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Optimised extraction and HPLC–DAD determination of steroidal glycoalkaloids in potato tubers: Analytical validation and environmental sustainability evaluation

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ABSTRACT

Steroidal glycoalkaloids (α-solanine and α-chaconine) are major potato toxins whose accurate quantification is often challenged by matrix effects and chromatographic selectivity. This study reports the optimisation and validation of a robust reversed-phase HPLC-DAD method for the determination of glycoalkaloids in potato tissues, combined with a systematic assessment of the environmental sustainability of the analytical workflow. Extraction conditions were optimized by integrating systematic solvent screening with a factorial experimental design to evaluate the effect of key operational variables on glycoalkaloid recovery and refine extraction performance The resulting extraction medium improved extract cleanliness and chromatographic stability. A subsequent C18 solid-phase extraction SPE step further reduced matrix effect and enhanced analytical selectivity. Chromatographic separation was achieved on a conventional C18 column using an acetonitrile-water gradient, providing baseline resolution (Rs > 1.5) of α-solanine and α-chaconine with stable diode-array detection (DAD) at 205 nm. The method was validated according to ICH Q2(R2) and AOAC guidelines, demonstrating excellent linearity ($R^2 > 0.995$), low limits of detection (0.462–0.767 μg/mL) and quantification (1.540–2.558 μg/mL), high precision (≤ 5% RSD), and recoveries of 90–105%. Robustness testing confirmed minimal sensitivity to routine variations in chromatographic parameters. Application to potato breeding lines demonstrated reliable quantification and revealed genotype-dependent variation in glycoalkaloid content. The environmental assessment of the analytical procedure was evaluated using Analytical Eco-scale, GAPI and AGREE metrics, demonstrating reduced solvent consumption, avoidance of strongly acidic extraction conditions, and a simplified sample preparation workflow. The proposed HPLC-DAD workflow provides a validated, high-performance, and environmentally conscious approach for routine GA quantification, suitable for food-safety monitoring, regulatory compliance and breeding programme applications.

1. Introduction

Steroidal glycoalkaloids (SGAs) are a structurally related group of secondary metabolites naturally accumulated in *Solanum tuberosum,* with α-solanine and α-chaconine as the predominant toxic metabolites [1,2]. Their ability to disrupt membrane sterol organisation, inhibit acetylcholinesterase, and induce systemic toxicity underscores their significance as chemical hazards in food safety assessments [3–5]. Current regulatory frameworks therefore impose strict limits on total GA concentrations in edible tubers and processed potato products, creating a sustained need for analytical methods capable of delivering accurate, compound-specific quantification across diverse matrices [6,7].

Despite the availability of numerous chromatographic procedures for GA determination, important limitations persist. Most established HPLC or UHPLC workflows rely on conventional reversed-phase C18 columns and hydro-organic extraction protocols [8–12]. However, the close structural similarity of α-solanine and α-chaconine, both based on the solanidane aglycone and differing only in trisaccharide composition, frequently results in incomplete resolution, peak distortion and detector drift [13]. These issues are exacerbated in potato matrices rich in phenolics, pigments, or membrane-derived lipids, which contribute to baseline instability and co-elution at low-UV detection wavelengths [14]. Additionally, reported extraction recoveries vary widely due to inadequate control of solvent polarity and pH, factors known to

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influence GA solubility and glycosidic integrity [15–17]. Consequently, several published methods fall short of contemporary validation requirements (ICH Q2(R1), ISO 17,025) [18], particularly with respect to linearity, accuracy, ruggedness and matrix effects. The growing analytical demands imposed by potato breeding programmes, post-harvest physiology research and food processing monitoring further emphasise the need for workflows offering enhanced selectivity, operational robustness, and high-throughput compatibility [19,20]. Beyond analytical performance, increasing attention has been directed toward the environmental sustainability of chromatographic workflows. The principles of Green Analytical Chemistry (GAC) advocate reducing solvent consumption, simplifying sample preparation, and minimising waste generation while maintaining analytical reliability and robustness [21–23]. In the context of glycoalkaloid (GA) determination, however, the integration of these principles remains limited. Conventional extraction protocols typically rely on relatively large volumes of methanol, acetonitrile, or acidified organic solvents and frequently incorporate multi-step clean-up procedures such as solid-phase extraction (SPE), which increase solvent consumption, generate additional waste streams, and reduce operational efficiency [8,10]. Despite the growing interest in green analytical chemistry, only a limited number of studies have addressed environmentally oriented extraction strategies specifically for steroidal glycoalkaloids in potato matrices. Most reported analytical workflows remain based on solvent-intensive liquid extraction, ion-pair chromatography, or QuEChERS-derived protocols that employ considerable volumes of acetonitrile and multiple sample-handling steps. Although recent approaches, including polarity-modulated aqueous solvent systems, miniaturised extraction formats, and QuEChERS-inspired methodologies, have demonstrated the potential to reduce solvent consumption without compromising extraction efficiency, their application to glycoalkaloid analysis remains relatively scarce [24]. Furthermore, systematic evaluations of the environmental performance of GA analytical methods using quantitative greenness metrics, such as the Analytical Eco-Scale, the Green Analytical Procedure Index (GAPI), or the AGREE framework, remain largely absent from the glycoalkaloid literature. As a consequence, the sustainability of existing analytical workflows is rarely assessed in a comprehensive and comparable manner. Integrating sustainability criteria into glycoalkaloid quantification is therefore essential not only to modernise extraction strategies but also to reduce solvent burden and align analytical methodologies with contemporary expectations for environmentally responsible analytical chemistry.

In this context, the present work describes the optimisation and validation of a reversed-phase HPLC method for the quantitative determination of α-solanine and α-chaconine in potato tubers, with particular emphasis on matrix selectivity, operational robustness, and environmental performance rather than on the introduction of a fundamentally new analytical principle. The workflow integrates three targeted innovations: (i) a polarity-tuned extraction medium that efficiently liberates GA from parenchymal tissue while minimising hydrolytic cleavage of glycosidic linkages and suppressing co-extraction of hydrophobic interferences; (ii) a chromatographic phase selected for enhanced affinity towards amphiphilic steroidal GAs, thereby improving partition selectivity and achieving robust resolution despite the minimal structural divergence of the two analytes; (iii) low-wavelength UV/DAD detection optimised for signal-to-noise performance and quantitative reliability. Method validation was performed in accordance with internationally recognised guidelines, including evaluation of specificity, linearity, sensitivity, precision, accuracy, robustness, and matrix effects across representative potato samples. In parallel, the environmental characteristics of the analytical workflow were quantitatively assessed using complementary green-analytical metrics and critically compared with representative literature procedures. Through the integration of rigorous validation, matrix-aware selectivity assessment, and transparent sustainability evaluation, this study aims to provide a technically reliable and operationally accessible analytical approach suitable for routine monitoring of potato glycoalkaloids in food safety, breeding, and post-harvest research contexts.

2. Experimental

2.1. Chemicals and standards

Analytical standards of α-solanine (≥ 98% HPLC purity) and α-chaconine (≥ 98% HPLC purity) were obtained from Extrasynthese (Genay, France). Methanol (HPLC grade), acetonitrile (gradient grade ≥ 99.9%), acetic acid (≥ 99%), and potassium dihydrogen phosphate (≥ 99%) were purchased from certified suppliers. Solid-phase extraction (SPE) cartridges (C18 silica, 500 mg, 1 mL; Waters, Milford, MA, USA) were used for matrix clarification. Primary stock solutions of each glycoalkaloid (1 mg/mL) were prepared in methanol, aliquoted into amber vials, and stored at −20 °C to prevent degradation. Working standards (10–100 μg/mL) were freshly prepared by serial dilution in methanol-water (50:50, v/v) immediately before analysis. Unless otherwise stated, calibration solutions and sample extracts underwent identical SPE cleanup to ensure matrix-consistent chromatographic behaviour. Prior to loading, SPE cartridges were conditioned with 1 mL methanol followed by 1 mL Milli-Q water, at a controlled dropwise flow (1 drop/*sec*). After sample application, cartridges were washed with 5 mL Milli-Q water and eluted with 10 mL methanol. Eluates were filtered through 0.22 μm PTFE syringe filters (Sigma-Aldrich, USA) directly into HPLC vials.

2.2. Plant material and sampling

Potato tubers (*Solanum tuberosum* L.) from a representative panel of the breeding line KIS 10–242/247–6 (white flesh, bright skin) and the variety KIS Blegoš (yellow flesh, yellow skin) were harvested in autumn 2024 at the experimental field station of the Agricultural Institute of Slovenia in Jablje (46°08′33.78″ N, 14°33′22.72″ E; 302 m a.s.l.). Dark green tuber tissues were additionally collected as a reference material with high GA content. Tubers were rinsed with distilled water, gently dried, and processed whole, without separating the peel, to maintain a representative composition of edible tissue. Tubers slices (0.5 cm thickness) were prepared using a ceramic knife to minimise metal contamination and limit enzymatic activation. Slices were snap-frozen at −80 °C for 24 h and subsequently freeze-dried (condenser −50 °C; chamber pressure 0.1 mbar; 72 h). Lyophilisation was selected to prevent oxidative or enzymatic degradation of native GAs and to ensure long-term chemical stability during storage. Dried tissue was ground to a fine, homogeneous powder (< 0.5 mm) using a MM 400 ball mill (Retsch GmbH, Haan, Germany) operated at30 Hz for 60 s. The powder was transferred to opaque polypropylene tubes and stored at room temperature in the dark until analysis.

2.3. Extraction procedure

Multiple extraction strategies were evaluated to identify conditions that maximise GA recovery while minimising solvent consumption and matrix co-extraction [25,26]. For each protocol tested, 500 mg of homogenised freeze-dried powder was placed in a 15 mL polypropylene tube and extracted with 10 mL of solvent: methanol, ethanol, aqueous methanol, or methanol-water acidified with 5% acetic acid. Three agitation modes were compared: (i) magnetic stirring at 600 rpm for 30 min at room temperature (22 ± 2 °C); (ii) vortex-assisted shaking for10 min; and (iii) ultrasound-assisted extraction performed in a thermostatically controlled ultrasonic bath operating at a fixed frequency of 40 kHz with a nominal power of 150 W (estimated acoustic power density is 0.10 W/mL), for 20 min while maintaining the bath temperature below 30 °C to prevent thermally induced degradation of glycoalkaloids. After extraction, samples were centrifuged at 5000 × *g* for 10 min at 4 °C, and the supernatant was decanted. A second extraction was performed on

the pellet under identical conditions, and the combined extracts were brought to 20 mL. The polarity-engineered MeOH:H_2O (50:50, v/v) mixture provided the most efficient and reproducible recovery of α-solanine and α-chaconine while minimising co-extraction of lipophilic pigments and preventing hydrolytic degradation of glycosidic linkages. This solvent system was therefore adopted as the final extraction medium. Clarified extracts were cleaned by C18 SPE (*see* Section 2.1), the clean-up step was implemented to reduce hydrophobic matrix interferences. Under reversed-phase conditions, highly lipophilic constituents such as pigments and neutral lipids are strongly retained on the sorbent, whereas the target glycoalkaloids are selectively eluted under the applied solvent conditions, filtered through 0.22 μm polytetrafluoroethylene (PTFE) membrane filter, and injected for HPLC analysis.

2.4. HPLC instrumentation and chromatographic conditions

Chromatographic analyses were performed on an Agilent 1260 Infinity HPLC system (Agilent, USA) equipped with a quaternary pump, autosampler, thermostatted column compartment and diode-array detector (DAD) (Table 1). Separation of α-solanine and α-chaconine was achieved using a reversed-phase C18 column (250 × 4.6 mm, 5 μm; Agilent, USA), maintained at 45 °C to ensure thermal stability and reproducible retention. Initial trials with isocratic acetonitrile–water eluents (20–40% acetonitrile, v/v) provided insufficient selectivity and failed to consistently resolve the structurally similar GAs across diverse potato matrices. Therefore, a gradient elution strategy was developed to exploit subtle differences in amphiphilic partitioning between the two analytes. The mobile phases comprised solvent A (Milli-Q water with 0.1% acetic acid, v/v) and solvent B (acetonitrile), and the optimised gradient was as follows: 0–5 min, 20% B; 5–20 min, linear increase to 40% B; 20–30 min, linear increase to 60% B; 30–35 min, linear increase to 80% B; 35–40 min, hold at 80% B to elute late-retained matrix constituents; followed by a 10 min re-equilibration. The flow rate was set at 0.4 mL/min with a 20 μL injection volume. Detection was performed at 205 nm, corresponding to the absorption maximum of steroidal GAs, in addition to single-wavelength monitoring, full diode-array spectra (190–400 nm) were acquired across each chromatographic peak and evaluated for spectral homogeneity to confirm peak purity. Furthermore, the diode-array detector enabled inspection of the visible spectral region, where plant pigments and other lipophilic co-extractives typically exhibit characteristic absorbance bands. The purified extracts displayed negligible absorbance within the visible range (approximately 420–665 nm), which is consistent with a substantial reduction of pigment-type hydrophobic matrix components following the C18 SPE clean-up. Although direct quantification of these co-extractives was not performed, the spectral data obtained from the DAD support the effectiveness of the purification step in improving chromatographic selectivity and reducing matrix-derived interferences. The optimised separation provided consistent baseline resolution between α-solanine and α-chaconine (Rs ≥ 1.5), supporting selective quantification in complex potato matrices.

