preparation of Sample Solutions:

For the quantification of the marketed dosage form, the complete contents of one lyophilized injection vial which labelled to contain 1500 mg of AZT and 500 mg of AVB were quantitatively shifted into a 500 mL volumetric flask. Nearly 400 mL of the diluent was added, and the mixture was fully sonicated for 25 minutes to confirm complete extraction of the active pharmaceutical ingredients from the formulation. The flask was then made up to the final volume with the diluent. To remove any undissolved particulate matter, the solution was filtered through a standard HPLC membrane filter, resulting in a sample stock solution producing concentrations of 3000 µg mL-1 for AZT and 1000 µg mL-1 for AVB. After all, to prepare the sample working solution corresponding to the 100% test level, a 0.4 mL volume of the filtered sample stock was transferred into a 10 mL volumetric flask and made up to the mark with the diluent. This provided a final sample concentration of 120 µg mL-1 for AZT and 40 µg mL-1for AVB, similar to the standard working solution.

2.4. Chromatographic conditions:

The optimized method utilised column CHS C18

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(2.1 x 100mm, 1.8µm) using temperature controller set at 30°C and the optimised mobile phase was phosphate buffer and Methanol taken in the ratio 60:40 by applying isocratic elution. The method was optimised using sample volume 1 µL with 0.3ml/min. flow rate and 254 nm detection wavelength which completes the separation in 3 min. runtime.

2.5. Method validation:

The proposed ultra-fast UPLC method was exposed to through validation in strict compliance with the International Council for Harmonisation (ICH) guidelines. The validation protocol systematically estimated critical analytical performance features, including linearity, specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and method robustness. For the carrying out of these validation parameters, particular calibration series were established on the target assay concentrations. The validation parameters were conducted over a concentration range of 30-180 µg mL-1for Aztreonam (AZT) and 10-60 µg mL-1for Avibactam (AVB).

2.6. Selectivity:

To evaluate the selectivity and specificity of the proposed procedure, individual and independent solutions of the blank diluent, standard solutions, and commercial sample preparations were injected into the UPLC system. The generated chromatograms were carefully superimposed and evaluated. This comparative analysis was made to verify the complete resolution of the targeted analytes and confirm absence of any co-eluting endogenous interferences or excipient peaks at the specific retention times of AZT and AVB.

2.7. Initial Risk Assessment and Factor Identification:

In line with AQbD principles, a complete initial risk estimation was done to systematically identify all probable variables that can impact the Critical Method Attributes (CMAs). To simplify this brainstorming activity and imagine the cause-and-effect associations, an Ishikawa (fishbone) diagram was structured Fig. (2). [16] The recognised analytical variables were classified into six key domains: Mobile Phase, Stationary Phase, Instrumental parameters, Materials, Analyst or human factors, and Greener Approach considerations.

Inside these categories, certain sub-factors were mapped. For example, instrumental parameters include flow rate, injection volume, and detection wavelength, while stationary phase variables involve column dimensions, particle size, and temperature. Additionally, highlighting study's commitment to White Analytical Chemistry (WAC) [17], ecological variables such as waste reduction, the utilization of greener solvents, and operational safety were openly incorporated into the risk assessment. This rigorous estimation provides the foundation for confirming the highest-risk parameters exclusively flow rate, organic phase proportion, and column temperature which is to be further assessed and optimized applying the Central Composite Design (CCD).

2.8. Analytical Method Optimization via Experimental Design[8, 18]

To bypass the incompetence and through material consumption related with conventional trial-and-error tactics, systematic method optimization was directed by a randomized Response Surface Methodology (RSM) Fig. (3). specifically, a Central Composite Design (CCD) managed by a quadratic model and consisting 20 experimental runs was used to express the analytical design space. Based on the produced mathematical models, the statistically predicted optimal chromatographic conditions were assessed as a flow rate of 0.30888 mL/min (evaluated range: 0.25-0.33 mL/min), an organic phase composition of 40.00% (evaluated range: 31-48%), and a column oven temperature of 30°C (evaluated range: 25-35°C). For laboratory performance, these parameters were rounded to operable values: a flow rate of 0.3 mL/min, 40% organic phase, and a temperature of 30°C. Upon testing the method under these practical optimized parameters, the retention times for Avibactam (AVB) and Aztreonam (AZT) were recorded at 0.871 min and 1.315 min, respectively, demonstrating unique association with the model's predicted times of 0.9048 min and 1.35 min. Furthermore, the experimental chromatographic resolution was determined to be 5.0, which perfectly matching the predicted value and ensuring complete separation. System efficiency was verified by theoretical plate counts of 2778 for AVB and 3207 for AZT, which closely matches with their respective predicted values of 2678 and 3194. Finally, the optimization yielded a perfect overall desirability function value of 1.000, clearly validating the robustness, reliability, and suitability of the developed UPLC method.

