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Designing a Green Chiral High-Performance Liquid Chromatography Method: Proof-of-Concept with Crizotinib

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ABSTRACT

With the growing emphasis on environmentally friendly analytical practices, green approaches to chiral separation have become vital for assessing the enantiomeric purity of chiral pharmaceutical agents. This study aimed to develop a green HPLC method for the enantioseparation of crizotinib, a tyrosine kinase inhibitor targeting anaplastic lymphoma kinase. The enantioselective performance of seven polysaccharide-based chiral stationary phases was systematically evaluated under polar organic conditions. Two chiral stationary phases—Lux Cellulose-3 and Lux Amylose-1—were identified as providing excellent enantiorecognition. Using experimental design-based approaches, we optimized three methods and retained them as final: (i) Lux Cellulose-3 with a methanolic mobile phase, (ii) Lux Cellulose-3 with an ethanol–water mobile phase, and (iii) Lux Amylose-1 with an ethanolic mobile phase. All three methods met ICH criteria: linearity ($r^2 \geq 0.9996$), precision (RSD $\leq$ 2%), and accuracy (~98%–101%), allowing quantification of the 0.02% distomer relative to the active enantiomer. Environmental performance was benchmarked using AGREE, Complex MoGAPI, Eco-Scale/Modified Eco-Scale, AMGS, and RGB-fast. All the developed methods showed superior environmental performance compared to the one method previously reported in the literature. However, it is also evident that applying green solvents does not inherently guarantee a cost-effective or sustainable approach. Overall, the developed methods provide an analytically robust and environmentally sustainable framework for the enantioseparation of crizotinib.

1 | Introduction

Green analytical chemistry represents a critical paradigm shift toward sustainability within the chemical sciences, placing emphasis on environmentally benign analytical practices [1]. Conventional analytical methodologies often rely on hazardous chemicals, excessive solvent consumption, and energy-intensive processes, all of which contribute to substantial environmental burdens. Consequently, increased attention has been dedicated toward the development and implementation of greener approaches designed to reduce environmental and health risks while preserving analytical reliability and efficiency [2, 3]. The pharmaceutical industry, with its heavy reliance on analytical methods for quality control, drug development, and regulatory compliance, stands to benefit substantially from adopting green analytical chemistry principles. Embracing these greener methodologies not only reduces environmental impact but also aligns pharmaceutical practices with contemporary regulatory expectations and global sustainability goals. Similarly, the focus of green chromatography is to reduce the environmental footprint of chromatographic processes by minimizing the use of toxic solvents, lowering energy consumption, and decreasing waste generation [4, 5].

Normal-phase chromatography, which commonly employs hexane as the primary eluent, is considered the most environmentally detrimental chromatographic technique. Nevertheless, the majority of chiral chromatographic methods and research today, especially in pharmacopeial applications, still relies on normal-phase conditions, despite the fact that many modern chiral stationary phases are fully compatible with polar organic and reversed-phase modes [6, 7]. Although hundreds of chiral columns are commercially available, polysaccharide-type chiral columns remain the most widely utilized [8, 9]. Their widespread application can be attributed largely to their multimodal nature, enabling effective use in normal-phase, polar organic, or even reversed-phase modes [10]. From an environmental standpoint, reversed-phase chromatography using an ethanol–water mixture represents one of the most favorable options. Beyond this, several newer green mobile phase systems and chromatographic approaches have been studied and used in recent years in routine practice, providing practical, lower impact alternatives to conventional solvents [11–13].

It should be emphasized, however, that altering the mobile phase also changes the enantiorecognition mechanism. Consequently, the development of chiral green methods is not as straightforward as merely substituting conventional solvents with greener alternatives but often requires an extended development process in which green solvents are prioritized from the outset. The sustainability of analytical methods can be systematically evaluated using established greenness assessment tools (e.g., NEMI, Eco-Scale, GAPI, MoGAPI, Complex MoGAPI, AGREE and AGREEprep), as comprehensively discussed in recent reviews on green profile tools, which consider parameters such as solvent hazards, waste generation, energy consumption, and sample requirements [14–17]. More recently developed frameworks such as the Click Analytical Chemistry Index (CACI) and the Analytical Green Star Area (AGSA), together with their associated pictograms, further extend this concept by enabling more nuanced, visual, and software-supported comparisons of methods in terms of both greenness and overall applicability [18, 19].

In parallel, 'whiteness' evaluation, rooted in the principles of White Analytical Chemistry (WAC), broadens the scope by integrating environmental sustainability (green) with analytical performance and fitness for purpose (red), and practicality or economic feasibility (blue) into a single whiteness score or RGB-style profile [20]. Greenness metrics provide a rapid and visual means of assessment but can over-simplify and overlook figures of merit (LOD, precision, robustness), as well as practical operational considerations. By contrast, whiteness offers a more balanced and decision-oriented perspective, yet it requires more extensive data, involves subjective weighing of criteria, and may produce inconsistent rankings across different tools. Both approaches present inherent limitations: hazard lists and scoring thresholds vary, upstream/embodied impacts (e.g., instrument manufacture) are rarely captured, and energy/source differences between laboratories are not accounted for [21]. In practice, the adoption of greener methods remains challenging due to several reasons, including regulatory lock-in arising from validated pharmacopeial methods, considerable cost and time required for the re-development and re-validation of methods, and potential compromises in method sensitivity or ruggedness. Further obstacles include limited availability to alternative solvents and instrumentation, as well as organizational constraints, such as staff training, standard operating procedure updates or supply chain limitations. Collectively, these factors hinder the timely replacement of established workflows [22, 23].

Crizotinib is a tyrosine kinase inhibitor predominantly prescribed for the treatment of ALK- or ROS1-positive nonsmall cell lung cancer (NSCLC) [24, 25]. The molecule contains a single chiral center (Figure 1) and is marketed as the *R*-enantiomer, which exhibits significantly higher pharmacological activity compared to the *S*-enantiomer [26]. Current regulatory guidelines require monitoring of the less active enantiomer (distomer), typically with a detection threshold of 0.1%. Achieving such sensitivity requires the development of highly selective and sensitive analytical methodologies. To date, only a single study has been reported in the literature addressing the enantioseparation of crizotinib. This method employed normal-phase chromatography using a hexane-based mobile phase and a Chiralcel OD column; however, it was not validated for the determination of the enantiomeric purity [27].

The aim of the present study was to develop and validate an environmentally friendly HPLC method for the baseline separation and sensitive detection of crizotinib enantiomers using polysaccharide-based chiral stationary phases (CSPs). The study emphasizes not only the achievement of high analytical performance but also the assessment of the method's environmental impact through comprehensive greenness and whiteness metrics, supporting the advancement of sustainable practices in pharmaceutical analysis.

2 | Materials and Methods

2.1 | Materials

Crizotinib (≥98%, HPLC; CAS No. 877399-52-5)and *S*-crizotinib (≥98%, HPLC; CAS No. 1 374 356-45-2) were obtained from Sigma–Aldrich (Budapest, Hungary). HPLC-grade solvents, including ethanol (EtOH), acetonitrile (ACN), and methanol (MeOH), were purchased from Merck KGaA (Darmstadt, Germany). Diethylamine (DEA) was sourced from Sigma–Aldrich (Budapest, Hungary). Ultrapure water was prepared using a Milli-Q Direct 8 system (Millipore, Milford, MA,

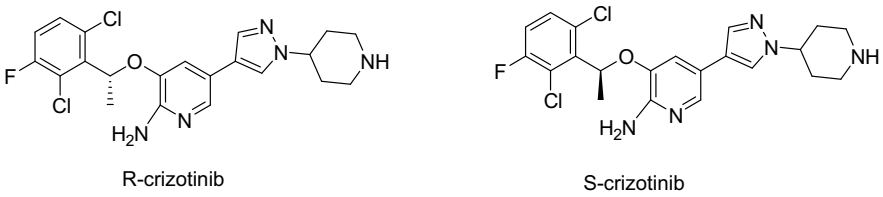


FIGURE 1 | The structures of *R*-crizotinib (left) as the eutomer and its enantiomer, *S*-crizotinib (right).