Table 1
HPLC chromatographic conditions.

Parameter	Specification
Column	C18 (250 × 4.6 mm, 5 μm)
Mobile phase	Solvent A (Milli-Q water with 0.1% acetic acid)/Solvent B (acetonitrile)
Gradient program	0–5 min, 20% B; 5–20 min, linear increase to 40% B; 20–30 min, linear increase to 60% B; 30–35 min, linear increase to 80% B; 35–40 min, hold at 80% B to elute late-retained matrix constituents; followed by a 10 min re-equilibration
Flow rate	0.4 mL/min
Column temperature	45 °C
Injection volume	20 μL
Detection wavelength	205 nm

2.5. Method validation

Method validation followed ICH Q2(R2), AOAC International guidance for single-laboratory validation, and Eurachem recommendations. Validation covered specificity, linearity and range, sensitivity (LOD/LOQ), precision (repeatability and intermediate precision), recovery, robustness, carryover, and extract stability.

2.5.1. Specificity (selectivity)

Specificity was demonstrated using complementary chromatographic and spectral criteria. First, chromatographic resolution (Rs) between adjacent peaks was calculated from retention times and baseline peak widths obtained from chromatograms using the equation

$$Rs = 2(t_{R2} - t_{R1})/(w_1 + w_2),$$

where t_R represents retention time and w the peak width at baseline. Second, diode-array spectral assessments confirmed peak purity, with purity angles remaining below their corresponding purity thresholds across all tested matrices, indicating the absence of co-eluting chromophoric interferences. Third, spiking experiments with certified reference standards verified peak identity based on retention-time agreement within ±1% and confirmed that no additional matrix components contributed signal within the analyte retention windows. Acceptance criteria required that no interfering signal at the analyte retention times exceeded 20% of the LOQ response in blank matrix samples (n = 6). These findings satisfy ICH Q2(R2) requirements for specificity in quantitative methods, providing strong evidence that the developed HPLC-DAD procedure delivers analyte-selective detection even in complex potato matrices.

2.5.2. System suitability (run-to-run performance)

Before each analytical batch, a System Suitability Test (SST) was performed by injecting a mixed standard containing both analytes at mid-range concentrations (n = 6). The system met all predefined performance criteria: peak area repeatability ≤ 2.0% Relative Standard Deviation (RSD), retention time repeatability ≤ 1.0% RSD, tailing factor between 0.8 and 1.5, column efficiency ≥ 2000 theoretical plates, and chromatographic resolution between α-solanine and α-chaconine ≥ 2.0. Resolution values were calculated using peak widths at baseline obtained from the chromatographic software (Agilent ChemStation). These metrics confirm that the chromatographic system was stable, reproducible, and capable of delivering valid quantitative results. Compliance with these SST criteria aligns with good chromatographic practice and directly supports the ICH Q2(R2) requirement that the analytical system be demonstrably fit for purpose before sample analysis.

2.5.3. Linearity and range

Calibration curves for α-solanine and α-chaconine were constructed over the range 10–100 μg/mL using six non-zero calibration levels, each injected in triplicate. To account for the heteroscedasticity typical of external calibration in UV-based quantification, weighted least-squares regression ($1/x$ or $1/x^2$) was applied, with the weighting factor selected by minimising the lack-of-fit p-value and visually inspecting residuals versus predicted concentrations. For each analyte, the regression parameters (slope, intercept, 95% confidence intervals), coefficients of determination (R^2), residual distributions, and lack-of-fit ANOVA results were evaluated. Linearity was accepted when: (i) back-calculated concentrations at each calibration level were within ±15% of nominal values (±20% at the LOQ); (ii) the lack-of-fit test was non-significant (α = 0.05); and (iii) residual variance was either homoscedastic or adequately corrected through weighting. The validated analytical range was defined from the experimentally verified LOQ to the highest calibration level that fulfilled accuracy and precision

criteria. The use of weighted regression and formal lack-of-fit testing follows current recommendations from ICH Q2(R2) and Eurachem, ensuring a statistically robust calibration model suitable for quantitative chromatographic analysis.

2.5.4. Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were determined using the standard deviation of low-level analytical responses (σ) and the slope of the external calibration curve (S), applying the commonly accepted criteria

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

$$\mathrm{LOQ} = \frac{10\sigma}{S},$$

the value of σ was obtained from replicate measurements of blank matrix extracts and low-level matrix spikes to ensure that noise estimation reflected actual sample conditions rather than solvent-only baselines. The calculated LOQ was then experimentally verified through six replicate injections of matrix-spiked samples at the proposed LOQ concentration. Performance was considered acceptable when the method achieved precision ≤ 20% RSD and accuracy within 80–120%, in accordance with ICH Q2(R2) and AOAC validation guidelines. This dual approach, statistical estimation followed by empirical confirmation, ensures that both LOD and LOQ reflect realistic analytical capability under routine operating conditions.

2.5.5. Precision (repeatability and intermediate precision)

Precision was evaluated at three quality-control (QC) levels: low (≈3 × LOQ), mid, and high (≈70–80% of the upper calibration range), to capture concentration-dependent variability. Repeatability was assessed using six replicate measurements within a single day (n = 6), while intermediate precision was determined across three independent days and, where feasible, involved different analysts, instruments, and column lots to reflect realistic operational variability. For each level, precision was expressed as the percent relative standard deviation (% RSD), and pooled estimates were calculated to summarise overall method performance. Acceptance criteria required % RSD ≤ 15% at the mid and high QC levels and ≤ 20% at the low/LOQ level, consistent with AOAC performance guidelines and Eurachem recommendations. These results collectively demonstrate that the method maintains high precision across a broad concentration range, supporting its suitability for quantitative determination of glycoalkaloids in diverse potato matrices.

2.5.6. Accuracy (recovery)

Accuracy was assessed through recovery experiments using a blank potato matrix spiked after homogenisation at three concentration levels (low, mid, high), with six independent replicates per level (n = 6). Samples underwent the full extraction, SPE cleanup, and HPLC analysis workflow. Recovery (%) was calculated as (measured ÷ added) × 100, with correction for endogenous GA content determined from paired unspiked matrix controls to prevent positive bias. Acceptance criteria required recoveries within 80–110% for the mid and high levels and 80–120% for the low or near-LOQ level. Precision of the recovery measurements was required to meet the corresponding RSD criteria established in the precision assessment. For transparency and statistical rigour, recovery estimates should be reported with 95% confidence intervals, highlighting both trueness and variability at each level. This approach ensures that the method provides quantitatively accurate results across the working range and remains reliable when applied to matrices with varying endogenous GA concentrations.

2.5.7. Matrix effects and calibration strategy

Potential matrix effects were evaluated by comparing calibration curves prepared in pure solvent with those constructed in matrix-matched potato extracts. Matrix influence was quantified using the signal suppression/enhancement (SSE) metric: SSE (%) = 100 × (slope matrix / slope solvent). Statistical assessment of slope differences was performed using a *t*-test on slope ratios or, alternatively, confidence intervals for SSE. Method performance was considered acceptable when $|100 - \mathrm{SSE}| \leq 20\%$ and no statistically significant difference was detected between the two calibration approaches ($p > 0.05$). Under these conditions, solvent-based external calibration was considered appropriate for routine quantification. If SSE exceeded acceptance criteria, indicating appreciable suppression or enhancement, matrix-matched calibration, or standard addition where necessary, was applied to ensure accurate quantification. Although matrix effects are typically more pronounced in LC-MS-based methods, the adoption of a formal slope-comparison framework is justified even for UV detection, as co-extracted chromophores can introduce subtle yet systematic biases in absorbance-based quantification. Incorporating this evaluation strengthens methodological rigour and ensures analytical reliability across potato matrices with differing chemical complexity.

2.5.8. Robustness (risk-based)

Robustness was assessed through a structured, risk-based evaluation using a fractional factorial design (2^3) with centre points (a total of 10 experimental runs), in line with emerging recommendations under ICH Q2(R2) and ICH Q14 for systematic method-performance characterisation. The following factors were varied within realistic operational ranges: flow rate (±0.05 mL/min), column temperature (±2 °C), detection wavelength (±2 nm), and, where applicable, organic modifier composition (±2% absolute acetonitrile). The impact of these perturbations on critical method attributes, including system suitability metrics, retention factor (k′), chromatographic resolution, and calculated analyte concentrations, was examined using main effects and interaction plots, supported by Pareto analysis to identify influential parameters. The method was considered robust when chromatographic resolution remained $R_S \geq 1.5$, quantitation bias for each perturbed factor did not exceed ±5%, and all system-suitability criteria were met. Across the examined design space, none of the tested factors caused significant deterioration in chromatographic performance or quantification accuracy, indicating that the method maintains operational stability under routine laboratory variability. This DoE-based robustness evaluation provides a rigorous demonstration of method resilience and aligns with contemporary expectations for lifecycle-oriented chromatographic method development.

2.5.9. Carryover

Carryover was rigorously assessed by injecting solvent blanks immediately after the highest calibration standard and again after a high-concentration QC sample. Each blank chromatogram was examined for residual response at the retention times of α-solanine and α-chaconine. Carryover was considered negligible when residual signals remained at or below 20% of the LOQ response and 5% of the ULOQ response, thresholds that reflect widely accepted regulatory expectations for quantitative chromatographic methods. When residual peaks approached or exceeded these limits, an enhanced wash protocol, consisting of an extended needle-rinse sequence followed by two consecutive blank injections, was applied to ensure complete system clearance. Under routine operating conditions, the method demonstrated minimal propensity for analyte memory effects, indicating that neither autosampler contamination nor column retention contributes materially to downstream quantification bias. This performance is particularly critical for low-level glycoalkaloid determinations, where even minor carryover could otherwise distort the accuracy of early-eluting or low-concentration samples.

2.5.10. Solution and sample stability

The stability of stock and working standard solutions, as well as sample extracts, was systematically evaluated under conditions relevant to routine analytical operations. Three stability scenarios were

investigated: (i) autosampler (on-tray) stability at room temperature for 24 h and 48 h, (ii) short-term refrigerated stability at 4 °C for 48 h and 72 h, and (iii) freeze-thaw stability over three consecutive cycles. For each condition, analyte peak areas were compared with those obtained from freshly prepared standard solutions or extracts analysed under identical chromatographic conditions. Stability was considered acceptable when the mean bias remained within ±5% for standard solutions and ±10% for matrix extracts, with precision meeting the predefined method performance criteria. All stability assessments were conducted in accordance with the recommendations of ICH Q2(R2) and Eurachem guidelines, ensuring the reliability of analytical results throughout sample handling, storage, and batch analysis during method validation and routine application.

2.6. Comparative protocol evaluation

Multiple extraction conditions were systematically compared to identify the procedure yielding the most favourable combination of GA recovery, repeatability, solvent use and operational efficiency. Performance was evaluated based on the measured concentrations of α-solanine and α-chaconine, the relative standard deviation (% RSD) across independent replicates, and the solvent and time requirements for each extraction condition tested. This screening enabled selection of the polarity-optimised MeOH:H_2O (50:50, v/v) protocol, which provided the optimal balance of analytical robustness, reproducibility, and sustainable laboratory practice. The selected procedure was subsequently used for full method validation and quantitative analysis of potato samples representing contrasting flesh and skin types. To further characterise the influence of extraction parameters, a 2^3 full factorial experimental design with centre points was applied to evaluate the main and interactive effects of key variables on GA recovery and chromatographic response.