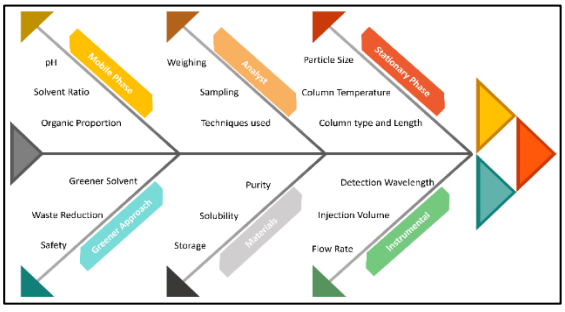

Figure 2 Ishikawa Fishbone Diagram for identifying parameters which affect method development [16]

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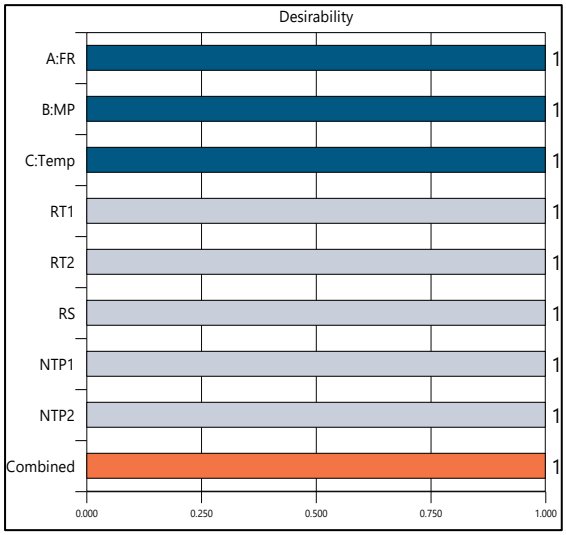

Figure 3 Desirability ramp showing RSM-optimized HPLC conditions

3. RESULT AND DISCUSSION:

3.1. System suitability test:

For confirmation of the optimal performance and reliability of the proposed analytical method before the sample analysis, system suitability parameters were carefully evaluated Table 1. This assessment was conducted by repeating injections of the standard working solution of concentrations 120 µg mL-1 of AZT and 40 µg mL-1of AVB. The obtained chromatograms were analysed for key chromatographic performance indicators, which peak symmetry with tailing factor, theoretical plate count, resolution, and retention time. The obtained results are detailed in Table 2, which demonstrated excellent peak symmetry and complete resolution between the AZT and AVB. Furthermore, the relative standard deviations (%RSD) for both peak areas and retention times among the replicate injections were consistently low and within the limit as per ICH. These outcomes confirm highly stable column efficiency and reproducible system performance, which set up the proposed UPLC method is appropriate for the routine and concomitant determination of AZT and AVB Fig. (4).

Table 1 System suitability parameters for optimized method

Mobile Phase	Phosphate buffer: Methanol (60:40) pH 4.0 by dil. Ortho Phosphoric Acid (OPA)
Stationary Phase	CHS C18 (2.1 x 100mm, 1.8µm)
Wavelength	254 nm
Injection Volume	1 µL
Oven Temperature	30°C
Flow rate	0.3 ml/min
Retention time for AVB	0.871min
Retention time For AZT	1.315min

Table 2 Results for System Suitability

System Suitability for AVB					**System Suitability for AZT**				
Concentration used 40 µg mL-1					**Concentration used 120 µg mL-1**				
Sr. No.	Peak Area	Theoretical Plate	Tailing Factor	Retention Time	Peak Area	Theoretical Plate	Tailing Factor	Retention Time	Resolution
1.	191521	2732	1.33	0.888	693457	3143	1.19	1.313	5.0
2.	191989	2739	1.32	0.888	692856	3140	1.19	1.313	5.0
3.	190004	2755	1.27	0.893	686948	3135	1.18	1.317	5.0
4.	189751	2739	1.27	0.898	687307	3160	1.18	1.321	4.9
5.	191668	2737	1.27	0.898	700452	3158	1.18	1.321	4.9
6.	191966	2725	1.26	0.899	686142	3168	1.18	1.322	4.9
Mean	191150			Mean	691194				
S.D.	1004.6			S.D.	5518.2				
%RSD	0.5			%RSD	0.8				

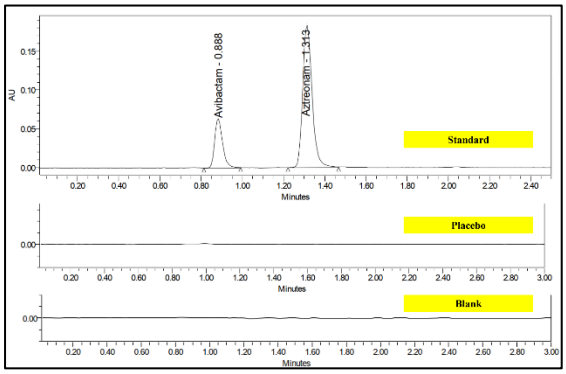

Figure 4 Method is selective and specific as shown in chromatograms of Placebo, Blank and eluted AVB and AZT