TABLE 1 | List of chiral columns used with abbreviation and their corresponding chiral selectors.

Column name	Chiral selector
Lux Amylose-1 (Am-1)	Amylose tris (3,5- dimethylphenylcarbamate)
Lux i-Amylose-1 (immobilized) (iAm-1)	Amylose tris (3,5- dimethylphenylcarbamate)
Lux Amylose-2 (Am-2)	Amylose tris (5–chloro-2-methylphenylcarbamate)
Lux Cellulose-1 (Cell-1)	Cellulose tris (3,5-dimethylphenylcarbamate)
Lux Cellulose-2 (Cell-2)	Cellulose tris (3-chloro-4-methylphenylcarbamate)
Lux Cellulose-3 (Cell-3)	Cellulose tris (4-methylbenzoate)
Lux Cellulose-4 (Cell-4)	Cellulose tris(4-chloro-3-methylphenylcarbamate)

USA). Polysaccharide-based CSP (150 × 4.6 mm, 5 µm) were supplied by Phenomenex (Torrance, CA, USA) and are detailed in Table 1.

2.2 | HPLC Analysis

Chiral screening of the columns was carried out using a high-performance liquid chromatography (HPLC) system comprising a Waters 2695 Separations Module coupled with a 2996 Photodiode Array (PDA) detector (Waters Corp., Milford, MA, USA). Instrument control, data acquisition, and spectral analysis were conducted via Empower 3 Chromatography Data Software.

The selected methods were further optimized using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) configured with a quaternary solvent delivery module (G1311A), an inline degassing unit (G1322A), a temperature-controlled autosampler (G1329A), a column thermostat (G1316A), and a diode array detector (G1315B), all operated via Agilent ChemStation software (version B04.03-SP2).

Preliminary chromatographic runs were conducted with a flow rate of 0.7 mL/min. The temperature was set at 25°C, and UV detection was performed at 268 nm. During the screening phase, the enantioseparation efficacy of seven polysaccharide-based HPLC columns (see in Table 1), was evaluated in polar organic mode using EtOH, ACN, and MeOH as eluents, each modified with 0.1% DEA, with the sample including enantio-enriched crizotinib at a ratio of approximately 3:1 *R*-crizotinib to *S*-crrizotinib. Injection volume was set at 2 µL.

During the optimization stage, parameters that influence separation were systematically evaluated using different full factorial experimental designs. The experimental matrix was generated with Design-Expert software (v7.0.0, Stat-Ease Inc., Minneapolis, MN, USA). The developed methods were validated for the determination of the enantiomeric purity of crizotinib. EtOH was used as the solvent for all stock solution preparations throughout the study. The final crizotinib test solution was prepared at a concentration of 1200 µg/mL, with the *S*-crizotinib impurity measured at 0.1% (1.2 µg/mL). Mobile-phase compositions are reported as volumetric ratios (v/v or v/v/v) of the main solvents; when an additive is used (e.g., 0.1% DEA), it is given as % (v/v) relative to the total mobile phase. Therefore, the values may formally sum to >100% (e.g., 92:8:0.1 or 100:0.1), which reflects standard notation in chiral chromatography.

2.3 | Greenness and Whiteness Evaluation

The evaluation of environmental sustainability and the 'whiteness' of chromatographic methods was conducted using five complementary tools, applied consistently for each run: the Analytical Eco Scale [28], the Green Certificate Modified Eco Scale [29], the complex modified green analytical procedure index (Complex MoGAPI) via the open–source Complex MoGAPI software (bit.ly/ComplexMoGAPI) [30], the analytical greenness calculator (AGREE) metric and software (https://mostwiedzy.pl/wojciech-wojnowski,174235-1/AGREE) [31], the red-green-blue (RGB) fast model [32], and the Analytical Method Greenness Score (AMGS) calculator provided by the ACS Green Chemistry Institute Pharmaceutical Roundtable (https://acsgcipr.org/amgs/) [33]. For each method, the inputs comprised solvent identity (pictograms, signal word, CAS number) and volume per run, additive concentrations, instrument energy relative to runtime and flow, waste streams, and any detoxification steps, as well as hazard information (GHS/SDS) for reagents; these data were consistently recorded across all frameworks to guarantee comparability. Calculations were executed utilizing software when accessible (AGREE, and Complex MoGAPI) or web–based calculator (AMGS) and the RGB quick tool as supplied; Eco Scale and Modified Eco Scale scores were derived in accordance with the referenced methodologies [28–32]. For mobile–phase additives, diethylamine was entered as a separate reagent in Eco–Scale and Green Certificate modified Eco–Scale, combined with the bulk organic solvent in AGREE and Complex MoGAPI, explicitly listed as an individual additive in RGBfast, and not included as a distinct input in AMGS, which was used with its predefined solvent options only.

A detailed description of the formulas, units, normalization procedures, and full step–by–step calculation workflow for Eco–Scale, Modified Eco–Scale, Complex MoGAPI, AGREE, RGBfast, and AMGS is provided in the Supporting Information.

3 | Results and Discussion

3.1 | Scouting Phase

A systematic screening of 21 enantioseparation systems using seven polysaccharide-based CSPs (Am-1, i-Am-1, Am-2, Cell-1, Cell-2, Cell-3, and Cell-4) in polar organic mode with three different mobile phases (MeOH, EtOH, and ACN, each modified with 0.1% of DEA) was conducted to determine their efficacy in resolving the enantiomers of crizotinib under conditions of 0.7 mL/min flow and a column temperature of 25°C, with injection volume set at 2 µL. The findings of the screening are summarized in Table 2.

Enantiomer recognition was observed in several cases, with considerable variance based on the column and eluent utilized. Using ACN Cell-2 and Cell-4 columns could separate crizotinib enantiomers; however, the enantiorecognition ability in the case

TABLE 2 | Chromatographic data obtained during the scouting phase. t_1 is the retention time of the first enantiomer, t_2 is the retention time of the second enantiomer, R_s is the resolution value, and S-R elution order means that the first eluted enantiomer is *S*-crizotinib).

Mobile phase[a]	Column	t_1 (min)	t_2 (min)	R_s	Elution order
MeOH	Am-1	7.91	—	—	—
	i-Am-1	4.49	—	—	—
	Am-2	4.55	—	—	—
	Cell-1	4.02	4.71	1.3	S-R
	Cell-2	5.16	—	—	—
	Cell-3	4.21	5.91	4.2	S-R
	Cell-4	4.08	—	—	—
ACN	Am-1	22.97	—	—	—
	i-Am-1	14.89	—	—	—
	Am-2	19.24	—	—	—
	Cell-1	13.08	—	—	—
	Cell-2	46.70	56.22	2.6	R-S
	Cell-3	6.68	—	—	—
	Cell-4	29.17	34.28	2.2	R-S
EtOH	Am-1	10.84	19.25	5.3	S-R
	i-Am-1	7.43	7.88	0.73	S-R
	Am-2	6.26	7.04	0.97	S-R
	Cell-1	8.20	9.18	1.0	S-R
	Cell-2	6.96	—	—	—
	Cell-3	6.94	9.55	1.5	S-R
	Cell-4	6.38	—	—	—

Note: - no enantiorecognition could be observed.
[a]All mobile phases contained 0.1% DEA as basic modifier.

of ACN is accompanied by high retention times. Using alcohol-type, protic eluents, more columns provided effective enantioseparation compared to ACN-based mobile phases. With MeOH, two columns resolved the enantiomers (Cell-1 and Cell-3); with EtOH, five columns (Am-1, i-Am-1, Am-2, Cell-1, and Cell-3) showed enantiorecognition. In ACN, the enantiomer elution order differs from that with alcohols. In alcohol-type eluents, a favorable elution order is observed: the distomer elutes before the eutomer peak. Some chromatograms from preliminary investigation are depicted in Figure 2. For further development two columns were chosen Cell-3 and Am-1.