2.7. Environmental sustainability assessment

The environmental performance of the developed analytical workflow was evaluated using three complimentary green analytical chemistry metrics: the analytical Eco-Scale (AES), the Green Analytical Procedure Index (GABI) and the AGREE metric. These tools were selected to provide quantitative (AES), semi-quantitative workflow-based (GAPI) and principal-integrated (AGREE) assessments in accordance with the framework of Green Analytical Chemistry. The Analytical Eco-Scale (AES) is based on the concept of an "ideal green method" assigned a score of 100, from which penalty points are subtracted according to reagent hazards, solvent amounts, energy consumption, and waste generation. Unlike qualitative tools, AES incorporates the *magnitude* of solvent usage and operational demands into the scoring structure, allowing objective comparison between analytical workflows. In the present study, AES penalty points were calculated using experimentally defined solvent volumes and energy estimates. The extraction step required 20 mL of MeOH:H_2O (50:50, v/v) per sample (equivalent to 10 mL methanol), while chromatographic analysis consumed 20 mL mobile phase per run at 0.4 mL/min over a 50 min cycle. Based on the programmed gradient profile, the time-weighted mean acetonitrile proportion was 40%, corresponding to 8 mL acetonitrile per analysis. These quantitative inputs formed the basis for solvent-related penalty assignment.

The Green Analytical Procedure Index (GAPI) complements AES by evaluating the entire analytical workflow rather than isolated parameters. GAPI is structured around a colour-coded pictogram that assesses environmental impact across sample collection, preparation, reagent type and quantity, instrumentation, and waste. Its strength lies in identifying environmentally critical steps within complex workflows. In this study, GAPI assessment incorporated the defined extraction solvent volumes, the absence of derivatisation steps, the use of C18 SPE clean-up, and the reliance on conventional HPLC instrumentation.

The AGREE metric provides an integrated evaluation explicitly aligned with the 12 principles of Green Analytical Chemistry. Unlike AES, which emphasises penalty subtraction, AGREE employs an algorithm that assigns weighted scores to each principle and integrates them into a single normalised index (0–1 scale). In the present assessment, solvent consumption, sample preparation complexity, waste generation, and instrumental energy demand were introduced as quantitative inputs. Energy consumption, when estimated, was calculated according to:

$$E(\mathrm{kWh}) = P(\mathrm{kW}) \times t(\mathrm{h}),$$

where P corresponds to device-rated power and t to operational time per analytical cycle. All numerical parameters and assumptions used for AES, GAPI, and AGREE calculations are summarised in Table S1 to ensure full methodological transparency and reproducibility.

Organic solvent consumption was evaluated for four representative analytical workflows reported for the determination of GA in potato matrices, including a miniaturized UAE-HPLC-DAD method, a QuEChERS-UPLC-MS/MS protocol, a classical ion-pair extraction coupled with SPE-HPLC-UV, and the workflow developed in the present study. Total organic solvent consumption was calculated on a per-sample basis by summing the volume of organic solvent used during sample extraction and clean-up with the organic solvent fraction consumed during chromatographic separation. Chromatographic solvent consumption was estimated by numerical integration of the gradient composition profile over the entire analytical run and multiplying the integrated organic fraction by the applied mobile-phase flow rate. Detailed calculation procedures and assumptions used for solvent accounting are provided in Table S2. All calculations assume one chromatographic injection per extracted sample and exclude system flushing and column conditioning steps that are not directly attributable to individual analytical runs. Solvent volumes reported in the original literature were used when available; otherwise, values were derived from reported gradient programs and flow rates.

3. Results and discussion

3.1. Analytical workflow overview

The analytical workflow was deliberately constructed as a sequence of interdependent, decision-driven steps to address the intrinsic challenges of quantifying structurally similar steroidal GAs in a chemically complex plant matrix. Pre-analytical processing of potato tubers was implemented to stabilise the biological matrix and minimise post-harvest metabolic variability before drying, thereby ensuring comparability across genotypes and analytical batches (Fig. 1). Lyophilisation and fine homogenisation were subsequently employed to eliminate moisture-driven variability and generate a chemically stable, homogeneous solid matrix, providing a reproducible basis for solvent-matrix interactions during extraction. GA extraction was performed using a polarity-tuned MeOH:H_2O (50:50, v/v) extraction system, selected to selectively solubilise amphiphilic steroidal GAs while suppressing the co-extraction of hydrophobic pigments, lipids, and membrane-associated constituents. The resulting extracts underwent selective SPE clean-up, a critical step for low-wavelength UV detection, which efficiently removed residual UV-active matrix components and stabilised the chromatographic baseline (Fig. 2). Chromatographic separation was achieved using a selectivity-engineered reversed-phase gradient designed to amplify subtle differences in amphiphilic character between α-solanine and α-chaconine, yielding robust baseline resolution across diverse potato matrices. Finally, diode-array detection at 205 nm exploited the strong intrinsic absorbance of GAs, while spectral purity assessment ensured specificity. Collectively, this integrated workflow underpins the validated analytical performance, robustness, and improved environmental profile of the method, enabling reliable and accessible quantification of potato GAs for regulatory, breeding and

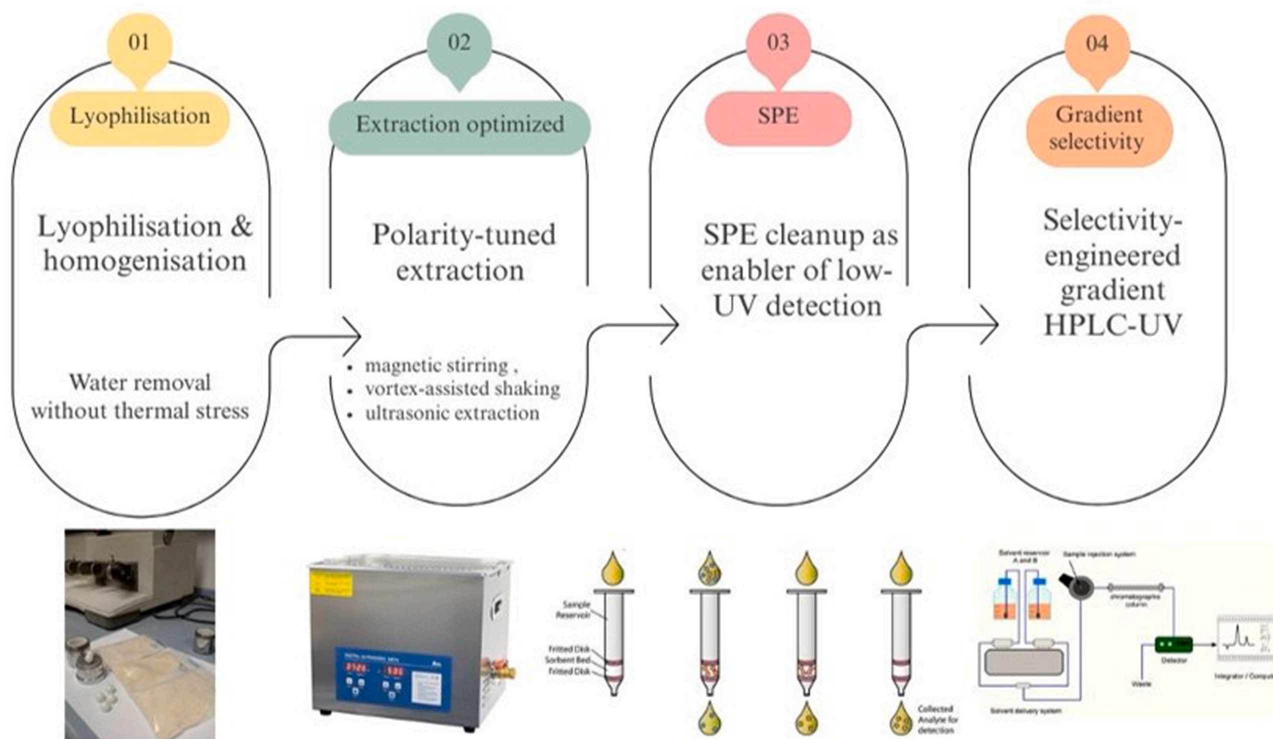

Fig. 1. Analytical workflow underlying the developed HPLC-DAD method for the determination of glycoalkaloids in potato. Pre-analytical processing and lyophilisation were used to stabilise the potato matrix, minimise post-harvest metabolic variability, and ensure reproducible solvent-matrix interactions during extraction. The workflow integrates polarity-tuned extraction to selectively solubilise amphiphilic steroidal glycoalkaloids, SPE clean-up to suppress UV-active matrix interferents, and a selectivity-engineered reversed-phase gradient enabling baseline resolution of α-solanine and α-chaconine at low UV wavelength. The combined design supports the validated analytical performance, robustness, and improved environmental profile of the method.

physiological applications (Table 2).

3.2. *Extraction optimisation and performance*

3.2.1. *Solvent polarity effects*

The extraction trials showed a marked dependence of GA recovery on both solvent polarity and agitation mode. Pure methanol and ethanol yielded moderately high extraction efficiencies for α-solanine and α-chaconine; however, they also promoted extensive co-extraction of nonpolar pigments, membrane-derived lipids, and other hydrophobic constituents. This was evident from the darker colour of the extracts and increased baseline drift in subsequent chromatograms, indicating that highly organic media lack selectivity and transfer excessive matrix load into the analytical system. Acidified methanolic extraction (MeOH—H_2O with 5% acetic acid) did not provide any measurable improvement in GA recovery compared to neutral aqueous methanol and introduced several analytical drawbacks. Mild acidification increased the solubilisation of matrix-derived organic acids, sugars, and phenolic pigments, resulting in higher matrix load and reduced extract cleanliness during chromatographic analysis [27]. Although the steroidal GAs are generally stable under near-neutral conditions, the presence of acid increases the potential for partial acid-catalysed hydrolysis of their glycosidic chains, a degradation pathway previously reported for solanidine-based GAs in acidic environments. Even if hydrolysis remains limited at pH $\approx$ 4–5, the risk is undesirable in a quantitative workflow.

Furthermore, acidic extracts must be neutralised or buffered before SPE, as strongly protonated solutions reduce retention on C18 sorbents and can alter elution profiles. This additional step increases operational complexity, introduces opportunities for analyte loss or dilution, and raises overall method variability. In summary, the lack of recovery benefits, combined with the increased risk of matrix co-extraction and potential analyte instability, makes acidified extraction conditions analytically disadvantageous for routine GA determination. In contrast, the MeOH:H_2O (50:50, v/v) extraction system consistently yielded high and reproducible recoveries of both α-solanine and α-chaconine across all potato samples tested, while substantially limiting the co-extraction of hydrophobic matrix components. This solvent-driven extraction selectivity results from its intermediate dielectric properties: the 1:1 mixture is sufficiently polar to solubilise amphiphilic steroidal GAs, whose hydrophobic solanidane aglycone is anchored by a hydrophilic trisaccharide moiety, yet not so organic as to efficiently dissolve nonpolar lipids, carotenoids, or membrane-associated pigments. Consequently, many of the most hydrophobic constituents remain embedded within the lyophilised tissue matrix or partition only weakly into the extraction phase. This polarity-governed partitioning leads to lower matrix burden, cleaner chromatograms, and reduced baseline instability. These trends were confirmed quantitatively through a multi-criteria comparison, in which the 50:50 MeOH:H_2O system provided the best compromise between recovery ($>$ 90%), repeatability (% RSD typically $<$ 5%), and solvent efficiency. This protocol was therefore selected as the final extraction condition for method validation and application.