3.2. Linearity and Calibration:

The linear analysis of the optimised method was evaluated across the working concentration ranges for both analytes. Calibration curves were drawn by plotting the analytical response in peak area on the y-axis against the corresponding drug concentration in µg/mL on the x-axis, with the calibration data and respective calibration plots presented in Table 3 and Fig. (5). Linear regression analysis of these plots calculated an equation of the line for each drug. For AVB, the slope and y-intercept were calculated which is 4794.2 and 847.93, respectively, come with a regression coefficient (R2) of 0.9998. Similarly, AZT exhibited a slope of 5696.6 and a y-intercept of 5069.3, along with a R2 value of 0.9996. These regression coefficients firmly authenticate a firmly proportional relationship between analyte concentration and detector response, this confirms the outstanding linearity and quantitative reliability

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of the proposed calibration model Fig. (6).

Table 3 Linearity results for AVB and AZT

AVB		AZT	
Concentration µg mL−1	**Peak Area**	**Concentration µg mL−1**	**Peak Area**
10	48958	30	175178
20	96297	60	343387
30	145234	90	528828
40	194153	120	693890
50	241803	150	864165
60	286280	180	1018884
R^2	0.9998	R^2	0.9996
Slope	4794.2	Slope	5696.6
Y intercept	847.93	Y intercept	5069.3

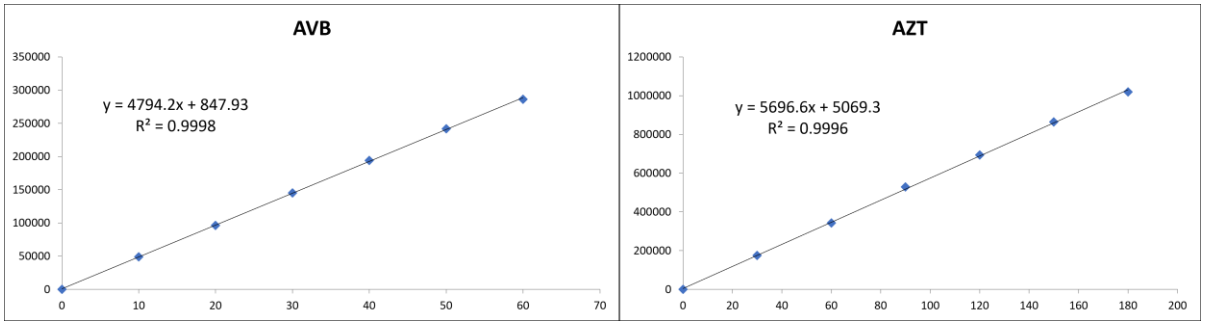

Figure 5 Linearity curve for AVB and AZT on x axis Peak area unit 5 mAU and on yaxis- Concentration unit µg ml -1)

3.3. Accuracy (Recovery Studies):

The accuracy of the proposed analytical procedure was validated by recovery studies using the standard addition protocol. Pre-analyzed sample solutions obtained were deliberately spiked with known, precise quantities of pure AVB and AZT reference standards. This reinforcement was fulfilled at three distinct concentration levels 50%, 100%, and 150% of the assay concentration with each level prepared and analyzed in triplicate. The percentage recoveries were consequently calculated by evaluating chromatographic peak areas against the theoretical concentrations expected. The method exhibited remarkable quantitative trueness, give way outstanding mean recovery values of 100.28% for AVB and 99.78% for AZT Table 4. These results of recovery metrics confirm the method's high accuracy and verified the absolute absence of any other matrix interference from the pharmaceutical formulation excipients.

Table 4 Accuracy results data of AVB and AZT

% Level	Quantity Spiked (µg mL−1)		Quantity Recovered (µg mL−1)		% Recovery		Mean % Recovery	
	AVB	AZT	AVB	AZT	AVB	AZT	AVB	AZT
50	20	60	19.87	59.847	99.3	99.74	100.28	99.78
	20	60	19.92	59.560	99.6	99.27		
	20	60	20.11	59.910	100.5	99.85		
100	40	120	39.63	119.688	99.1	99.74		
	40	120	39.75	119.299	99.4	99.42		
	40	120	40.15	119.302	100.4	99.42		
150	60	180	60.52	180.803	100.9	100.45		
	60	180	60.17	178.937	100.3	99.41		
	60	180	60.14	181.238	100.2	100.69		

3.4. Precision:

The precision of the optimized analytical method was systematically validated by rating repeatability (intra-day precision), intermediate precision (inter-day precision), and reproducibility. Assessing these parameters is important to confirm the reliability and evenness of the data measurement across various runs. Robust precision guarantee that the optimized procedure presents uniformly despite possible normal variations in operating conditions or carrying out by different analysts. Consequently, high degree of precision ensures that the proposed method can be positively and repeatedly adopted for routine quality control analysis, reliably yielding accurate results that are fit for its intended purpose.