The most rapid enantioseparation was observed on Cell-3 with MeOH as the mobile phase ($R_s = 4.2$; 5.91 min). A method using an EtOH–water mixture on the same column was also developed due to the less advantageous safety and sustainability characteristics of MeOH. Am-1 with EtOH yielded the best resolution ($R_s = 5.3$); however, this is accompanied by a prolonged analysis time (19.25 min) and elevated solvent usage, rendering it unsuitable as the standard for high throughput. The objective of the method development in this case was to reduce the analysis time. As a conclusion of preliminary screening three candidate systems were chosen for further method development (i) Cell-3 with MeOH, (ii) Cell-3 with EtOH–water mixture, and (iii) Am-1 with EtOH.

3.2 | Method Optimization

As a first step of method optimization the effect of water was analyzed in the ethanolic mobile phase on the Cell-3 column. Adding water to the mobile phase can improve the resolution and the sustainability of the method; however, it usually increases analysis time simultaneously. The enantioresolution and retention behavior of crizotinib enantiomers on the Cell-3 CSP were found to be strongly influenced by the water content in EtOH, as illustrated in Figure 3.

The relationship between water content and the resolution and retention times followed a U-shaped pattern. Upon initial addition of water to the mobile phase, a hydrophilic interaction chromatography (HILIC)-like retention behavior was observed for both enantiomers, as retention times decrease with increased water percentage (up to about 15% water in EtOH). As the water percentage continued to increase, the tendency flipped, leading to better enantioresolution and more retention, probably due to a reversed-phase retention mechanism. At around 40% water content, the maximum R_s could be observed. Similar results were previously also described on polysaccharide-based CSPs [34, 35].

In the next step, the role of additive was clarified. The inclusion of DEA was crucial for attaining satisfactory peak shape and resolution; elevating the DEA percentage of the mobile phase above 0.1% v/v yielded no further advantages; thus, 0.1% was established as the standard for all experiments. A full factorial design-based optimization was applied to all three methods. For the reversed-phase method, three factors—EtOH content in water, column temperature, and flow rate—were optimized. For the polar organic methods, temperature and flow rate were further optimized. The chosen response variables for optimization included resolution and retention time of the second-eluting peak (analysis time). After sequential model selection was done automatically (by maximizing adjusted R^2), in most cases quadratic, 2-factor with interaction, or linear models were fitted to each response. Model significance was evaluated by ANOVA (F value, R^2, adjusted R^2), with detailed statistics provided in the (Design_ANOVA_detailed_statistics /pdf/). Tables 2A, 2B, and 2C summarize the experimental matrix and the associated outcomes for the three optimization designs for reversed-phase method, for Cell-3 with methanolic, and Am-1 with ethanolic eluent, respectively. The tables display the original input data alongside a parallel set, which was randomized to enhance the robustness and reproducibility of statistical analyses and outcomes. The optimization objectives were uniform across all platforms: attain baseline enantioseparation and reduce analysis time (≤10 min when possible); as longer analysis times were observed on the Am-1-EtOH system, resolution was emphasized while minimizing runtime as much as possible. For simultaneous multiresponse optimization, a Derringer–Suich–type desirability approach was used, following the formulation of Myers and Montgomery [36]. Each experimental condition is evaluated through an overall objective function $D(X)$, called the desirability function, which combines the individual desirabilities d_i associated with each response. The individual desirabilities are defined

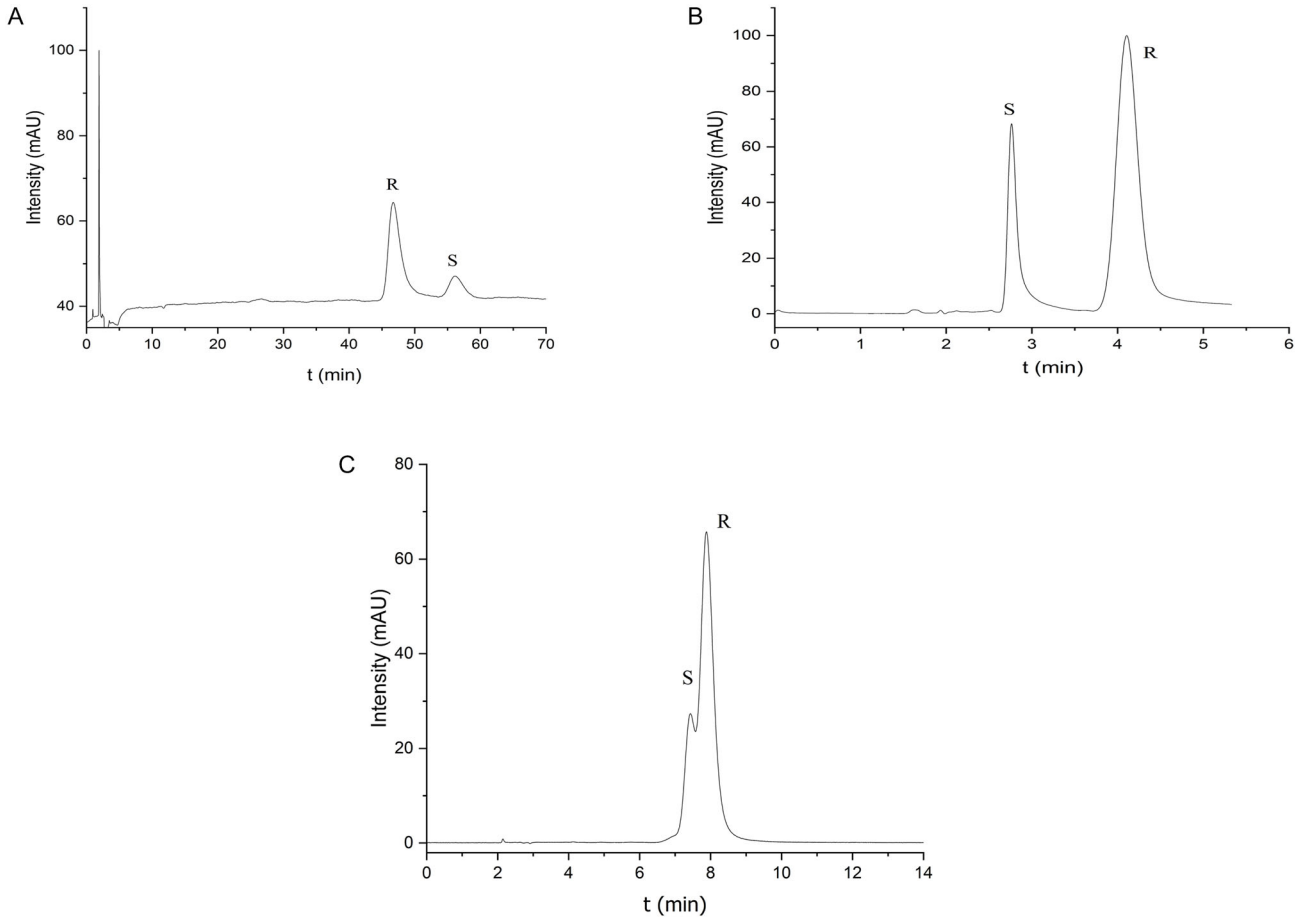


FIGURE 2 | Representative chiral HPLC chromatograms obtained during the method development phase. Separations were carried out on (A) Cellulose-2 (Cell-2), (B) Cellulose-3 (Cell-3), and (C) immobilized amylose-1 (i-Am-1) chiral stationary phases. The mobile phases consisted of acetonitrile containing 0.1% DEA for Cell-2, methanol containing 0.1% DEA for Cell-3, and ethanol containing 0.1% DEA for i-Am-1. All chromatographic analyses were performed at 25°C with a flow rate of 0.7 mL min^{-1}, and UV detection was conducted at a wavelength of 268 nm.