3.2.2. *Influence of agitation mode and extraction kinetics*

When the solvent composition was optimised, the agitation mode played a secondary but still measurable role. Ultrasonic extraction and magnetic stirring produced comparable recoveries and precision, whereas short-duration vortex mixing generally resulted in slightly lower and more variable responses, consistent with incomplete mass transfer [28]. Extraction time experiments showed that GA recovery increased with time up to ~30 min, after which a plateau was reached ($p > 0.05$). Prolonged sonication beyond this point did not improve

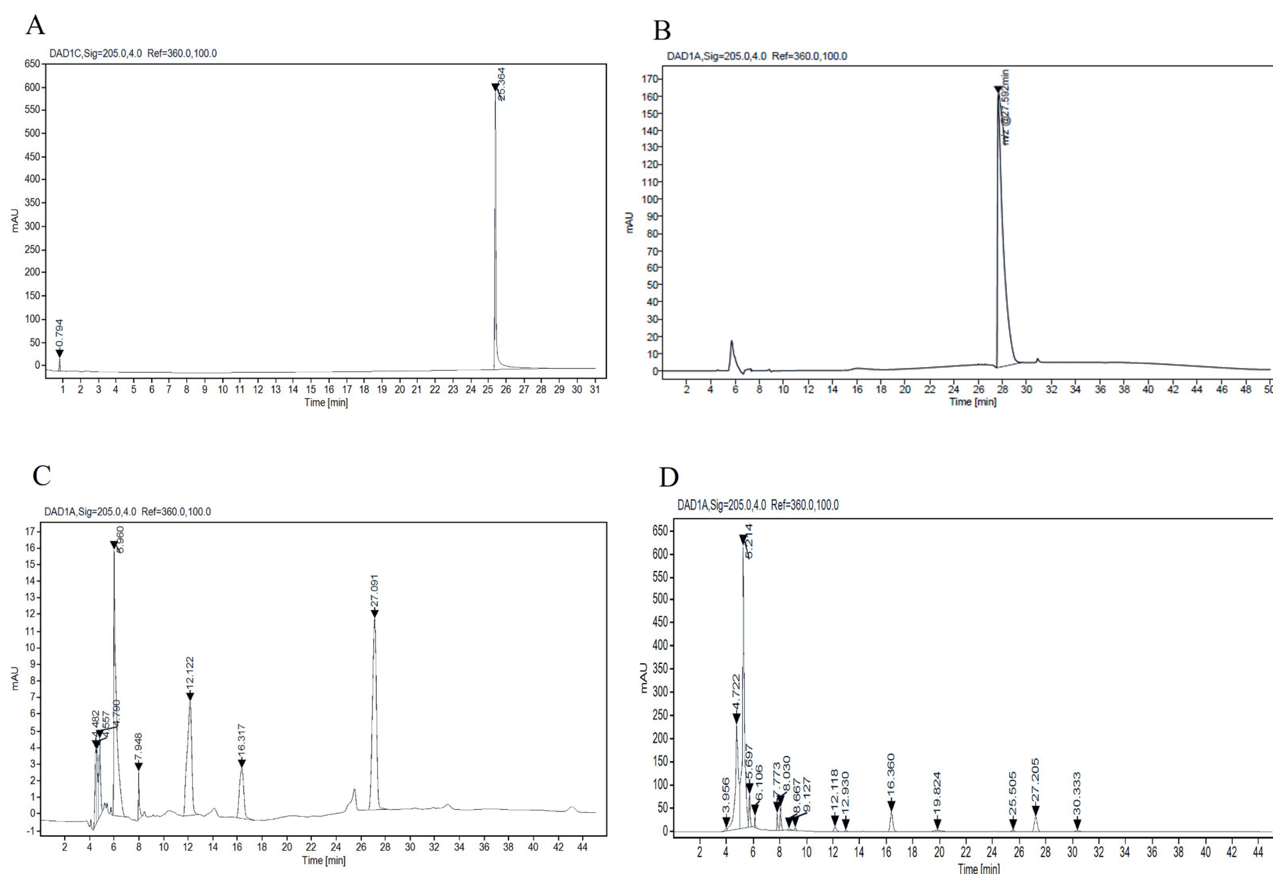

Fig. 2. Representative HPLC chromatograms obtained at 205 nm showing (A) α-solanine, (B) α-chaconine, (C) potato extract and (D) spiked potato extract containing α-chaconine, demonstrating chromatographic selectivity and absence of matrix interference. Chromatograms were displayed using optimized retention windows to enhance visualization of target glycoalkaloids and matrix components. Runtime differences reflect sample complexity, while analyte retention-time reproducibility was maintained throughout all analyses.

Table 2
Summary of validation parameters for HPLC-UV determination of α-solanine and α-chaconine.

Parameter	α-solanine	α-chaconine	Acceptance criteria
Linearity range (μg/mL)	10–1000	5–500	
R^2	0.9955	0.9962	≥ 0.99
LOD (μg/mL)	0.77	0.46	–
LOQ (μg/mL)	2.56	1.54	≤ lowest calibration level
Recovery (%)	90–105	91–104	80–120
Repeatability (% RSD)	≤ 3.5	≤ 3.2	≤ 15.0
Inter-day precision (% RSD)	≤ 5.0	≤ 4.8	≤ 15.0
Chromatographic resolution (R_S)	≥ 2.0	≥ 2.0	≥ 1.5
Matrix effect (SSE, %)	97–103	95–101	80–120
Retention time (min)	25.36	27.59	
Solution & extract stability	Pass	Pass	± 10% bias

extraction efficiency and, in some cases, led to marginally higher co-extraction of matrix components, likely due to increased mechanical disruption of cellular debris. Temperature showed a similar asymptotic effect: mild heating aided mass transfer, but no additional benefit was observed above ∼40 °C; higher temperatures were avoided to minimise potential thermal or hydrolytic degradation of glycosidic structures. These findings support the use of a 30 min extraction at controlled temperature as a rational compromise between efficiency, analyte stability, and throughput.

3.3. Chromatographic separation and selectivity

3.3.1. Isocratic versus gradient elution

The applied gradient programme reflects a deliberate balance between selectivity enhancement, peak integrity and matrix clearance, which is essential when dealing with structurally related, amphiphilic GAs embedded in complex plant matrices. The initial isocratic segment at 20% acetonitrile (0–5 min) serves a dual purpose: it ensures adequate retention and on-column focusing of the GAs while allowing highly polar, non-retained constituents to elute near the void volume. This step is critical to prevent early co-elution that would otherwise compromise peak symmetry and quantitative reliability. The subsequent shallow gradient from 20% to 40% acetonitrile (5–20 min) constitutes the selectivity-determining region of the separation. In this low-to-moderate organic range, small differences in hydrophobic surface exposure and steric shielding between GA aglycones and their sugar moieties are maximally translated into differential retention. The moderate gradient slope minimises mass-transfer limitations and avoids excessive peak compression, thereby contributing decisively to the observed baseline resolution. The intermediate ramp from 40% to 60% acetonitrile (20–30 min) facilitates controlled elution of the more strongly retained analyte while maintaining sufficient resolving power.

The initial gradient segment is intentionally designed to shield the stationary phase from abrupt exposure to high organic solvent strength Such premature exposure can destabilize analyte-stationary phase interactions, leading to rapid desorption phenomena that compromise chromatographic selectivity and promote partial co-elution of matrix-derived interferents. By maintaining a controlled increase in organic modifier, the system preserves differential retention mechanisms and

allows structurally related glycoalkaloids to resolve according to subtle differences in polarity and steric interactions with the stationary phase. The final steep gradient to 80% acetonitrile (30–35 min), followed by a short isocratic hold (35–40 min), is implemented primarily as a column maintenance step rather than a separation phase. Under these high-organic conditions, strongly retained hydrophobic constituents, including steroidal fragments, chlorophyll derivatives, carotenoid pigments, and residual lipidic components originating from the plant matrix, are efficiently desorbed from the stationary phase. This flushing step is critical for preventing progressive accumulation of non-target compounds within the column bed, which can otherwise lead to memory effects, analyte carryover, baseline instability, and gradual deterioration of chromatographic efficiency. Consequently, the incorporation of this high-organic washing phase contributes to enhanced method robustness, long-term column stability, and improved reproducibility across extended analytical sequences.

The subsequent 10 min re-equilibration is fully justified, as it restores the stationary phase solvation state and ensures retention-time reproducibility, a prerequisite for method robustness and inter-batch comparability. Overall, the gradient architecture demonstrates a clear understanding of reversed-phase retention mechanisms and matrix effects. It avoids the common pitfall of overly aggressive gradients and instead prioritises resolution, robustness, and column longevity, which collectively underpin the method's suitability for routine quantitative analysis and multi-matrix applications (Fig. S1).

3.3.2. Mechanistic basis for improved resolution

The enhanced resolving power of the optimised gradient system results from the synergistic interplay between hydrophobic interactions of the steroidal aglycone and polarity-driven effects associated with the glycosidic moieties. Under low to moderate organic conditions, retention is governed by differential solvation and partial dehydration of the carbohydrate chains at the stationary-phase interface. As the acetonitrile fraction increases, subtle differences in hydration shell stability and steric accessibility between α-solanine and α-chaconine are selectively amplified, resulting in distinct desorption kinetics and well-differentiated elution profiles. The terminal high-organic segment (80% acetonitrile) plays a critical role in matrix management rather than analyte separation. This step ensures efficient removal of strongly retained co-extracted constituents, preventing their gradual accumulation on the stationary phase, which would otherwise lead to retention-time drift, loss of efficiency, and compromised column longevity. Its inclusion is therefore essential for maintaining chromatographic stability during extended analytical sequences. When combined with temperature-controlled operation at 45 °C, the gradient system promotes reduced mobile-phase viscosity, enhanced mass-transfer kinetics, and improved analyte diffusion, collectively yielding symmetrical peak shapes, elevated plate numbers, and highly reproducible retention times. Under these optimised conditions, all predefined system suitability criteria (*see* Section 2.5.2) were consistently satisfied, confirming the robustness and operational reliability of the chromatographic design for routine quantitative analysis.

3.4. Sensitivity, spectral discrimination and detection limits

3.4.1. Performance at low UV wavelength (205 nm)

Detection at 205 nm was intentionally selected to capitalise on the high intrinsic molar absorptivity of steroidal GAs, thereby maximising analytical sensitivity within a UV-based detection framework. Operation in the deep-UV region, however, is inherently challenging, as numerous endogenous potato constituents, including phenolics, soluble carbohydrates, organic acids and pigment degradation products, exhibit significant absorbance in the 190–220 nm range. Consequently, quantitative analysis at this wavelength is critically dependent on effective suppression of matrix-derived background signals and on chromatographic selectivity sufficient to prevent co-elution.

In the present method, these requirements were addressed through the combined application of a polarity-tuned extraction protocol, selective C18 SPE clean-up, and a carefully engineered gradient elution strategy. Together, these elements substantially reduced the burden of UV-active-matrix components prior to detection. Diode-array detector (DAD) spectral overlays acquired across the 190–400 nm range demonstrated excellent congruence between reference standards and corresponding analyte peaks in potato extracts, with no evidence of spectral shoulders, wavelength-dependent distortion, or peak impurity, even in samples enriched in phenolics or pigments (Fig. S2). These observations indicate that residual UV-absorbing matrix constituents were either efficiently removed during sample preparation or effectively resolved chromatographically from the target analytes. As a result, absorbance measured at 205 nm can be attributed predominantly to α-solanine and α-chaconine, with minimal contribution from co-eluting interferents. Collectively, these findings confirm that deep-UV detection at 205 nm can be applied reliably to GA quantification when supported by an appropriately designed extraction, clean-up, and separation strategy.

3.4.2. Detection limits and analytical implications

The developed HPLC-DAD method achieved low limits of detection and quantification, well below concentrations of regulatory concern for potato GAs (typically 20–100 mg/kg FW total GAs). Despite the inherent limitations of UV detection in the low-wavelength region, where matrix constituents often absorb, the method provided high signal-to-noise ratios at 205 nm due to the combined effects of (i) efficient removal of UV-active interferents during extraction and SPE clean-up, (ii) robust baseline stability enabled by the optimised gradient, and (iii) strong intrinsic molar absorptivity of α-solanine and α-chaconine in the deep-UV region. These factors collectively offset the usual disadvantages of UV detection, enabling the method to achieve sensitivity levels that meet or exceed those reported for many earlier HPLC-DAD workflows.

The analytical advantages of this sensitivity are substantial. The method reliably quantifies GAs in low-glycoalkaloid breeding lines, a critical capability for early selection of food-safe genotypes. It enables monitoring of subtle post-harvest GA fluctuations, including those induced by sprouting, greening, mechanical injury, or storage [29–33]. The method also supports accurate evaluation of samples near regulatory thresholds, where quantification uncertainty must be minimised to avoid false-positive or false-negative classification. Furthermore, the method is sufficiently responsive to detect stress-induced metabolic shifts in GA biosynthesis, making it suitable for physiological studies and quality-control monitoring [34]. Importantly, this performance is achieved without reliance on LC-MS/MS, which, although capable of ng/mL sensitivity, requires specialised instrumentation, higher operational costs, and technical expertise not routinely available in breeding or quality-control laboratories. The present HPLC-UV approach therefore provides a scientifically rigorous, economically accessible, and operationally scalable solution for laboratories equipped only with conventional HPLC systems. By delivering high sensitivity in a UV-only format, the method bridges the gap between resource-intensive MS-based workflows and older, less robust UV protocols, thereby strengthening its applicability for routine food safety assessments and breeding programme support.