Table 5 Data for Method precision (Repeatability) results for AVB and AZT.

Sr. No	AVB		AZT	
	Conc. (µg/mL)	Peak area	Conc. (µg/mL)	Peak area
1.	40	192195	120	697255
2.	40	192738	120	692186
3.	40	193338	120	700893
4.	40	190879	120	694733
5.	40	193196	120	701489
6.	40	192595	120	691890
AVG		192490	AVG	696408
SD		891.3	SD	4187.9
% RSD		0.5	% RSD	0.6

3.5. Method Precision (Repeatability):

To measure method precision (intra-day repeatability), six independent preparations of the

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sample solution were injected into the UPLC system under identical, optimized chromatographic conditions within a single working day. The chromatograms peak areas for both analytes, were carefully recorded. Then calculated percentage %RSD across all six replicate injections was found to be below the 2.0% threshold. This extremely low variation fully assures the universally accepted ICH acceptance criteria for assay precision, validating the method's excellent repeatability. The comprehensive statistical data for these evaluations are sum up in Table 5.

3.6. Limit of Detection (LOD) and Limit of Quantification (LOQ):

To evaluate the sensitivity of the proposed analytical method, the Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined. The LOD is defined as the lowest concentration of an analyte that can be distinguished from background baseline noise, while the LOQ represents the minimum concentration that can be accurately and precisely quantified. Establishing these is a critical validation step, ensuring that the method possesses the requisite sensitivity to accurately sense trace level drug concentrations during routine quality control applications.

These sensitivity parameters were experimentally verified using the signal-to-noise (S/N) ratio methodology in accordance with standard guidelines. By sequentially analyzing gradually diluted standard solutions of known concentrations, the LOD and LOQ were calculated at S/N ratios of approximately 3:1 and 10:1, respectively. For AVB, the method demonstrated very good sensitivity, yielding a computed LOD of 0.06 µg/mL and an LOQ of 0.19 µg/mL. For AZT, the respective values were noted as 0.52 µg/mL for the LOD and 1.56 µg/mL for the LOQ. Ultimately, these low threshold values validate the high sensitivity and robust performance of the proposed UPLC procedure for the simultaneous estimation of AZT and AVB.

3.7. Robustness:

In harmony with ICH Q2 (R2) guidelines, the robustness of an analytical method is defined as its capacity to remain unaffected by small, deliberate variations in method parameters, thereby providing an indication of its assurance during routine usage. To critically evaluate the robustness of the optimized UPLC method, sensitive but deliberate alterations were introduced to the established critical chromatographic parameters, which contains, the flow rate (± level), mobile phase composition (± level), and column oven temperature (± level) these were slightly varied from their optimal points Table 6.

Standard working solutions of AZT and AVB were injected by set up of these intentionally modified conditions, and the stability of the analytical response that is peak area was closely monitored. The method exhibited outstanding flexibility across all evaluated varied parameters. For AVB, the %RSD of the peak areas under the varied conditions ranged from 0.3% to 1.0%. Similarly, for AZT, the calculated %RSD values spanned a notably narrow range of 0.2% to 0.8%. Because the maximum %RSD observed under any altered condition should remained below the stringent acceptance limit of 2.0%, the proposed analytical method shows effective, reliable quantitative data despite minor operational fluctuations integral to routine laboratory settings.

Table 6 Robustness Data

Parameter Varied	Variation	AVB (%RSD)	AZT (%RSD)
Flow Rate (0.3 mL/min)	- 0.1(0.2)	0.6	0.4
	+ 0.1(0.4)	0.6	0.3
Mobile Phase (40%)	- 5(35)	0.6	0.2
	+ 5(45)	0.5	0.8
Temperature (30°C)	- 3(27)	1.0	0.5
	+ 3(33)	0.3	0.5

3.8. Comprehensive Greenness and White Analytical Chemistry Assessment: [11-16]

To thoroughly verify the environmental sustainability, occupational safety, and practical feasibility of the developed UPLC method, a demanding multi-metric assessment was conducted Fig. (7). This evaluation applied a suite of industry-standard qualitative as wll as quantitative metrics to provide a holistic profile of the analytical procedure. The method's in line with the principles of Green Analytical Chemistry (GAC) is visually demonstrated by the AGREE (A) and AGREEprep (B) pictograms, which gives outstanding overall scores of 0.82 and 0.81, respectively.[13,19] These metrics verify the method's high throughput, minimal operator exposure, and excellent energy efficiency. The ecological footprint across the entire analytical workflow starting from sample preparation to final determination of analytes is illustrated by the predominantly green GAPI pentagram (C) and the modified GAPI (mo-GAPI) diagram (G). [14, 20.] Particularly, the mo-GAPI visually integrates the method's ultra-low Analytical E-factor (0.825 g/run), emphasising negligible waste generation.