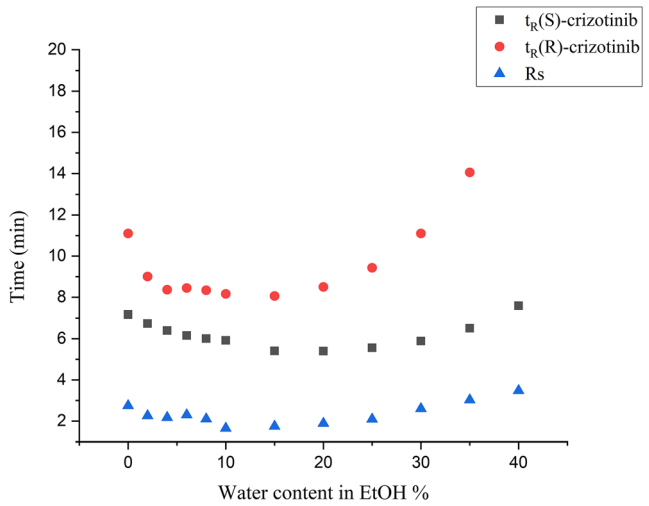


FIGURE 3 | Plots showing the retention times and resolution as a function of the water concentration in EtOH on the Cellulose-3 (Cell-3) chiral column. Mobile phase: EtOH-water mixtures containing 0.1% DEA; flow rate 0.7 mL/min; column temperature 25°C; and UV detection conducted at a wavelength of 268 nm.

on a 0–1 scale, where 0 corresponds to a completely unacceptable response value and 1 to a fully satisfactory (target) value. Values between 0 and 1 represent partially acceptable outcomes. In this study, resolution and analysis time were assigned the same weight and the same importance in optimization. Within the investigated design space (flow rate and temperature kept within their experimental ranges, resolution set to be maximized, and analysis time targeted to be minimized, preferably to values ≤10 min), with its desirability decreasing as the run time increases within the studied range. The overall desirability for a given factor combination was calculated as the geometric mean of the individual desirabilities [37]:

$$D = (d_1 \cdot d_2 \cdot \ldots \cdot d_n)^{\frac{1}{n}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}} \quad (1)$$

where n denotes the total number of responses considered in the optimization. If any individual response (or its associated factor level) falls outside the predefined acceptable range, the desirability assigned to that response is set to zero, which in turn reduces the overalldesirability function D to zero (Figure 4).

The optimized method parameters, where the highest resolution value with lowest retention time can be achieved are summarized

TABLE 2A | Full factorial design and its outcomes utilized for the optimization of the analytical EtOH/Cell 3 method, considering key response parameters.

Run NO.	EtOH in water, %[a] (factor 1)	Temperature, °C (factor 2)	Flow rate, ml/min (factor 3)	Analysis time, min	R_s
1	84	40	0.6	5.61	2.91
2	96	30	0.6	6.89	2.77
3	88	40	0.7	4.72	2.57
4	84	30	0.6	6.68	2.93
5	84	40	0.5	6.74	3.14
6	88	30	0.5	7.78	3.1
7	88	40	0.6	5.54	2.79
8	84	30	0.5	8.01	3.19
9	96	30	0.7	5.91	2.62
10	88	20	0.6	7.83	2.29
11	92	20	0.7	6.67	2.02
12	96	40	0.7	5.04	2.48
13	92	40	0.5	6.71	2.92
14	88	20	0.5	9.41	2.51
15	84	40	0.7	4.79	2.68
16	84	20	0.6	8.19	2.35
17	88	20	0.7	6.68	2.13
18	92	20	0.5	9.36	2.43
19	88	40	0.5	6.64	3
20	92	30	0.7	5.57	2.55
21	96	40	0.6	5.89	2.64
22	92	30	0.6	6.51	2.82
23	92	40	0.6	5.59	2.73
24	96	20	0.7	7.01	2.03
25	84	30	0.7	5.67	2.62
26	92	20	0.6	7.79	2.2
27	92	40	0.7	4.79	2.49
28	92	30	0.5	7.82	3.03
29	96	20	0.5	9.86	2.5
30	96	20	0.6	8.25	2.28
31	96	40	0.5	7.07	2.8
32	88	30	0.6	6.45	2.81
33	96	30	0.5	8.27	2.99
34	84	20	0.7	6.97	2.16
35	84	20	0.5	9.83	2.55
36	88	30	0.7	5.53	2.59

[a]Contained 0.1% DEA as basic modifier.

in Table 3. The chromatograms of optimized systems were depicted in Figure 5.

3.3 | Method Validation

All three developed analytical methods were validated for the quantification of *S*-crizotinib as an enantiomeric impurity in compliance with the International Council for Harmonization (ICH) Q2(R2) recommendations. The principal validation objectives were the key performance characteristics: sensitivity, linearity, accuracy (trueness), and precision. Summaries of the obtained results are presented in Table 4. Sensitivity was evaluated using signal-to-noise ratios of 3:1 and 10:1, resulting in LOD values of 0.076, 0.080, and 0.087 μg/mL, and LOQ values of 0.23,

TABLE 2B | Diminished two-factor design and results-MeOH/Cell-3 system.

Run NO.	Flow rate, ml/min (factor 1)	Temperature, °C (factor 2)	Analysis time, min	R_s
1	0.9	30	4.44	2.69
2	0.5	30	8.08	3.41
3	0.9	40	3.75	2.31
4	0.7	40	4.85	2.57
5	0.7	30	5.74	3.05
6	0.5	20	9.68	3.68
7	0.7	20	6.87	3.15
8	0.9	20	5.33	2.75
9	0.5	40	6.84	2.78

TABLE 2C | Diminished two-factor design and results-EtOH/Am-1 system.

Run NO.	Flow rate, ml/min (factor 1)	Temperature, °C (factor 2)	Analysis time, min	R_s
1	1.2	45	6.56	5.36
2	1.2	35	8.65	4.8
3	1	45	7.98	5.82
4	1	40	9.10	5.61
5	0.8	35	13.30	5.82
6	1	35	10.48	5.25
7	1.2	40	7.47	5.14
8	0.8	45	10.09	6.34
9	0.8	40	11.52	6.88

0.24, and 0.26 μg/mL for Method-1, Method-2, and Method-3, respectively. In accordance with ICH Q2(R2), the LOQ levels were further validated by accuracy and precision experiments: five replicate preparations at the LOQ concentration were analyzed for each method, giving relative standard deviations below 2% and mean recoveries within 98–102% of the nominal value, confirming suitable precision and trueness at the quantitation limit. Linearity was confirmed throughout the range of 0.017%–0.27% relative to target concentrations of 1200 μg/mL across five calibration levels, with correlation values $r^2 \geq 0.9996$ for all three approaches. The residuals exhibited a normal distribution, and the 95% confidence intervals surrounding the y-intercepts included zero. Accuracy (mean recovery) varied from 99.05%–101.07%, 98.28%–101.34%, 99.51%–100.55% with an RSD% less than 2 for Method-1, Method-2, and Method-3, respectively. Repeatability (RSD%) ranged from 0.63%–0.99%, 0.4%–1.17%, 0.58%–1.04% for Method-1, Method-2, and Method-3, respectively; intermediate precision over 2 days yielded an RSD% of less than 2.0 independently of the method. Repeatability and intermediate precision were assessed intra-day and inter-day, respectively, at three concentration levels within the linearity range, with each level examined in quintuplicate. The results together indicate that the three optimized techniques are appropriate for routine analytical applications, each consistently quantifying *S*-crizotinib as a chiral impurity with sufficient sensitivity, linearity, accuracy, and precision.

3.4 | Greenness and Whiteness Evaluation

A thorough multicriteria evaluation was performed on four HPLC methods for enantiomeric resolution of crizotinib, the three previously discussed methods developed in our laboratory and one previously published normal-phase method (Table 5) using five complementary frameworks: Analytical Greenness Metric (AGREE) [31], Complex Modified Green Analytical Procedure Index (Complex MoGAPI) [30], Analytical Eco-Scale [14, 28],Green Certificate Modified Eco-Scale [29], and the White Analytical Chemistry RGB-fast model [32]; and the Analytical Method Greenness Score (AMGS) [33]. The results are summarized in Table 6, providing a comparative overview of the greenness profiles of the evaluated methods.

The sustainability and holism measures aligned to form a cohesive ranking, while also revealing discrepancies due to their unique scopes and weightings. AGREE scores (approximately 0.65, 0.50, 0.63, and 0.45 for method-1, method-2, and method-3, and previously published method, respectively) demonstrated that adherence to the 12 principles of green analytical chemistry was highest when a benign solvent base (EtOH) was partially replaced by water and solvent throughput was minimized, intermediate when MeOH was employed at elevated flow rates, and least effective for the normal phase.