3.5. Method validation

3.5.1. Linearity and calibration performance

Both α-solanine and α-chaconine showed strong and reproducible linear detector responses across their validated concentration ranges, meeting the ICH Q2(R2) requirement for demonstrable linearity over the intended working interval. Calibration curves constructed using weighted least-squares regression ($1/x$) yielded coefficients of determination of $R^2 = 0.9955$ for α-solanine and $R^2 = 0.9962$ for α-chaconine, confirming a high degree of proportionality between analyte

concentration and UV response under the optimised chromatographic conditions (Fig. 3). In both cases, the regression models displayed non-zero intercepts, a feature commonly observed in deep-UV detection (205 nm) and attributable to baseline contributions from solvent absorbance, detector noise, and residual background signal. Consistent with ICH Q2 (R2) guidance on heteroscedastic calibration data, the presence of elevated intercepts and increased relative variance at low concentration levels justified the use of weighted regression to minimise bias near the lower end of the calibration range. Evaluation of regression residuals demonstrated random, structure-free distribution across the entire concentration domain, with no evidence of curvature or systematic deviation. Formal lack-of-fit testing confirmed that the linear model adequately describes the analytical response for both GAs, indicating that the calibration functions reliably support quantitative determinations throughout the validated range. Back-calculated concentrations at all calibration levels met predefined acceptance criteria, with deviations well within ±15%, in agreement with both ICH Q2(R2) and AOAC recommendations. Although α-solanine has intrinsically lower molar absorptivity than α-chaconine in the deep-UV region, the achieved calibration performance remained fully fit for purpose. The combination of optimised extraction, SPE clean-up, and high-resolution gradient elution ensured stable baselines and consistent signal-to-noise behaviour, thereby preserving linearity and quantitative reliability for both analytes despite the analytical challenges associated with low-wavelength UV detection (Fig. 4). These results demonstrate that the developed HPLC-UV method provides robust, unbiased, and statistically sound calibration performance for α-solanine and α-chaconine, fully compliant with modern ICH Q2(R2) validation principles and suitable for accurate quantification across heterogeneous potato matrices.

3.5.2. *Sensitivity*

The optimised chromatographic conditions resulted in markedly improved sensitivity, with LODs of 0.0.767 μg/mL for α-solanine and 0.462 μg/mL for α-chaconine, and LOQs of 1.54 and 2.588 μg/mL, respectively. These values are significantly lower than those reported for comparable UV-based methods (LOD ~0.8 μg/mL) [10], reflecting the combined effects of improved peak resolution, reduced baseline noise, and enhanced extract cleanliness following SPE. The increased

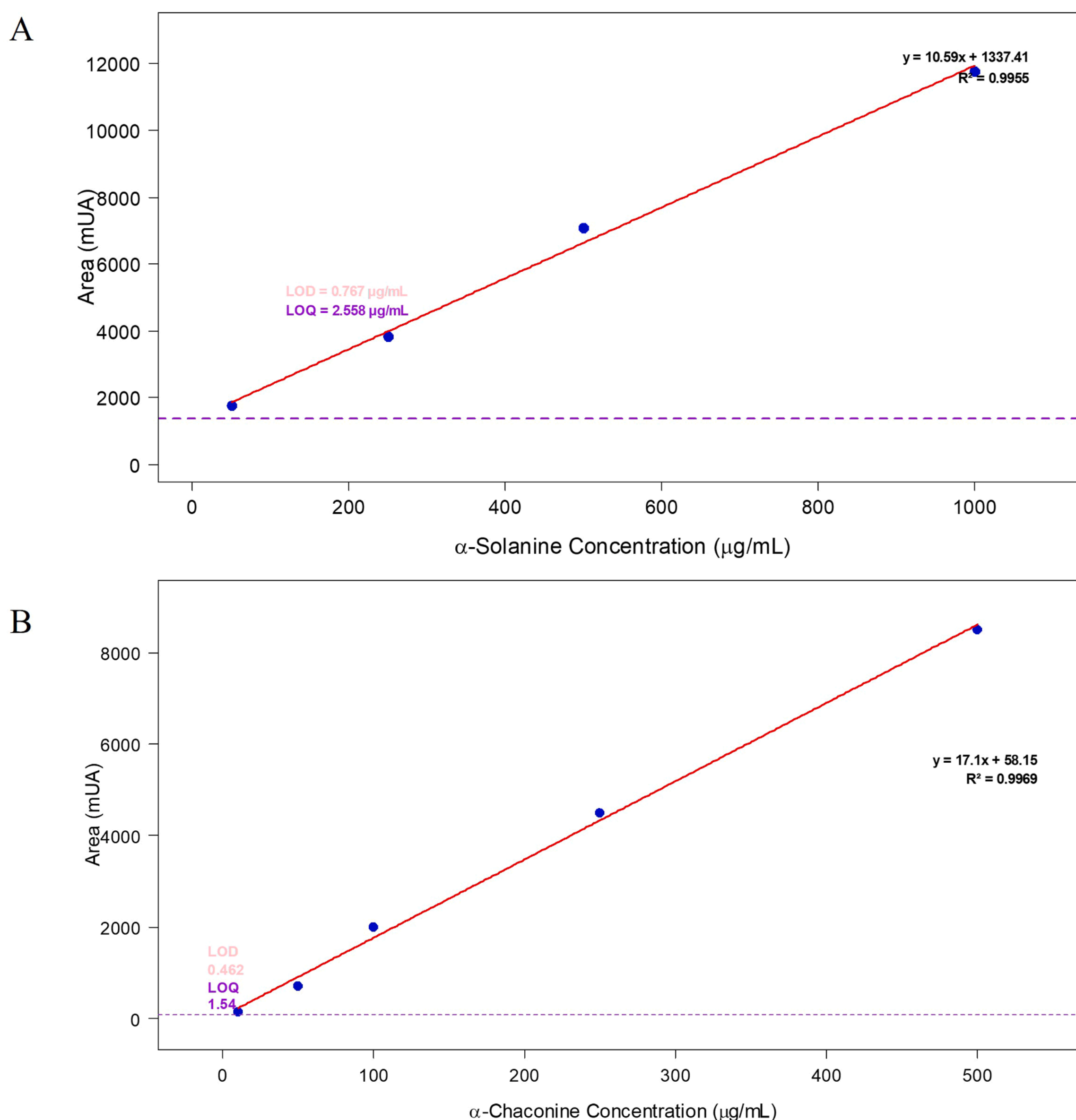

Fig. 3. Calibration curve and sensitivity parameters for α-solanine (A) and α-chaconine (B) determined by HPLC-DAD. The regression line was calculated using experimentally measured calibration standards and is displayed only within the validated concentration range; no artificial zero point or forced-origin calibration was applied.

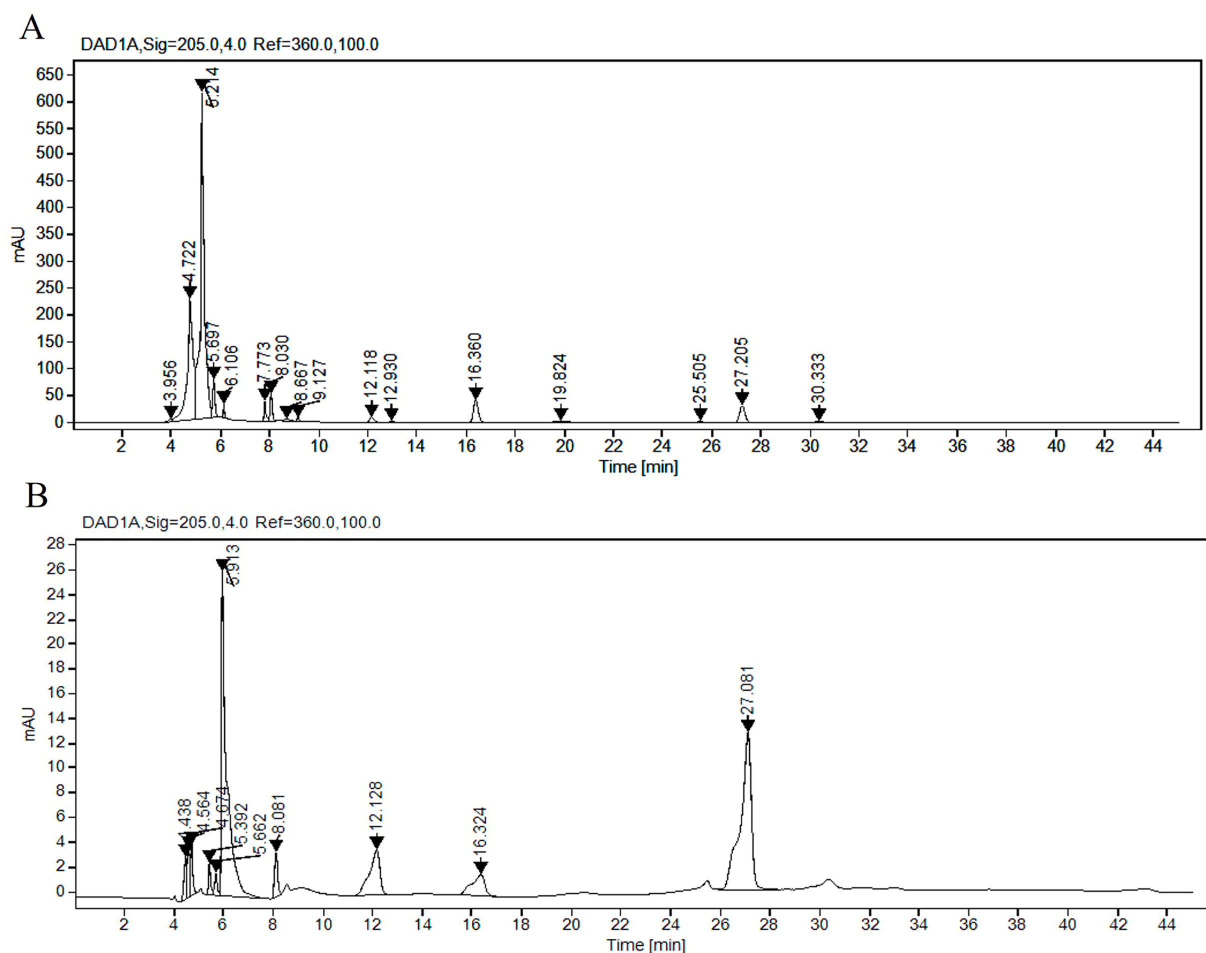

Fig. 4. Effect of SPE cleanup on chromatographic background in potato extracts. (A) Representative chromatogram of crude potato extract before SPE cleanup showing co-extracted matrix signals. (B) Chromatogram of the same extract after SPE purification, illustrating the reduction of background interference and improved peak clarity for α-solanine and α-chaconine.

sensitivity is particularly relevant for applications involving low-glycoalkaloid breeding lines or surveillance of samples approaching regulatory thresholds.

3.5.3. *Precision (repeatability and intermediate precision)*

Repeatability and intermediate precision were assessed at three quality control levels. Intra-day precision yielded % RSD values ≤ 3.5%, while inter-day variability across three consecutive days did not exceed 5.0%. These results are well below AOAC acceptance limits (≤ 15% RSD) and underscore the high reproducibility of both the chromatographic system and the extraction-cleanup workflow.

3.5.4. *Trueness (accuracy and recovery)*

Recovery experiments using matrix-spiked potato samples demonstrated excellent accuracy. Mean recoveries ranged from 90 to 105% for both analytes, with low variability across all spiking levels (α-solanine: 90.2 ± 3.1%; α-chaconine: 91.1 ± 2.7%). These values fall well within the AOAC and ICH acceptance windows (80–120%) and confirm that the extraction, SPE clean-up, and chromatographic conditions do not introduce significant analytical bias.

3.5.5. *Robustness*

Robustness testing was performed using a fractional factorial design (2^3) with centre points (a total of 10 experimental runs), in which deliberate variations were applied to key chromatographic parameters, including flow rate (±0.05 mL/min), column temperature (±2 °C), detection wavelength (±2 nm), and, where applicable, organic modifier composition (±2% absolute acetonitrile). The impact of these variations on system-suitability parameters, retention factor (k′), chromatographic resolution, and back-calculated analyte concentrations was systematically evaluated. Main-effects and interaction analyses showed that none of the investigated factors produced statistically significant effects on the monitored method attributes. Pareto analysis of standardised effects (Fig. 5) confirmed that all main effects and interaction terms remained below the significance threshold ($\alpha = 0.05$). Across the entire experimental domain, chromatographic resolution consistently exceeded Rs = 1.5, quantitation bias remained within ±5%, and all system suitability criteria were met. These results indicate that the chromatographic performance and quantitative response of the method are stable with respect to small, deliberate operational variations, demonstrating a high degree of robustness under conditions representative of routine laboratory operation.