Further eco-friendly and safety profiling was achieved using the Analytical Greenness Profile (AGP) (H) and the NEMI pictogram (I). The AGP (Raynie and Driver tool) exhibited four green segments, with only a single yellow penalty sparking the moderate inherent flammability of the organic modifier.[21.] Similarly, the NEMI profile successfully met three out of four stringent criteria, with the

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hazardous quadrant remaining uncolored due to the unavoidable inclusion of methanol. The Analytical Eco-Scale (J) provided a robust quantitative support to these visuals, allocating minimal penalty points for reagents (-5) and waste generation (-4), concluding in a final score of 91, which officially declares the method's greenness as "Excellent."[22]

The Rationale and Benefits of Multi-Metric Greenness Assessment:

Historically, analytical methods were evaluated for "greenness" using isolated, singular metrics, which frequently introduced assessment biases. For example, the **Analytical Eco-Scale** [12] provides excellent semi-quantitative penalty scoring for chemical waste and reagent toxicity but completely ignores sample preparation throughput and operator ergonomics [12]. Conversely, **AGREE** comprehensively models the 12 principles of Green Analytical Chemistry (GAC) on a 0–1 scale but does not account for the financial or operational practicality of the instrument[13]. Furthermore, qualitative tools like **NEMI** and **GAPI** provide rapid visual profiles but lack the mathematical granularity required for precise regulatory benchmarking [14].

By deploying an integrated, multi-metric assessment battery (Fig. 6, A–J), this study entirely eliminates individual metric limitations. The visual GAPI pentagrams confirm low energy usage across the analytical lifecycle, while the modified mo-GAPI explicitly integrates the method's exceptional micro-waste generation (**E-factor: 0.825 g/run**). Most importantly, the deployment of the **White Analytical Chemistry (WAC)** framework mathematically reconciles GAC mandates with industrial reality[15]. The resulting WAC White Score of **71.7** proves that the proposed UPLC method achieves an uncompromised, optimal balance between superior analytical precision (Red Score: **77.5**), environmental safety (Green Score: **72.1**), and operational economic efficiency (Blue Score: **65.6**).

Finally, to evaluate the method beyond environmental impact, the Blue Applicability Grade Index (BAGI, D) and Red Analytical Procedure Index (RAPI, E) were assessed along with the widespread White Analytical Chemistry (WAC) framework (F).[23-25]. The WAC evaluation fruitfully bridged ecological safety with functional utility, yielding a highly recommendable overall White Score of 71.7. This score confirms an optimal equilibrium between analytical robustness (Red Score: 77.5), environmental responsibility (Green Score: 72.1), and operational or economic efficiency (Blue Score: 65.6). Collectively, these convergent multi-metric profiles (A–J) unequivocally confirms that the proposed UPLC validated procedure as a highly sustainable, analytically flawless, and practically competent tool for routine pharmaceutical quality control.

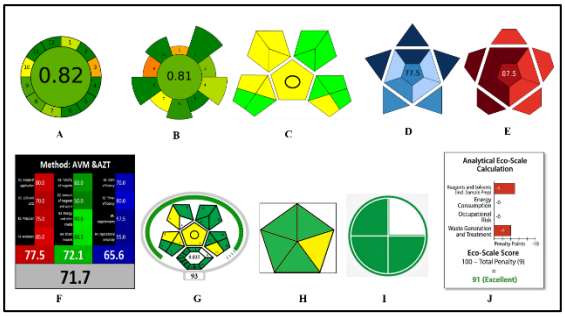

Figure 6 Multi-metric sustainability and White Analytical Chemistry (WAC) assessment of the UPLC method: (A) AGREE, (B) AGREEprep, (C) GAPI, (D) BAGI, (E) RAPI, (F) WAC, (G) mo-GAPI, (H) AGP, (I) NEMI, and (J) Analytical Eco-Scale.

3.9. Comparative Assessment with Existing Analytical Methods:

A meticulous comparative analysis benchmarking the proposed UPLC procedure against recently published academic literature unequivocally proves its functional and ecological superiority (Table 7). Current literature addressing the AZT/AVB combination is heavily hindered by methodological compromises. For instance, Trozzi et al. (2026)[4] demonstrated that achieving simultaneous quantification was so complex that they were forced to utilize highly expensive UHPLC-qTOF MS/MS instrumentation and run two distinct, sequential analytical methods totalling **20.0 minutes** per sample [4]. Similarly, conventional HPLC methods developed by Sreelatha et al. (2026) [5] and Haritha et al. (2025) [6] rely on high solvent flow rates (1.0 mL/min) and toxic acetonitrile concentrations reaching up to 85% [5,6].