The Eco-Scale scores and Modified Eco Scale (76, 70, 76, and 34) enhanced this differentiation by focusing on chemical hazards and mass intensity, significantly penalizing n-hexane and the total volumes of flammable organics in the former published method while favoring EtOH and aqueous substitution in Method-1 and—despite increased flow—Method-3. The complex MoGAPI, which assesses the entire workflow from sampling to waste, yielded tighter numerical clustering (≈69, 68, 69, and 65) due to the uniformity of the instrumental platform and sample-handling steps across all four procedures, with only a few variables (solvent identity/amount and waste) differing; nonetheless, its pictograms confirmed the same directional trends.

AMGS illustrates the trade–offs among the three developed methods and the published procedure. For the four systems examined, the AMGS greenness scores were 1656.94 for Method–1, 1098.58 for Method–2, 2114.38 for Method–3, and 1824.84 for the published method. In line with the AMGS definition, lower scores correspond to greener methods [38]; thus, Method–2 appears most favorable from the AMGS perspective, followed by Method–1, whereas the Method-3 and the published method are penalized by longer runtimes and higher solvent and energy demand. This ranking is consistent with the observation that AMGS is particularly sensitive to total solvent throughput and instrument energy usage and therefore tends to favor very fast separations even when they rely on less benign solvents, while ethanol–water systems gain more advantage in metrics that emphasize solvent hazard and overall sustainability rather than energy and solvent mass alone.

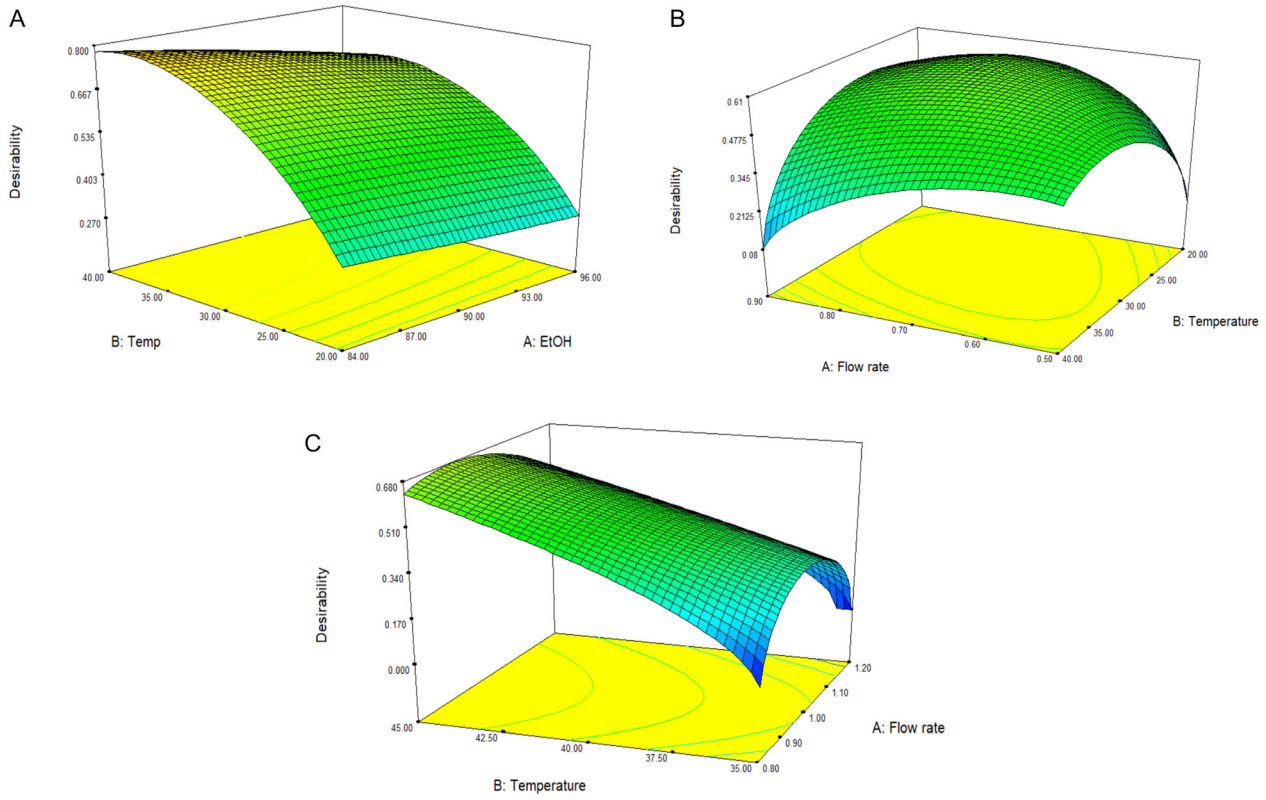


FIGURE 4 | 3D response surface plots of overall desirability for three optimized methods. Mobile phase compositions and conditions: (A) Method-1: Cellulose-3 (Cell-3) column, mobile phase EtOH:H_2O:DEA 92:8:0.1 (v/v/v), 0.5 mL/min, 40°C. (B) Method-2: Cellulose-3 (Cell-3) column, mobile phase MeOH:DEA 100:0.1 (v/v), 0.7 mL/min, 27.5°C. (C) Method-3: Amylose-1 (Am-1) column, mobile phase EtOH:DEA 100:0.1 (v/v), 0.9 mL/min, 45°C. UV detection at 268 nm for all methods.

TABLE 3 | The final parameters of optimized systems.

	Column	Mobile phase	Flow	Temperature	R_s	Analysis time
Method-1	Cell-3	EtOH:H_2O:DEA 92:8:0.1 (v/v/v)	0.5	40	2.86	8.0
Method-2	Cell-3	MeOH:DEA (100:0.1, v/v)	0.7	27.5	3.08	5.8
Method-3	Am-1	EtOH:DEA (100:0.1, v/v)	0.9	45	6.1	8.6

The RGB fast model, which combines analytical aspects (red), environmental greenness (green), and practicality/economics (blue) into a singular whiteness (albedo) score, elucidates why a procedure may be chemically 'green' yet not entirely ideal. The acquired assessment results are presented in (Figures 6 and 7); all data are also accessible in the accompanying supplement (Excel sheet).

The results unequivocally demonstrate that the Method-1 approach, utilizing EtOH:water, is the most environmentally friendly and the most effective in achieving whiteness. It achieved the highest saturation values for green (65.2) (ChlorTox rating 74.4 due to EtOH with partial aqueous substitution, low hazard, and reduced solvent mass) and a moderate blue color (50.9), while the red criteria were comparable to the Method-2 and 3 and superior to the published values. Its whiteness attained a value of 57. Method-2 exhibits the second greatest level of whiteness (55). Although the AGREE score is lower due to the mobile phase, this method offers a considerable advantage over alternatives in terms of expedited analysis time (notable blue color 52.2) and cost-effectiveness. Method-3 demonstrated a commendable Eco-Scale and high AGREE, owing to the inherently benign characteristics of EtOH; however, it experienced a decrease in whiteness (≈50) due to the ChlorTox score (substantial mobile phase volume) and longer analysis duration (low blue color 45) compared to alternative methods, while its red criteria were marginally elevated, keeping in mind that this is the most costly method. The previously published method exhibits a whiteness not reaching 50, indicating that it appears inferior to the three previously proposed methods. It is important to highlight that the approach employing a mobile phase of n-hexane and a ternary organic mixture containing 0.5% DEA increases hazard potential (the lowest rate of ChlorTox is 36.4), outweighing any energy advantages from ambient operation and ultimately diminishing both the red and blue RGB components.