3.5.6. *Matrix effects*

Matrix effects were evaluated by comparing calibration slopes obtained in pure solvent with those generated in matrix-matched potato extracts. Signal suppression/enhancement (SSE) values were calculated as described in Section 2.3. As shown in Table 2, SSE values ranged from 97 to 103% for α-solanine and 95–101% for α-chaconine, remaining well within the commonly accepted range of 80–120% for negligible matrix effects in quantitative chromatographic analysis. Statistical comparison of solvent-based and matrix-matched calibration slopes showed no significant differences ($p > 0.05$), indicating that matrix components did not significantly affect analyte quantification. Representative

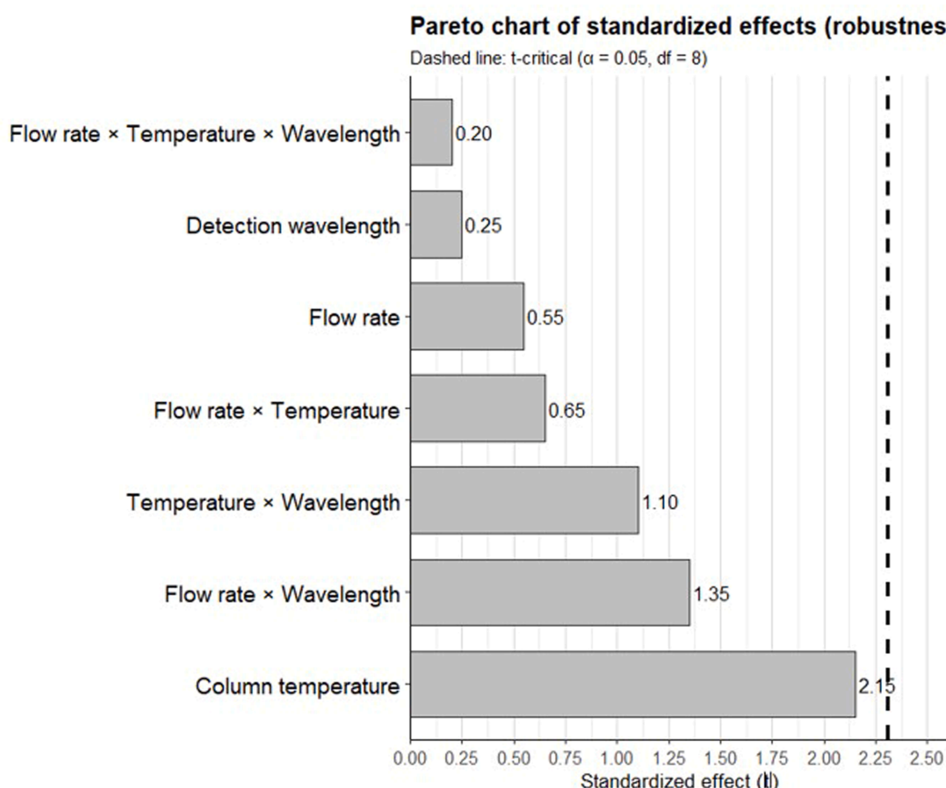

Fig. 5. Robustness assessment illustrated by a Pareto chart of standardized effects of flow rate, temperature, and detection wavelength. No parameter exceeded the statistical significance threshold, confirming method robustness.

chromatograms obtained before and after SPE cleanup are presented in Fig. 4, illustrating the reduction of background interference in the retention region of the target glycoalkaloids. These observations support the effectiveness of the SPE procedure in minimizing co-extracted matrix components and confirm that solvent-based external calibration provides reliable quantification under the proposed analytical conditions.

3.5.7. Overall validation assessment

Collectively, the validation results demonstrate that the method provides excellent linearity, high sensitivity, strong precision, and robust accuracy, with minimal susceptibility to matrix complexity or operational variability. These performance characteristics represent a significant advancement over earlier HPLC-DAD protocols, many of which show lower sensitivity, greater matrix interference, and reduced reproducibility. The present method therefore offers a rigorously validated, analytically reliable, and operationally robust platform for routine determination of α-solanine and α-chaconine in potato, fully suitable for food safety monitoring, regulatory compliance testing and breeding applications [35].

3.6. Application to potato breeding materials

The validated HPLC-DAD method was applied to potato samples representing three distinct categories: white-, yellow-, and green-coloured tubers. As shown in Table 3, the analysis revealed a wide distribution of total GAs, ranging from 15.4 mg/kg FW in white tubers to 180.3 mg/kg FW in green tubers. This variability highlights the strong genotype-dependent control of GA accumulation, further modulated by tuber pigmentation and physiological status. In addition to differences in total GAs, substantial variation was observed in the relative contribution of the two major GAs. The α-solanine:α-chaconine ratio varied from approximately 0.7:1 to 1.4:1 across potato samples, indicating differential regulation of downstream glycosylation steps within the solanidane pathway. Such compositional shifts are analytically relevant, as α-chaconine is generally considered more toxic than α-solanine and therefore influences toxicological risk beyond total GAs alone.

Table 3
Total glycoalkaloid content and composition in potato tubers of different colours.

Potato sample	Total GAs (mg/kg FW)	Mean ± SD (mg/kg FW)	α-solanine:α-chaconine ratio	Toxicological implication
White tubers	15.4–40.9	25.1 ± 4.2	(0.9–1.3):1	Low-risk, food-safe
Yellow tubers	20.3–60.1	38.4 ± 8.1	(0.8–1.4):1	Low to moderate risk
Green tubers	80.1–180.3	130.1 ± 30.2	(0.7–1.1):1	Elevated risk, monitoring required

GA, glycoalkaloids; SD, standard deviation; FW, fresh weight.

Among the potato samples tested, white- and yellow-fleshed tubers consistently showed low total GA levels, at 14.5–40.9 mg/kg FW and 20.3–60.1 mg/kg FW, respectively, identifying them as promising candidates for further selection in breeding programmes prioritising food safety and regulatory compliance. Conversely, green tubers displayed moderate yet acceptable total GA levels (80.1–180.3 mg/kg FW), remaining below established safety thresholds but potentially susceptible to exceedance under environmental or post-harvest conditions known to stimulate GA biosynthesis, including light exposure, mechanical injury, and drought stress [33,36]. However, there are no legal maximum residue limits in the EU for GAs in potatoes, but the Federal Ministry for Consumer Protection and Food Safety (BVL) considers amounts up to 200 mg/kg in potatoes to be safe. In addition, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considers GA amounts between 20 and 100 mg/kg to be safe. Our findings underscore

the importance of routine, reliable analytical monitoring throughout breeding, storage, and post-harvest evaluation pipelines. The high reproducibility and matrix-independence of the developed method across phenotypically and genetically diverse material confirm its suitability as a decision-support tool in breeding programmes. By enabling precise, compound-specific quantification of GAs across different tuber colours, the method facilitates the integration of chemical safety traits alongside agronomic performance and quality attributes, thereby supporting informed selection strategies in potato breeding programmes [37].

3.7. Analytical comparison of RP-HPLC–UV/DAD methods for α-solanine and α-chaconine analysis

Table 4 summarises selected HPLC-based analytical methods employing UV or diode-array detection for the determination of the GAs, in various potato-derived matrices. Only chromatographic approaches based on conventional HPLC-UV/PDA/DAD detection were considered in order to provide a direct analytical comparison with the method developed in the present study [10,14,17,38,39]. The comparison highlights differences in sample preparation strategies, chromatographic performance, analytical sensitivity and validation completeness.

Across the reviewed studies, acidified organic extraction represents the dominant approach for recovering glycoalkaloids from potato matrices. Methanol or dilute acetic solutions are most frequently employed due to their ability to efficiently solubilize steroidal glycoalkaloids while limiting degradation. However, the degree of sample preparation complexity varies substantially among reported methods. Earlier HPLC method often rely on multi-step purification procedures, including ammonium hydroxide precipitation, liquid-liquid partitioning, nitrogen evaporation and solvent reconstitution steps. For example, the RP-HPLC method reported in [17] requires several sequential clean-up stages prior to chromatographic analysis. While such procedures can produce high recovery values (≈99% for both analytes), the analytical workflow becomes time-consuming and may increase experimental variability during routine analyses. Other methods employ a solid-phase extraction (SPE) using C18 cartridge, which represents a more standardized approach for removing pigments and co-extracted matrix compounds from potato tissue extracts [10,38]. Although SPE improves extract cleanliness, it introduces additional sample handling steps and increases consumable costs. More recent studies have attempted to simplify extraction procedures, for instance by applying ultrasound-assisted extraction for potato peel matrices [14]. While such miniaturized extraction strategies improve throughput and reduce solvent consumption, their effectiveness strongly depends on matrix composition and may require additional selectivity assessment to ensure accurate quantification [40]. Despite differences in extraction protocols, the reviewed methods show strong convergence toward reversed-phase chromatographic separation using C18 stationary phases, reflecting the moderately hydrophobic nature of steroidal glycoalkaloids. Conventional columns (typically 250 × 4.6 mm, 5 μm) remain widely used in these methods. Mobile phases generally consist of acetonitrile combined with water or buffered aqueous solutions, operated either in gradient or isocratic mode. Some early analytical approaches employ ion-pair chromatography, using triethylammonium phosphate buffers to improve peak shape and retention behaviour [10]. Although ion-pair reagents can enhance chromatographic resolution, their use may introduce operational disadvantages such as column contamination and extended system equilibration times.

Retention times reported for α-solanine and α-chaconine vary considerably across studies, ranging from approximately 5 to 23 min depending on column type, mobile phase composition, and elution conditions. For instance, relatively rapid separations were achieved in the reversed-phase method reported in [38], whereas other approaches required longer chromatographic runs to ensure adequate peak resolution. Such variability highlights differences in chromatographic optimization strategies among laboratories.

All reviewed methods rely on UV-based detection within the narrow wavelength range of approximately 200–208 nm, which corresponds to the absorption maximum of the steroidal alkaloid chromophore. Although this spectral region provides sufficient sensitivity for glycoalkaloid detection, it also increases susceptibility to matrix interferences, particularly when analysing complex matrices such as potato peel or processed products. Consequently, adequate chromatographic resolution and effective extract clean-up are essential to avoid co-eluting compounds that could compromise quantification accuracy. Reported limits of detection (LOD) vary among methods, typically ranging from approximately 1.0–1.2 μg/mL in earlier studies [10] to sub-microgram levels (≈0.3–0.48 μg/mL) in more optimized chromatographic systems [39]. Differences in sensitivity can be attributed to variations in detector configuration, chromatographic resolution, and sample preparation efficiency.

A critical observation emerging from the comparison is the heterogeneity in the depth of analytical validation reported across published HPLC methods. Several studies provide only partial validation information, such as calibration linearity and recovery, without evaluating additional parameters essential for assessing analytical reliability [41]. For example, although the method described in [39] reports acceptable calibration linearity, it does not include evaluation of matrix effects or robustness, which are important considerations for complex food matrices. Similarly, the RP-HPLC method presented in [17] reports excellent recovery values but lacks assessment of robustness or matrix interference. An important exception is the collaboratively validated HPLC method reported in [38], which underwent extensive interlaboratory evaluation across multiple laboratories, providing strong evidence of reproducibility and analytical reliability. However, this method does not provide detailed sensitivity parameters such as limits of detection or quantification.

Within the context of these previously reported HPLC approaches, the method developed in the present study provides several methodological advantages. The analytical procedure combines methanol-water extraction with C18 SPE clean-up, followed by reversed-phase chromatographic separation on a conventional RP-C18 column. This configuration enables consistent baseline separation of α-solanine and α-chaconine (Rs > 1.5) under optimized chromatographic conditions. The method demonstrates limits of detection between 0.46 and 0.76 μg/mL, which are comparable to or lower than those reported for several previously published HPLC-UV methods. Importantly, the method incorporates comprehensive validation, including evaluation of recovery, intra- and inter-day precision, matrix effects (SSE 95–103%), and robustness assessed using a design-of-experiments (DoE) approach. Such systematic validation remains relatively uncommon among previously reported HPLC methods for glycoalkaloid determination.

Taken together, the comparison highlights that HPLC-UV/DAD methods remain the most widely applied analytical approach for routine determination of potato glycoalkaloids, owing to their robustness, accessibility, and compatibility with standard laboratory instrumentation. However, the literature reveals significant variability in sample preparation procedures, chromatographic optimization, and validation practices. The method developed in this study contributes to this field by combining analytical simplicity, adequate sensitivity, reliable chromatographic separation, and comprehensive validation, thereby addressing several limitations identified in previously reported HPLC methods for the determination of α-solanine and α-chaconine in potato matrices.