In stark contrast, the proposed AQbD-driven UPLC method achieves true simultaneous, baseline separation of AZT and AVB in a single run of just 3.0 minutes. Mathematically interpreted, this represents an approximate 85% reduction in analytical run time compared to sequential MS/MS [4], and a 60% reduction compared to conventional HPLC [5,6]. From an environmental engineering standpoint, operating at a precisely controlled micro-flow rate of 0.3 mL/min reduces total chemical effluent to a negligible 0.9 mL per analysis—an overwhelming 91% reduction in organic waste compared to legacy HPLC procedures that generate 10.0 to 12.0 mL of toxic effluent per run [4–6]. This micro-waste footprint drives the method's stellar Analytical Eco-Scale score of 91 ("Excellent") and an AGREE index of 0.82.

Industrial Applications and Manufacturing Usefulness:

Beyond academic novelty, the present work offers

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immense practical utility for the commercial pharmaceutical manufacturing sector. In high-throughput industrial environments, the ultra-fast short minute run time allows quality assurance (QA) departments to process hundreds of commercial batch samples per day, drastically increasing operational productivity. Furthermore, the method's strict adherence to ICH Q14 and ICH Q2(R2) guidelines establishes a highly robust Method Operable Design Region (MODR). This regulatory compliance directly supports Real-Time Release Testing (RTRT) and continuous in-line process monitoring during the formulation of advanced AZT/AVB nanocarriers and sterile injectable dosage forms. Finally, replacing expensive mass-grade acetonitrile with standard chromatographic methanol yields massive financial cost savings for industrial quality control laboratories while ensuring absolute compliance with international occupational safety standards.

Table 7 Data of Analytical and ecological comparison of the proposed method versus existing literature.

Metric	Proposed Study (Proposed Method)	Trozzi et al., 2026[4]	Sreelatha et al., 2026[5]	Haritha et al., 2025[6]
Analytes	AVB & AZT	AVB & AZT	AVB & AZT	Ceftazidime & AVB
Technique	**UPLC (Simultaneous)**	UHPLC-qTOF MS/MS (Sequential)	RP-HPLC (Simultaneous)	RP-HPLC (Simultaneous)
Mobile Phase	Methanol : Buffer (40:60)	Gradient (ACN/Methanol utilized)	Acetonitrile : Formic Acid (20:80)	Acetonitrile : Water (85:15)
Flow Rate	**0.3 mL/min (Micro-flow)**	0.5 mL/min	1.0 mL/min	1.0 mL/min
Run Time	**3.0 minutes**	20.0 minutes (Sequential)	5.0 minutes	12.0 minutes
Total Waste/Run	**0.9 mL**	10.0 mL	5.0 mL	12.0 mL
Analytical Eco-Scale	**91 (Excellent)**	~ 70 (Acceptable)	~ 75 (Acceptable)	~ 60 (Inadequate)
AGREE Score	**0.82 (Highly Green)**	~ 0.45 (Moderate/Red)	~ 0.50 (Moderate)	~ 0.40 (Red/Yellow)
NEMI Profile	**Green Circle**	Non-Green (ACN utilized)	Non-Green (ACN is hazardous)	Non-Green (85% ACN)
GAPI Pentagram	**Mostly Green**	Mixed Red/Yellow (High Energy)	Significant Red/Yellow (ACN)	Mostly Red (High Waste/ACN)
mo-GAPI Score	**91**	~ 45	~ 55	~ 40
BAGI (Practicality)	**85 (High Utility)**	< 50 (Prohibitive MS/MS cost)	~ 60 (Standard HPLC)	~ 55 (Standard HPLC)
WAC (White Score)	**71.7**	~ 58.0 (High Red, Low Blue/Green)	~ 55.0	< 50.0

4. CONCLUSION:

Results: In this study, a highly robust, ultra-fast, and ecologically sustainable Ultra-Performance Liquid Chromatography (UPLC) procedure was developed and validated for the concomitant quantification of Aztreonam (AZT) and Avibactam (AVB). Strictly adhering to the ICH Q14 enhanced analytical development framework, a Central Composite Design (CCD) was utilized to optimize critical parameters, establishing an operable design region with a perfect desirability score of 1.000. Operating at a micro-flow rate of 0.3 mL/min with a methanol-buffer mobile phase (40:60, v/v), the method achieved sharp baseline resolution (**Rs = 5.0**) in an analytical run time of just 3.0 minutes. Validation executed per ICH Q2(R2) criteria confirmed exceptional linearity (**R2 > 0.999**), high quantitative accuracy near unity (99.78%–100.28%), and robust precision (**%RSD < 0.8%**).

Limitations: The primary limitation encountered during this study involves the strict operational boundaries of the Method Operable Design Region (MODR). Because AVB is an extremely polar molecule lacking a strong chromophore, any deliberate variation in mobile phase organic modifier or pH alterations can induce peak broadening and reduce chromatographic resolution. Furthermore, the backpressure generated by stationary phases (1.8 µ) restricts the deployment of this method exclusively to ultra-high-pressure LC platforms, limiting its immediate transferability to older, standard HPLC instruments operating in legacy laboratories.