It is important to acknowledge that the five evaluative techniques are deliberately nonisomorphic. AGREE systematically aligns with the 12 GAC principles, demonstrating a strong emphasis on procedural simplification and solvent substitution; Eco-Scale

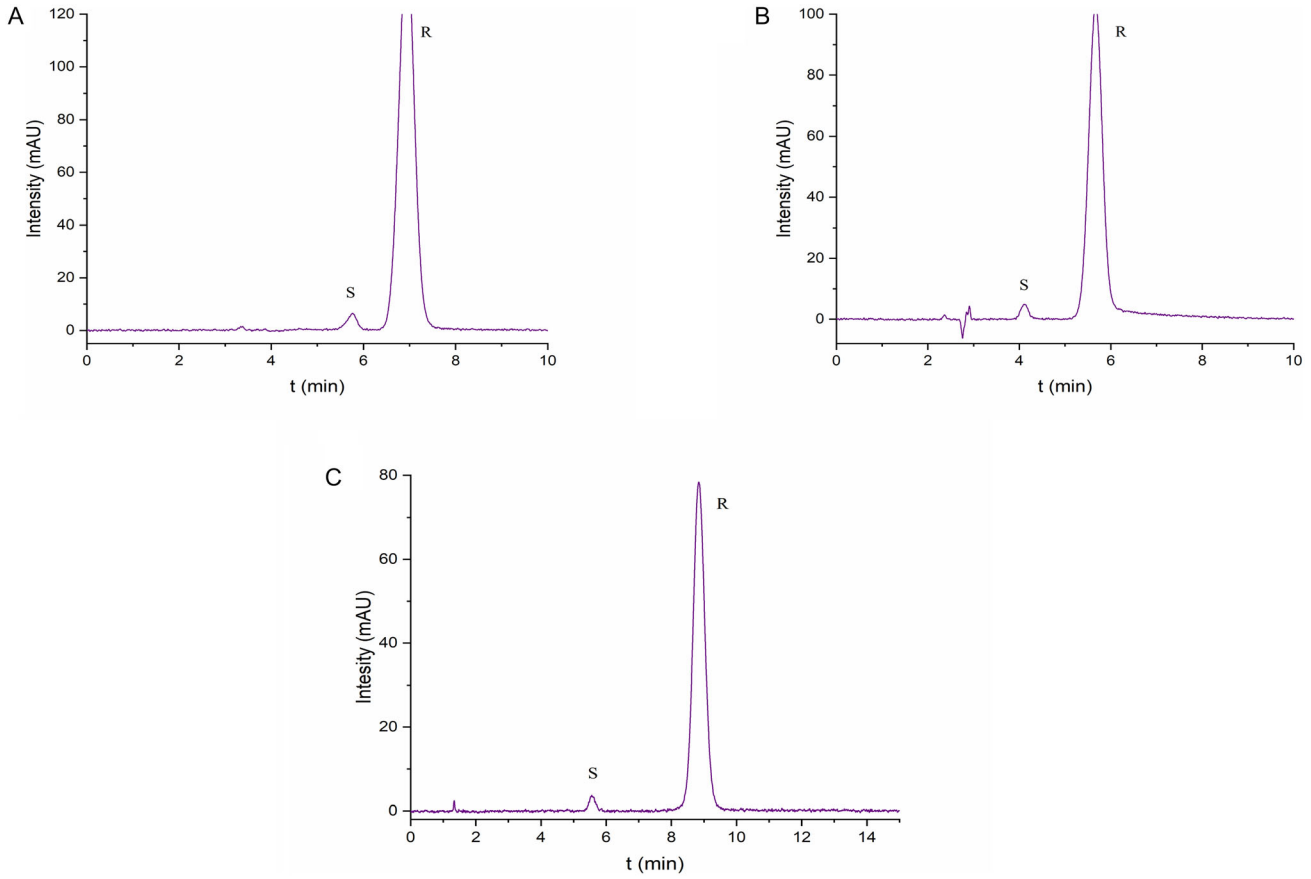


FIGURE 5 | Chromatograms demonstrating the resolution of crizotinib from its enantiomeric impurity (0.1% spiked level) acquired under the finalized analytical conditions established during method optimization and validation. Mobile phase compositions and conditions: (A) Method-1: Cellulose-3 (Cell-3) column, mobile phase EtOH:H_2O:DEA 92:8:0.1 (v/v/v), flow rate 0.5 mL/min, 40°C. (B) Method-2: Cellulose-3 (Cell-3) column, mobile phase MeOH:DEA 100:0.1 (v/v), flow rate 0.7 mL/min, 27.5°C. (C) Method-3: Amylose-1 (Am-1) column, mobile phase EtOH:DEA 100:0.1 (v/v), flow rate 0.9 mL/min, 45°C. UV detection at 268 nm for all methods.

TABLE 4 | Summary of data acquired during methods validation for the concurrent assessment of enantiomeric purity in a 1200 μg/mL crizotinib sample.

		***S*-crizotinib**		
Parameter	**Level**	**Method-1**	**Method-2**	**Method-3**
Range, μg/ml		0.2–3.2	0.2–3.2	0.2–3.2
Equation		120.52x + 8.2275	79.267x + 5.552	68.115x + 11.05
r^2		0.9997	0.9997	0.9996
LOD, μg/ml		0.076	0.080	0.087
LOQ, μg/ml[a]		0.23	0.24	0.26
Accuracy (trueness)	I (0.05%)	101.07%	98.28%	100.20%
	II (0.4%)	99.05%	100.65%	99.51%
	III (0.8%)	100.15%	101.34%	100.55%
Intraday precision, RSD%	I (0.05%)	0.63%	0.86%	0.58%
	II (0.4%)	0.99%	1.17%	1.04%
	III (0.8%)	0.87%	0.84%	0.67%
Intermediate precision, RSD%	I (0.05%)	1.02%	0.81%	0.59%
	II (0.4%)	0.86%	1.1%	0.91%
	III (0.8%)	0.95%	0.87%	1.13%

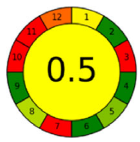
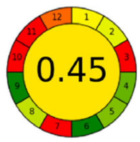
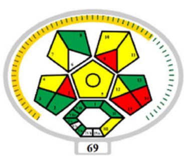
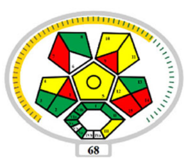
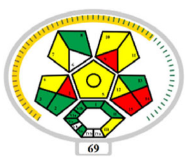
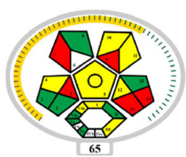
[a]The 0.2 μg/mL level is included for calibration regression only; the LOQ (0.23–0.26 μg/mL, method-dependent) defines the lower limit for reliable quantification. Quantification below LOQ is not recommended.

TABLE 5 | Crizotinib elution conditions, costs, and waste from published and developed methods.

Method	Elution conditions	Cost[a], USD	Waste[a], g	Reference
Published	HPLC-DAD using Daicel Chiralcel OD column. Isocratic elution using n-hexane:2-propanol:MeOH:DEA (40:30:30:0.5, v/v/v/v) as mobile phase	45.62	587.41	[27]
Method-1	HPLC-DAD using Cell-3 column. Isocratic elution using EtOH:H_2O:DEA (92:8:0.1, v/v/v) as mobile phase	37.22	282.01	This work
Method-2	HPLC-DAD using Cell-3 column. Isocratic elution using MeOH:DEA (100:0.1, v/v) as mobile phase	22.13	360.321	This work
Method-3	HPLC-DAD using Am-1 column. Isocratic elution using EtOH:DEA (100:0.1, v/v) as mobile phase	104.03	701.026	This work

[a]For one hundred samples.

TABLE 6 | Comparison between the greenness profiles of the developed methods and a published method [27] via Eco, Modified Eco scale, Complex MoGAPI, AGREE, and AMGS tools.