3.8. Integrated greenness assessment of the analytical workflow

The environmental sustainability of the developed HPLC-DAD method for determining α-solanine and α-chaconine was evaluated using a triangulated greenness assessment that combined Complex MoGAPI, AGREE, and Analytical Method Greenness Score (AMGS)

Table 4
Normalized comparison of analytical strategies for potato glycoalkaloid quantification distinguishing detection physics, validation depth and matrix relevance.

HPLC method	Matrix	Extraction/clean-up	Column/elution (key conditions)	Detection (λ)	Separation performance	Sensitivity (LOD/LOQ)	Validation depth/performance	Reference
HPLC-DAD	Freeze-dried potato tubers	MeOH:H_2O extraction + C18 SPE clean-up	RP-C18 (250 × 4.6 mm, 5 µm); ACN-water gradient, 45 °C; 0.4 mL/min	DAD 205 nm	α-chaconine 27.59 min α-solanine 25.36 min	LOD 0.46–0.76 µg/mL; LOQ 1.45–2.55 µg/mL	Recovery 90–105%; intra-day RSD ≤3.5%, inter-day ≤5%; SSE 95–103%; robustness (DoE)	This work
Ion-pair HPLC-UV	Fresh potato tuber	Methanol-acetic acid extraction followed by SPE cleanup	Zobrax RX-C18column (5 µm, 4.6 × 250 mm), TEAP (pH 3): MeCN (80:20, v/v) gradient; 50 °C; 2.0 mL/min	UV 202 nm	α-chaconine 16.0 min α-solanine 17.5 min	LOD 1.0–1.2 µg/mL; LOQ 3.3–3.8 µg/mL	Recovery 82.7–93.6%	[10]
HPLC-DAD	Potato peel waste	UAE in MeOH; miniaturised approach; selectivity assessed by spiked/non-spiked + peak purity	InfinityLab Poroshell 120 EC—C18 column (3.0 mm i.d. × 150 mm, 2.7 µm); ACN/0.01 M sodium phosphate buffer, pH 7.2 – MeOH (60:30:10) in isocratic mode; room temperature; 1.0 mL/min	DAD 202 nm	α-chaconine 9.4 min α-solanine 8.3 min	LOD 0.3 mg/L; LOQ 1.0 mg/L	Validation reported (linearity 0.99; recovery 100–103%; precision with RSD values)	[14]
RP-HPLC with UV–Vis detection	Peel and flesh of potato	5% acetic acid extraction; NH_4OH precipitation; butanol partitioning; N_2 evaporation; reconstitution in MeOH	C18 (25 × 4.6 mm, 5 µm); ACN/0.05 M ammonium phosphate (30:70, pH 6.5), isocratic mode; 25 °C; 1.5 mL/min	UV 200 nm	α-chaconine 14.4 min α-solanine 12.87 min	NR	Recovery 99.6 ± 0.52% for α-solanine and 99.4 ± 0.38% for α-chaconine; no robustness or matrix-effect evaluation	[17]
Reversed-phase HPLC	Fresh potato tuber tissue	Dilute acetic acid extraction from potato tissue. Solid-phase extraction (SPE) clean-up on Sep-Pak C18 cartridges.	Hypersil C18 (25 × 4.6 mm, 5 µm); isocratic aqueous buffer/acetonitrile (composition adjusted to 60% acetonitrile); 40 °C; 1.5 mL/min	UV 202 nm	α-chaconine 7.3 min α-solanine 5.9 min	NR	Large interlaboratory collaborative validation across 10 labs; precision (repeatability and reproducibility) extensively evaluated with naturally incurred potato samples	[38]
RP-HPLC with PDA detection	Potato peel/potato flesh/whole potato/dehydrated potato flakes	5% acetic acid extraction (40 mL); ultrasonication; NH_4OH precipitation; centrifugation	Inertsil ODS-3 V column (5 µm, 4.0 × 250 mm), ACN/20 mM KH_2PO_4 (80:20, v/v) gradient; 30 °C; 1.0 mL/min	PDA 208 nm	α-chaconine 13.5 min α-solanine 23.5 min	LOD 0.31–0.48 µg/mL	Calibration linearity (R^2 = 0.995–0.996); no robustness, matrix-effect or full ICH validation	[39]

To avoid misleading cross-method ranking, Table 4 normalizes the comparison by explicitly separating detection physics, validation depth, and matrix representativeness. Consequently, lower detection limits observed for MS-based techniques reflect instrumental selectivity rather than superior overall analytical suitability for routine glycoalkaloid monitoring. Abbreviations: SPE, solid-phase extraction; UAE, ultrasound-assisted extraction; Org, organic solvent volume (MeOH/ACN); Aq, aqueous/buffer volume; NR, not reported in the source.

metrics. The deliberate use of complementary tools enabled distinction between workflow-level environmental performance, step-specific bottlenecks, principle-based compliance, and energy-centric contributions, thus providing a transparent and context-aware sustainability profile of the entire analytical chain. To further contextualize the environmental positioning of the proposed workflow, a horizontal comparison with representative green-oriented HPLC approaches was performed (Fig. 8). Parameters including organic solvent consumption per sample, normalized solvent intensity (mL/g), total solvent handled, and analysis time were extracted from the respective publications. This comparison allows an objective assessment of solvent efficiency independent of detector class (Tables S1 and S2).

3.8.1. Workflow-level benchmarking using ComplexmoGAPI

The overall environmental performance of the analytical workflow was quantified using Complex MoGAPI, which extends the visual diagnostic capability of ComplexGAPI by introducing a global numerical score that encompasses pre-analytical, analytical, and post-analytical operations [42]. The developed method achieved a Complex MoGAPI score of 71 (Fig. 6). It should be emphasised that the Complex MoGAPI pictogram does not represent a simple visual average of green, yellow, and red fields; rather, the final score results from weighted numerical integration of individual procedural domains, each contributing differently to the overall environmental footprint. Consequently, the presence of localised red segments, primarily associated with solvent-dependent chromatographic elution and consumable-based cleanup, does not disproportionately penalise the final score, as these steps reflect analytically indispensable operations with limited alternatives under UV-based detection.

Green and yellow segments predominated in sample handling, extraction, and instrumental analysis domains, indicating controlled solvent consumption, moderate energy demand, and the absence of environmentally aggressive reagents or derivatisation steps. In contrast, red segments were largely confined to chromatographic elution and off-line clean-up, highlighting analytical constraints intrinsic to reversed-phase separations of structurally homologous GA, rather than shortcomings in method design.

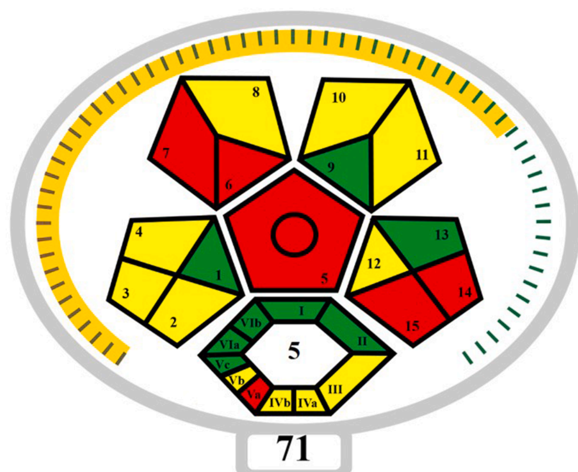

Fig. 6. ComplexMoGAPI assessment of the developed HPLC-DAD workflow for α-solanine and α-chaconine. The five coloured pentagons represent distinct stages of the analytical chain: pre-analytical operations, sample preparation and extraction, reagent and solvent use, chromatographic separation and detection, and waste generation. Individual numbered fields correspond to specific procedural elements evaluated according to ComplexMoGAPI guidelines. Colour coding indicates relative environmental impact (green: low; yellow: moderate; red: high or unavoidable). The global score of 71 represents the weighted numerical integration of all evaluated domains and reflects the overall environmental performance of the method.

3.8.2. Step-specific diagnostics using GAPI

To complement the integrative ComplexMoGAPI score, the Green Analytical Procedure Index (GAPI) was used to provide a stepwise evaluation of the environmental performance of the analytical workflow, enabling critical identification of procedure-specific environmental burdens and strengths [43]. This assessment clearly identified the extraction stage, traditionally the most environmentally burdensome component of GA analytical workflows, as predominantly green to yellow. This favourable profile is directly attributable to the adoption of a streamlined, single-step liquid extraction, characterised by moderate solvent consumption, the absence of thermal or chemical intensification, and the complete elimination of derivatisation or hydrolytic pre-treatment. Collectively, these features represent a marked improvement over classical protocols relying on Soxhlet extraction, acid-assisted hydrolysis, solvent evaporation, or multi-stage liquid--liquid extraction, all of which are associated with increased energy demand, solvent usage, and waste generation.

In contrast, red-coded elements were mainly associated with chromatographic elution and off-line SPE clean-up. The former reflects the unavoidable reliance on acetonitrile-based gradient systems and extended run times required to achieve baseline resolution of structurally related GAs under UV detection. The latter results from the use of single-use polymeric sorbents, which contribute to solid waste generation. Importantly, these limitations are intrinsic to the current state of validated HPLC-DAD methodologies for steroidal GAs and are driven by fundamental requirements for selectivity, sensitivity, and matrix interference control rather than by avoidable methodological inefficiencies.

3.8.3. Principle-based evaluation using AGREE

The AGREE assessment, which evaluates compliance with the 12 principles of Green Analytical Chemistry, produced a global score of 0.46 (Fig. 7) indicating an intermediate level of overall greenness. Such values are typical of well-optimised chromatographic methods operating under inherent physicochemical and selectivity constraints, especially when targeting non-volatile, amphiphilic analytes with UV detection. High subscores were associated with minimal sample manipulation, elimination of derivatisation, reduced solvent volumes compared to legacy ion-HPLC protocols, moderate extraction-related energy demand, and a streamlined analytical process with few unit operations. Lower scores were primarily linked to the continued requirement for organic modifiers, extended gradient elution to resolve α-solanine and α-chaconine, and off-line clean-up necessary to stabilise the deep-UV baseline and suppress co-eluting matrix constituents, features that remain analytically essential to ensure specificity, sensitivity, and trueness. The individual AGREE contributions ranged from strong compliance in derivatisation avoidance, sample-size minimisation and solvent-volume reduction to weaker performance in solvent safety, waste generation and process integration, collectively constraining the overall greenness score. Compared with previously reported ion-pair RP-HPLC methods for potato glycoalkaloids, which typically employ higher flow rates, larger solvent volumes and amine-containing buffers, the present protocol therefore represents a measurable advancement in analytical sustainability [10,44,45]. Future improvements in greenness may realistically arise from UHPLC miniaturisation, substitution of acetonitrile with bio-based ethanol eluents, or implementation of microextraction strategies capable of eliminating SPE and further reducing solvent and energy consumption.