Scope for Future Work: The outstanding sensitivity (**LOD: 0.06 µg/mL** for AVB; **0.52 µg/mL** for AZT) and rapid turnaround time of the proposed method establish a broad foundation for future commercial and clinical research. Future investigations will focus on successfully transferring this AQbD-UPLC platform to clinical settings for real-time Therapeutic Drug Monitoring (TDM) and pharmacokinetic (PK) bioequivalence trials in human plasma and cerebrospinal fluid. Additionally, future analytical work will explore interfacing this sub-2-micron separation chemistry with automated, green micro-extraction techniques (e.g., solid-phase microextraction or AGREEprep-guided liquid-liquid extraction) to further streamline raw material inspection and biological sample processing.

Societal Benefits and Global Impact: This research delivers an immediate, profound benefit to global public health and society. By delivering a fast, cost-effective, and mass-spectrometry-free

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analytical method, this study empowers standard, resource-limited quality control laboratories in developing nations to rigorously monitor the quality of commercial AZT/AVB antibiotic co-formulations. Ensuring the quantitative trueness and chemical integrity of these life-saving drugs directly protects vulnerable patients from substandard or degraded medicines, thereby aiding in the global fight against antimicrobial resistance (AMR). Concurrently, reducing organic solvent waste by over 90% protects laboratory personnel from toxic chemical exposure and actively preserves the broader ecosystem, exemplifying the necessary transition toward green, sustainable analytical practices in the pharmaceutical sciences.

Ethical Approval:
This research did not involve any studies with human participants or animal subjects performed by any of the authors. Therefore, ethical committee approval was not required for this study.

Informed Consent:
Not applicable

Consent to Participate:
Not applicable

Consent for Publication:
Not applicable

Data Availability:
All the data is available with the authors and shall be provided upon request

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

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Abbreviations:

Abbreviation	Full Form
AGP	Analytical Greenness Profile
AGREE	Analytical GREEnness
AQbD	Analytical Quality by Design
AVB	Avibactam
AZT	Aztreonam
BAGI	Blue Applicability Grade Index
CCD	Central Composite Design
CMP	Critical Method Parameter
GAC	Green Analytical Chemistry
GAPI	Green Analytical Procedure Index
HPLC	High-Performance Liquid Chromatography
ICH	International Council for Harmonisation
LOD	Limit of Detection
LOQ	Limit of Quantification
MODR	Method Operable Design Region
NEMI	National Environmental Methods Index
RAPI	Red Analytical Procedure Index
RSM	Response Surface Methodology
RSD	Relative Standard Deviation
UPLC	Ultra-Performance Liquid Chromatography
WAC	White Analytical Chemistry

REFERENCES:

1. World Health Organization. (2024). Global antimicrobial resistance and use surveillance system (GLASS) report. Geneva, World Health Organization. https://www.who.int/publications/i/item/9789240093461
2. Mauri, C., Maraolo, A.E., Di Bella, S., Luzzaro, F. and Principe, L. (2021). The revival of aztreonam in combination with avibactam against metallo-β-lactamase-producing Gram-negatives: A systematic review of in vitro studies and clinical cases. *Antibiotics*, 10(8): 1012. https://doi.org/10.3390/antibiotics10081012
3. Falcone, M., Daikos, G.L., Tiseo, G., Bassoulis, D., Giordano, C., Galfo, V., et al. (2021). Efficacy of ceftazidime-avibactam plus aztreonam in patients with bloodstream infections caused by metallo-β-lactamase-producing Enterobacterales. *Clin. Infect. Dis.*, 72(11): 1871-1878. https://doi.org/10.1093/cid/ciaa586
4. Trozzi, I., Giorgi, B., De Paola, R., Gatti, M. and Pea, F. (2026). Accurate and sensitive UHPLC-tandem mass spectrometry sequential methods for therapeutic drug monitoring of aztreonam/avibactam in human plasma. *Pharmaceutics*, 18(3): 377. https://doi.org/10.3390/pharmaceutics18030377
5. Sreelatha, G., Kolli, N. and Mallik, A. (2026). RP-HPLC analytical method development and validation of avibactam and aztreonam. *Asian J. Pharm. Res. Dev.*, 14(1): 15-19. http://dx.doi.org/10.22270/ajprd.v14i1.1688
6. Haritha, V., Latha, P.V.M., Suvarna, K. and Devi, P.U. (2025). Method development and validation for the simultaneous estimation of ceftazidime and avibactam in bulk and pharmaceutical dosage forms by RP-HPLC method. *Int. J. Innov. Res. Technol.*, 12(7): 2614-2618. https://ijirt.org/publishedpaper/IJIRT188524_PAPER.pdf
7. Lier, C., Kees, F., Witowski, A., Rahmel, T., Pockes, S. and Dorn, C. (2025). Simultaneous determination of ceftazidime and avibactam in patients by isocratic ion-pair liquid chromatography with photometric detection. *J. Chromatogr. Open*, 7: 100212. https://doi.org/10.1016/j.jcoa.2025.100212
8. International Council for Harmonisation. (2023). *ICH harmonised guideline: Analytical procedure development Q14*. Geneva, International Council for Harmonisation. https://database.ich.org/sites/default/files/ICH_Q14_Document_Step2_Guideline_2022_0324.pdf
9. Raman, N.V.V.S.S., Mallu, U.R. and Bapatu, H.R. (2015). Analytical quality by design approach to test method development and validation in drug substance manufacturing. *J. Chem.*, 2015: 435129. https://doi.org/10.1155/2015/435129
10. International Council for Harmonisation. (2023). *ICH harmonised guideline: Validation of analytical procedures Q2(R2)*. Geneva, International Council for Harmonisation. https://database.ich.org/sites/default/files/ICH_Q2%28R2%29_Guideline_2023_1130.pdf
11. Gałuszka, A., Migaszewski, Z. and Namieśnik, J. (2013). The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. *TrAC, Trends Anal. Chem.*, 50: 78-84. https://doi.org/10.1016/j.trac.2013.04.010
12. Van Aken, K., Strekowski, L. and Patiny, L. (2006). EcoScale, a semi-quantitative tool to select an organic preparation based on economical and ecological parameters. *Beilstein J. Org. Chem.*, 2: 3. https://doi.org/10.1186/1860-5397-2-3