Greenness profile	Method-1	Method-2	Method-3	Published method
AGREE[a,b,c]				
Eco-Scale[a,b]	76 (Excellent level)	70 (Acceptable level)	76 (Excellent level)	34 (Inadequate level)
Modified Eco-Scale[a,b]				
Complex modified GAPI[a,b]				
AMGS[b,d]	1656.94	1098.58	2114.38	1824.84

[a]The detailed description methods used for greenness evaluation are illustrated in Supporting information (Supplementary Tables S1–S15).
[b]For one hundred samples.
[c]The full method reports are listed in AGREE report sheets (Pdf).
[d]The full method reports are listed in AMGS Spreadsheets (Pdf).

employs a penalty-point system to evaluate reagent hazards and mass; the Modified Eco-Scale enhances this by factoring in hazard severity, offering a more visual and comprehensive evaluation of a procedure's environmental sustainability; the complex MoGAPI provides a broad, workflow-level categorization that minimizes distinctions among methods utilizing the same HPLC platform; and RGB-fast adopts a holistic approach, penalizing any discrepancies among analytical performance, environmental impact, and practicality. The principled distinctions elucidate why Eco-Scale distinctly differentiates ethanol-based proposals from the normal-phase procedure, why Complex MoGAPI exhibits narrower numerical spreads while preserving the same trend, and why neat EtOH operation can maintain elevated green scores despite a reduction in whiteness when increased flow and temperature raise solvent and energy requirements. AMGS, which prioritizes solvent hazards, cumulative energy demand, instrument energy usage, and total solvent waste (with lower values indicating greener methods), consequently yields a slightly different ordering of the ethanol-based methods; this divergence is consistent with the distinct criteria embedded in each metric and should be interpreted as a complementary, rather than conflicting, perspective on method greenness. Collectively, the metrics should be interpreted as complementary rather than adversarial: their

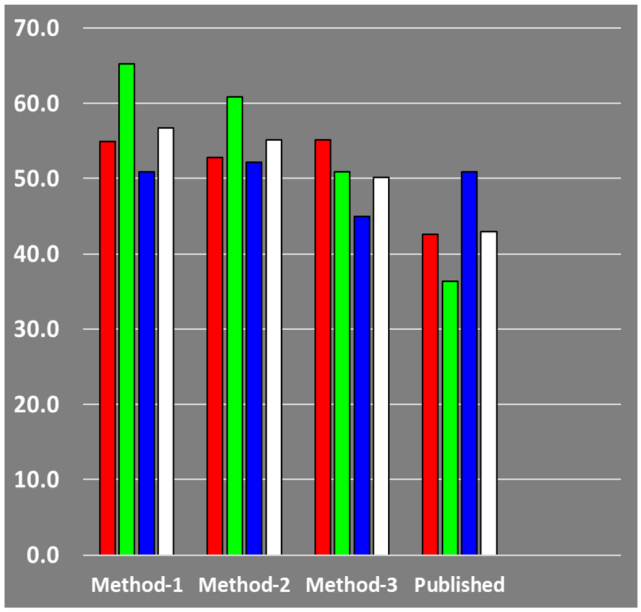


FIGURE 6 | Comparative analysis of four HPLC methods for the chiral resolution of crizotinib based on RGB color saturation and resultant whiteness as a holistic evaluation metric.

convergence on the same qualitative hierarchy—Method-1 ≥ Method-2 > Method-3 ≫ published method by whiteness, and Method-1 ≈ Method-3 ≥ Method-2 ≫ published method by green-only indices—confirms that replacing the conventional normal phase with ethanol-centric systems substantially reduces hazards and waste without compromising analytical performance or practicality. Partial aqueous substitution and controlled flow in Method-1 afford the most advantageous balance of R, G, and B components, Method-2 offers a cost-minimizing alternative with only a modest reduction in 'albedo,' and Method-3 would benefit from further optimization of the mobile phase composition to translate its chemical greenness into genuine 'whiteness.' The published protocol, while analytically robust, remains environmentally burdensome and operationally inferior to the proposed solutions in laboratories equipped with modern cellulose/amylose CSPs and EtOH–water polar organic elution.

3.5 | Comparison of Greenness and Whiteness Metrics Used in This Chiral HPLC Study

Analytical Eco–Scale, and its Green Certificate–modified variant, remain attractive because they are conceptually simple and yield quantitative scores and enable straightforward comparison of the greenness of different analytical methods, while explicitly treating each reagent so that low–level additives such as diethylamine in our study can be fully included. In these tools, greenness is expressed as a dimensionless score out of 100 obtained by subtracting cumulative penalty points assigned on the basis of quantities with physical units, such as solvent volume, reagent mass, and energy use from an ideal method, so the final value has no physical unit and functions as a semi–quantitative index rather than a physically defined measure. However, the three–level classification of solvent consumption in both Eco–Scale and the modified Eco–Scale does not capture the impact of very high reagent use or the benefits of ultra–low volumes, and neither includes an explicit component for assessing greenness associated with sample collection. Complex MoGAPI provides a comprehensive greenness metric with both qualitative and numerical outputs that can be applied to all stages of the analytical workflow, reflecting the 12 principles of green analytical chemistry more deeply than the original GAPI and allowing explicit inclusion of individual reagents and additives such as diethylamine. In this approach, each step of the method is assigned categorical scores which are aggregated into a dimensionless total score and pictogram, so the overall Complex MoGAPI index is unitless even though the underlying inputs include masses, volumes, and energy–related quantities; this enhanced descriptive power

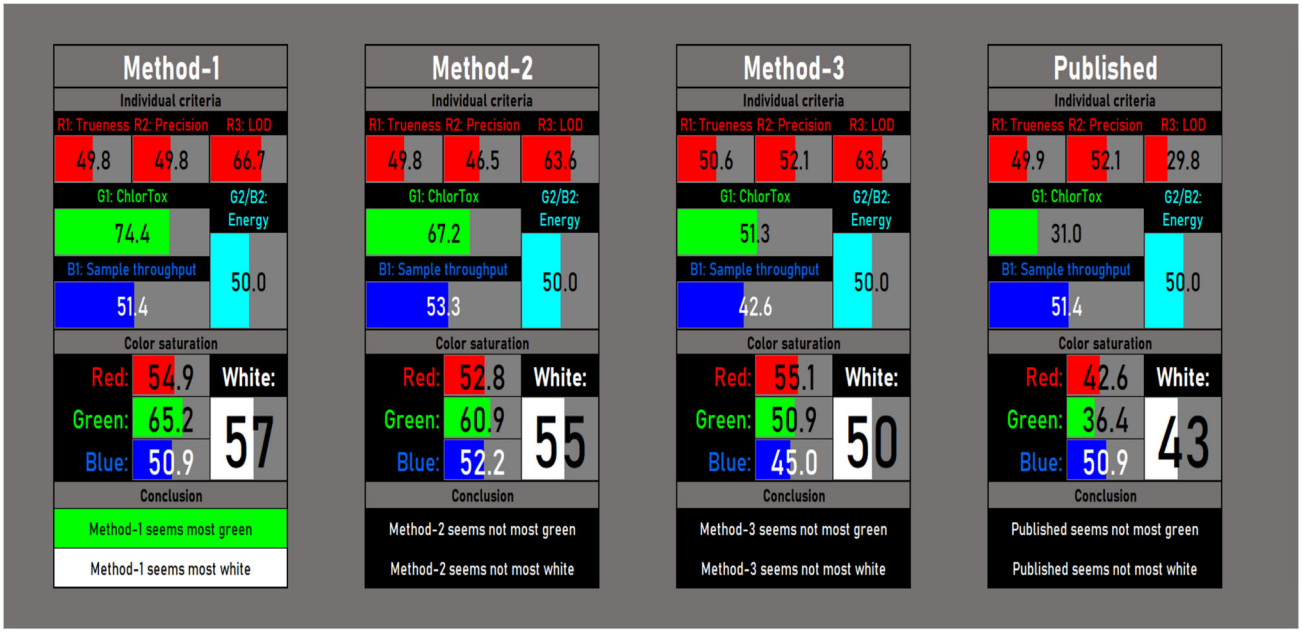


FIGURE 7 | Visualization of the compared methods via automatically generated tables and pictograms using the RGBfast model.