13. Pena-Pereira, F., Wojnowski, W. and Tobiszewski, M. (2020). AGREE—Analytical GREEnness metric approach and software. *Anal. Chem.*, 92(14): 10076-10082. https://doi.org/10.1021/acs.analchem.0c03180
14. Płotka-Wasylka, J. (2018). A new tool for the evaluation of the analytical procedure: Green analytical procedure index. *Talanta*, 181: 204-209. https://doi.org/10.1016/j.talanta.2018.01.013
15. Nowak, P.M., Wietecha-Posłuszny, R. and Pawliszyn, J. (2021). White analytical chemistry: An approach to reconcile the principles of green analytical chemistry and functionality. *TrAC, Trends Anal. Chem.*, 138: 116223. https://doi.org/10.1016/j.trac.2021.116223
16. Smartsheet. (2026). *Free fishbone (Ishikawa) diagram templates*. https://www.smartsheet.com/free-fishbone-diagram-templates
17. Nowak, P.M., Kościelniak, M. and Tobiszewski, M. (2021). White Analytical Chemistry: An approach to reconcile the principles of green analytical chemistry and functionality. *Green Chem.*, 23(9): 3263-3274. https://doi.org/10.1039/D1GC00856C
18. Attar, M.S., Sakhare, R.S. and Vijayendra Swamy, S.M. (2026). QbD-driven development of a sustainable UPLC method for xanomeline and trospium chloride: A multi-metric greenness evaluation. *J. Chem. Lett.*, 7(1): 71-80. https://doi.org/10.22034/jchemlett.2026.578234.1386
19. Wojnowski, W., Tobiszewski, M., Pena-Pereira, F. and Psillakis, E. (2022). AGREEprep—Analytical greenness metric for sample preparation. *TrAC, Trends Anal. Chem.*, 149: 116553. https://doi.org/10.1016/j.trac.2022.116553
20. Płotka-Wasylka, J. and Wojnowski, W. (2021). Complementary green analytical procedure index (mo-GAPI) and data analysis tools. *Green Chem.*, 23(21): 8657-8665. https://doi.org/10.1039/D1GC03393A
21. Raynie, D.E. and Driver, J.P. (2009). Green assessment of chemical methods. *LCGC North Am.*, 27(3): 218-220. https://www.chromatographyonline.com
22. Gałuszka, A., Migaszewski, Z.M., Konieczka, P. and Namieśnik, J. (2012). Analytical Eco-Scale for assessing the greenness of analytical procedures. *TrAC, Trends Anal. Chem.*, 31: 18-29. https://doi.org/10.1016/j.trac.2012.03.013
23. Płotka-Wasylka, J., Wojnowski, W. and Tobiszewski, M. (2023). BAGI—Blue applicability grade index for evaluation of practical utility of analytical procedures. *Talanta*, 256: 124294. https://doi.org/10.1016/j.talanta.2023.124294
24. Nowak, P.M., Wojnowski, W. and Płotka-Wasylka, J. (2024). Red Analytical Procedure Index (RAPI) as a tool for evaluating the analytical performance of procedures. *Talanta*, 268: 125332. https://doi.org/10.1016/j.talanta.2024.125332
25. Keith, L.H., Gron, L.U. and Young, J.L. (2007). Green analytical methodologies. *Chem. Rev.*, 107(6): 2695-2708. https://doi.org/10.1021/cr068359e