comes at the cost of requiring detailed procedural information and makes the evaluation more time–consuming and demanding than with simpler metrics. The Analytical Method Greenness Score (AMGS) yields a single, comparable value by combining solvent health–safety–environment attributes, energy demand, instrument energy use, and solvent waste for chromatographic separation methods, but it is less broadly applicable than the other metrics, its calculation is relatively complex, and it does not allow the free specification of individual mobile–phase additives (such as diethylamine in our study), instead relying on a predefined solvent list that currently omits several emerging green eluents, including Cyrene and dimethyl carbonate [11, 39]. From a mathematical standpoint, AMGS aggregates contributions with different physical dimensions (for example, solvent mass, mass–weighted safety/health/environment terms, and mass scaled by cumulative energy demand expressed in kg–solvent per MJ) into a single sum that is then normalized only by the number of analytes, so the resulting score has no unique physical unit and is best regarded as an empirical composite index for ranking methods rather than as an absolute, physically interpretable measure of greenness. AGREE is straightforward to apply and generates both color–coded and numerical outputs that are easy to interpret and—analogously to Eco–Scale and Complex MoGAPI—can, in principle, accommodate low–level mobile–phase additives such as diethylamine when these are explicitly specified. In this metric, all 12 green analytical chemistry criteria are mapped onto a common 0–1 scale and combined into a single, dimensionless greenness index, so the final AGREE score is unitless and intended purely as a normalized, comparative measure; however, the weighting scheme used to aggregate the criteria lacks a fully transparent justification, and the emphasis of the first GAC principle on direct analysis has, in practice, contributed to the frequent omission of a rigorous assessment of the sample–preparation step. To overcome this shortcoming, the AGREEprep tool was introduced in 2022 to specifically assess the greenness of sample preparation [40], thereby complementing AGREE and helping to identify realistic strategies such as online sample preparation and solvent–volume reduction to enhance overall method greenness. RGBfast combines analytical performance (red), environmental greenness (green), and practicality/economics (blue) into a unified framework in which each group of criteria is converted to a 0–100 index and then merged into an overall whiteness score; all three outputs (red, green, and blue) are therefore dimensionless and represent relative performance rather than physical quantities. Within this scheme, trade–offs between sensitivity, cost, and environmental impact are made explicit, and the internal ChlorTox component enables the contribution of individual solvents and mobile–phase additives, including diethylamine, to chemical risk to be captured in the green channel; nevertheless, application of RGBfast requires extensive input data and expert judgment, and modest changes in the weighting or scoring rules can influence the final ranking of the evaluated methods. Collectively, these strengths and limitations indicate that no single metric is sufficient: AGREE, Eco–Scale, modified Eco–Scale, Complex MoGAPI, RGBfast, and AMGS each capture distinct facets of greenness and whiteness, with AMGS being dedicated to chromatographic separations while the other tools can be applied more broadly, and only their combined application can furnish abalanced assessment of the environmental and practical performance of the evaluated methods [41].

4 | Conclusion

Three suitable HPLC techniques were developed for the enantioseparation and quantification of crizotinib through a systematic procedure that integrated extensive screening, experimental design-based optimization, and multicriteria sustainability evaluation. The extensive preliminary screening sequence revealed that alcohol-based eluents are often more effective than ACN for enantiorecognition on polysaccharide CSPs. Among the primary contenders, Cell-3 with MeOH provided the fastest enantioseparation, Am-1 with EtOH had the greatest resolution, and Cell-3 with an EtOH:water mixture proved to be the most viable green alternative. Optimization elucidated the primary levels for performance and sustainability. On Cell-3, the incorporation of water into EtOH resulted in the anticipated nonmonotonic (U-shaped) response in retention and resolution, facilitating an equilibrium between speed and selectivity at partial aqueous substitution; a concentration of 0.1% DEA was established as essential for optimal peak shape, with no advantage observed beyond this threshold. The design of experiments (full factorial for the EtOH-water system, reduced two-factor designs for polar organic systems) and response surface modeling, complemented by Derringer desirability, produced operational parameters that attain baseline separation while reducing analysis time, with platform-specific trade-offs. All three improved methods satisfied ICH Q2(R2) validation criteria, exhibiting low LOD/LOQ, exceptional linearity ($r^2 > 0.9996$), and accuracy and precision within acceptable bounds throughout the calibration range. This validates their appropriateness for the routine quantification of *S*-crizotinib as a chiral impurity. An extensive evaluation of greenness and whiteness (AGREE, Complex MoGAPI, Eco-Scale/Modified Eco-Scale, RGB-fast, and AMGS) revealed a consistent hierarchy: the EtOH-water method on Cell-3 exhibited the most advantageous overall profile (highest whiteness) by integrating environmentally benign solvent selection with minimized solvent mass and brief runtime; the MeOH method ranked second overall, capitalizing on speed and cost-effectiveness despite a less favorable solvent; and the ethanol method on Am-1 maintained chemical greenness but demonstrated lower whiteness due to increased solvent consumption and prolonged processing times. The single previously published normal-phase technique ranked lowest in both green-only and holistic measures. Waste and expense assessments validated these rankings. The EtOH-water Cell-3 method is endorsed as the prevailing, sustainable approach for routine quality control and stability assessments; the MeOH Cell-3 method can be viewed as a rapid, cost-effective alternative when high-throughput is critical; and the EtOH Am-1 method is recommended for confirmatory analyses or impurity control necessitating the highest enantioresolution.

Author Contributions

Ali Mhammad: conceptualization (supporting), investigation (lead), methodology (lead), writing – original draft (equal), writing – review and editing (equal). **Gergely Dombi:** conceptualization (supporting), data curation (lead), investigation (supporting), methodology (supporting), writing – original draft (supporting), writing review and editing (equal). **Máté Dobó:** conceptualization (supporting), investigation (equal), methodology (equal), writing – review and editing (supporting). **Eliza Tóth:** investigation (supporting), methodology (supporting), project administration (lead), resources (equal), software (equal). **Gergely Molnár:** investigation (supporting),

methodology (supporting), validation (equal), visualization (equal), writing – review and editing (supporting). **Arash Mirzahosseini:** data curation (equal), investigation (equal), methodology (equal), project administration (equal), resources (equal). **Zoltán-István Szabó:** conceptualization (equal), resources (lead), supervision (equal), writing – original draft (supporting), writing – review and editing (equal). **Gergő Tóth:** conceptualization (lead), funding acquisition (lead), supervision (lead), writing – original draft (equal), writing – review and editing (lead).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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Supporting Information

Additional Supporting Information can be found online in the Supporting Information section. Detailed descriptions of the greenness-evaluation methods are presented in Supplementary Tables S1–S15 (Supporting Information/word file/). In addition, the full AGREE method reports (PDF sheets), the complete AMGS outputs (PDF spreadsheets), and the RGBfast assessment (Excel workbook) are also provided. **Supporting Table S1:** Calculation of Analytical Eco-Scale Penalty Points (PPs). **Supporting Table S2:** Detailed Description of the Analytical Eco-Scale for Evaluating the Greenness of Method-1. **Supporting Table S3:** Detailed Description of the Analytical Eco-Scale for Evaluating the Greenness of Method-2. **Supporting Table S4:** Detailed Description of the Analytical Eco-Scale for Evaluating the Greenness of Method-3. **Supporting Table S5:** Detailed Description of the Analytical Eco-Scale for Evaluating the Greenness of the Published Method. **Supporting Table S6:** Description of the Twelve Criteria Used in the AGREE Assessment. **Supporting Table S7:** Detailed Description of AGREE for Evaluating the Greenness of Method-1. **Supporting Table S8:** Detailed Description of AGREE for Evaluating the Greenness of Method-2. **Supporting Table S9:** Detailed Description of AGREE for Evaluating the Greenness of Method-3. **Supporting Table S10:** Detailed Description of AGREE for Evaluating the Greenness of the Published Method. **Supporting Table S11:** Detailed Description of ComplexMoGAPI Parameters. **Supporting Table S12:** Detailed Description of the Complex modified GAPI for Evaluating the Greenness of Method-1. **Supporting Table S13:** Detailed Description of the Complex modified GAPI for Evaluating the Greenness of Method-2. **Supporting Table S14:** Detailed Description of the Complex modified GAPI for Evaluating the Greenness of Method-3. **Supporting Table S15:** Detailed Description of Complex-GAPI for Evaluating the Greenness of the Published Method.