3.8.4. Energy-centric evaluation using AMGS

To disentangle the relative contributions of instrumental energy demand and solvent-related impacts within the overall sustainability profile, the Analytical Method Greeness Score (AMGS) was applied as a complementary, energy-resolved metric alongside workflow and principal-based greens tool. In contrast to holistic indicators such as AGREE or GAPI, AMGS quantitatively integrates instrumental energy consumption, solvent energy demand and solvent environmental-

The 12 AGREE Criteria (Green Analytical Chemistry Principles)
1. Direct analytical techniques
2. Minimal sample size and number of samples
3. In situ measurements
4. Minimal sample preparation
5. Automation and miniaturization
6. Avoidance of derivatization
7. Generation of minimal waste
8. Multi-analyte or multiplex capability
9. Use of safer solvents and reagents
10. Energy efficiency
11. Use of renewable resources
12. Operator safety

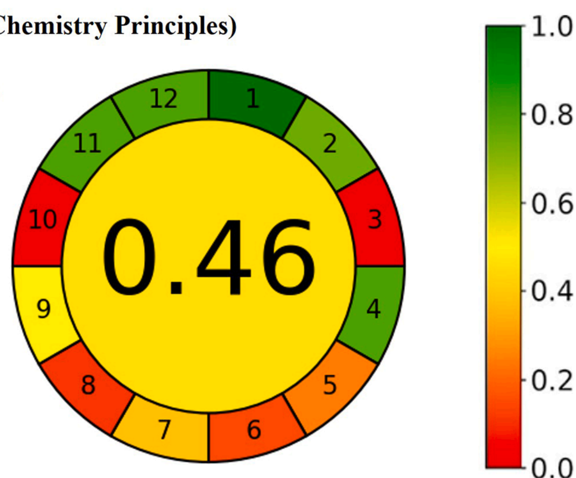

Fig. 7. AGREE assessment of the developed HPLC-DAD method for α-solanine and α-chaconine. The global greenness score (0.46) reflects intermediate overall sustainability. Green sectors indicate compliance with Green Analytical Chemistry principles, yellow sectors denote partial adherence, and red sectors highlight domains limited by the intrinsic requirements of reversed-phase HPLC, including organic solvent consumption and off-line operation. The combined assessment supports the method as environmentally improved relative to classical glycoalkaloid workflows while indicating clear paths for future greening.

health-safety (EHS) characteristics into a single cumulative score, in which lower values correspond to greener analytical performance. According to the interpretative framework proposed for AMGS-based evaluation, values below ~100 are generally associated with highly energy-efficient green methods, 100–200 with analytically acceptable but moderately energy-intensive procedures and above 200 with environmentally burdensome workflows dominated by instrumental or solvent impacts. The developed chromatographic method yielded an AMGS value of 175.76, positioning it within the intermediate "acceptable-yet-energy-demanding" domain. Decomposition of the score revealed that instrumental energy consumption accounted for 73.28% of the total environmental burden, markedly exceeding the contributions of solvent energy demand (9.24%) and solvent EHS characteristics (17.48%). Such a distribution is characteristic of conventional full-scale HPLC separations, where high analytical precision, robustness and reproducibility are achieved through continuous pump operation, extended run times and thermostatted detection system, inevitably increasing electrical energy requirements. Conversely, the comparatively minor solvent-energy contribution reflects efficient solvent management and optimized chromatographic conditions while the EHS fraction primarily arises from the necessary use of standard orgonic eluents required to preserve glycoalkaloid stability, selectivity and chromatographic resolution.

To contextualize this value within a rigorous analytical and sustainability framework, comparison with previously reported liquid-chromatographic methods for potato glycoalkaloids determination indicates that ultra-high-performance LC-MS/MS or micro-flow strategies typically achieve markedly lower AMGS-equivalent energy burdens. This enhanced performance arises from the combined effects of substantially shortened analysis times, reduced volumetric flow rates and minimized solvent throughput, operational characteristics that frequently enable such system to approach the 100-score threshold associated with highly energy-efficient green analytical procedures. In contrast the conventional HPLC-UV methodologies and SPE-intensive sample [46].

In contrast, conventional HPLC-UV methodologies and SPE-intensive sample-preparation workflows are intrinsically predisposed to higher cumulative AMGS values, reflecting the energetic and material costs imposed by prolonged chromatographic runtimes, elevated solvent consumption and multistep extraction and clean-up sequences. These structural features collectively amplify instrumental electricity demand and solvent-related environmental burdens, thereby constraining overall sustainability performance.

Within this comparative continuum, the present method occupies a transitional yet scientifically coherent sustainability position. It delivers substantive environmental improvement relative to traditional high-consumption HPLC protocol, while not reaching the extreme energy efficiency characteristic of stat-of-art UHPLC or miniaturized-flow system. Importantly, this positioning reflects a deliberate methodological design strategy, wherein chromatographic conditions were optimized to preserve instrumental accessibility operational robustness and quantitative reliability alongside progressive, though not maximal, and reductions in energy demand [47]. Such a calibrated balance embodies a pragmatic paradigm of sustainable analytical chemistry, prioritising real-world applicability and methodological transferability while achieving meaningful, mechanistically grounded reductions in environmental impact, an approach fully aligned with contemporary directions in green separation science and food safety-oriented chromatographic analysis.

3.8.5. Horizontal comparison with reported green HPLC-based methods

To complement algorithm-based greenness indices (AES, GAPI, AGREE), a quantitative benchmarking of representative LC workflows for the determination of α-solanine and α-chaconine was conducted (Fig. 8). By converting methodological design choices into measurable parameters, this analysis enables a transparent comparison of extraction strategies and chromatographic operating conditions across commonly reported analytical approaches.

To provide a balanced and transparent evaluation of the environmental positioning of the developed HPLC-DAD workflow, a horizontal comparison was performed against representative green and conventional strategies for glycoalkaloid determination, including QuEChERS-UPLC-MS/MS [48], ion-pair extraction coupled with SPE-HPLC-UV [10], and recently reported miniaturised UAE-HPLC-DAD approaches [14]. When normalised per analysed sample and per injection, the proposed method exhibits a reduced total organic solvent demand relative to QuEChERS-based workflows, which are inherently dependent on acetonitrile-intensive salting-out extraction and dispersive clean-up steps, typically requiring substantial solvent volumes in addition to chromatographic consumption. In contrast, the present method achieves a lower cumulative solvent input through simplified extraction and a controlled chromatographic system, while avoiding auxiliary reagents such as ion-pairing agents that contribute to chemical persistence and instrumental contamination. From a temporal perspective, QuEChERS and ion-pair-based methodologies are characterized by multi-step and time-demanding preparation procedures, including partitioning,

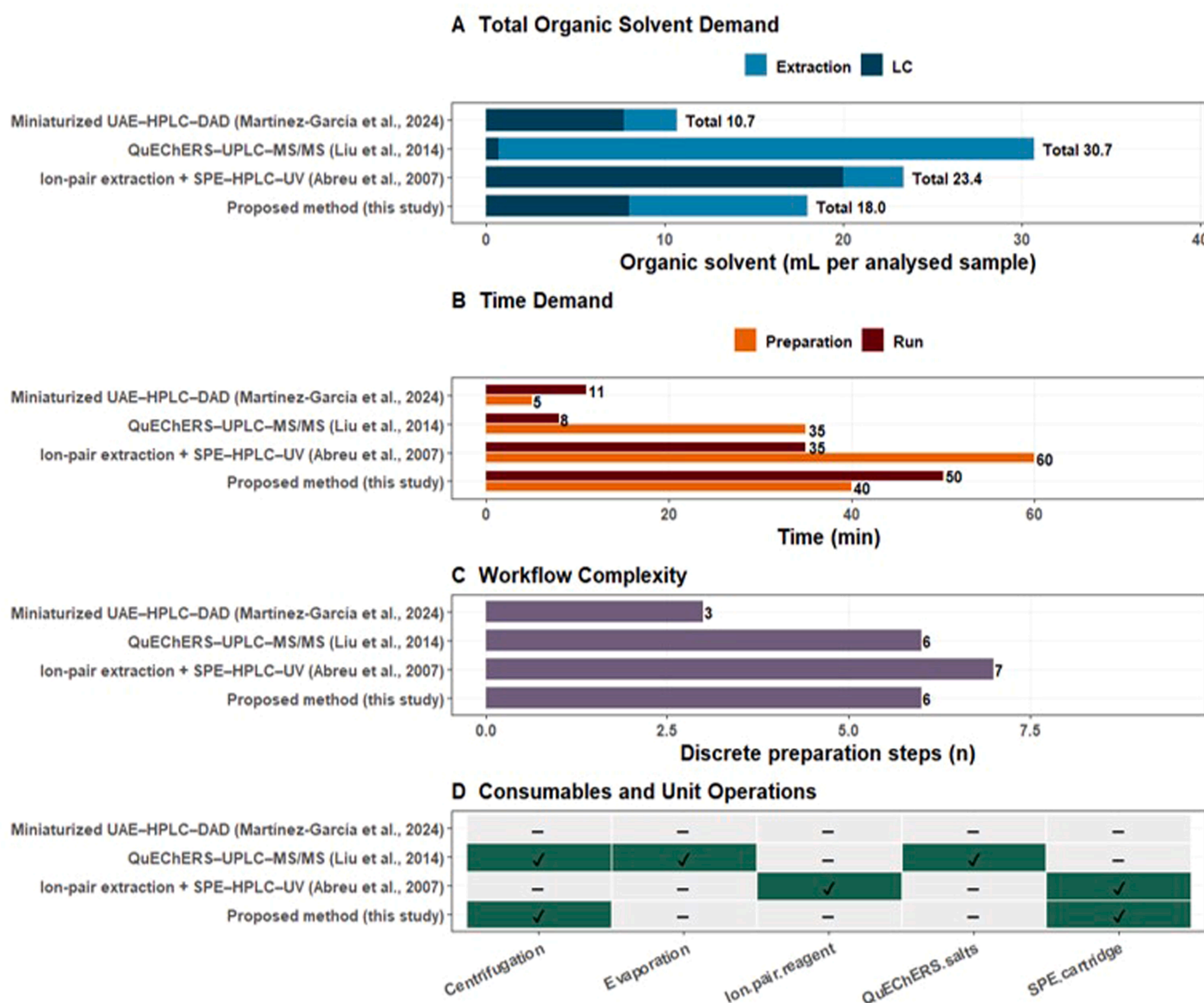

Fig. 8. Horizontal benchmarking of representative LC workflows for the determination of α-solanine and α-chaconine in potato matrices. (A) Total organic solvent demand per analysed sample, expressed as the sum of extraction-related and chromatographic organic solvent consumption; stacked bars distinguish extraction and LC contributions, with numerical labels indicating cumulative totals (mL per sample). (B) Operational time demand, showing sample preparation time and chromatographic runtime (min). (C) Workflow complexity, expressed as the number of discrete preparation steps (n). (D) Consumables and unit operations required for each workflow, including QuEChERS salt mixtures, SPE cartridges, evaporation steps, centrifugation, and ion-pair reagents (✓, required; –, not required). Organic solvent consumption during chromatographic separation was calculated by numerical integration of the gradient organic fraction over time multiplied by the applied flow rate for each method (Table S1). This quantitative benchmarking complements greenness index scoring by providing direct operational metrics of solvent consumption, time requirement, and consumables.

clean-up, and conditioning steps, which increase variability and reduce throughput. Although the developed method does not reach the extreme rapidity of miniaturised UAE-based protocols, it reduces unnecessary unit operations and maintains a more controlled and reproducible workflow, with chromatographic runtimes remaining within the range required for regulatory-grade separation. Importantly, the avoidance of evaporation and ion-pair chemistry further enhances method reliability by limiting analyte loss and matrix-induced interferences. From an operational and economic standpoint, QuEChERS protocols rely on proprietary salt mixtures and dispersive sorbents, while ion-pair methods require additional reagents and SPE cartridges, collectively increasing per-sample cost, material consumption, and waste generation. The developed workflow, by contrast, minimizes reliance on disposable materials and eliminates hazardous reagents, thereby improving sustainability in routine laboratory implementation. Taken together, this integrated comparison demonstrates that the environmental advantage of the proposed method is not merely relative to conventional solvent-intensive approaches but remains evident when benchmarked against contemporary green analytical strategies. Rather than maximizing a single green metric, the method achieves a strategic balance between solvent reduction, chemical safety, workflow simplification, and analytical robustness, which represents a more realistic and sustainable trajectory for glycoalkaloid analysis in complex plant matrices.

3.8.6. *Convergent interpretation and perspectives for further greening*

Across all greenness metrics, a coherent and internally consistent trend emerges: the developed HPLC-DAD method demonstrates a significantly improved environmental profile compared with classical GA workflows, while maintaining full analytical and regulatory validity. Importantly, the residual environmental burden does not result from lax design choices but from the fundamental analytical challenge of resolving structurally homologous steroidal GAs within a UV-based chromatographic framework, where greener substitutions or substantial runtime reductions are chemically non-trivial. Nevertheless, the integrated assessment identifies realistic and scientifically grounded

opportunities for further greening, including transition to UHPLC platforms to reduce solvent consumption and runtime, cautious partial substitution of acetonitrile with greener organic modifiers where selectivity allows, implementation of on-line or in-line clean-up strategies to eliminate disposable SPE cartridges, and miniaturisation of extraction protocols to proportionally reduce solvent and energy use. Taken together, the combined Complex MoGAPI, AGREE, and AMGS evaluation demonstrates that the developed HPLC-DAD method achieves a deliberate and analytically justified compromise between environmental responsibility and performance requirements. Although solvent and energy consumption remain unavoidable constraints of high-selectivity reversed-phase chromatography, the developed workflow represents a substantive and well-justified advancement towards environmentally responsible, regulatory-grade GA analysis and is well suited for routine deployment in breeding, food, safety, and quality-control applications.

4. Conclusions

This study presents the optimisation and full validation of a reversed-phase HPLC-DAD method for the quantitative determination of the principal potato steroidal GAs α-solanine and α-chaconine, integrating polarity-adjusted hydro-organic extraction, selective C18 solid-phase clean-up, and gradient chromatoraphic separation adapted to the amphiphilic nature of steroidal GAs. The 50:50 MeOH:H_2O extraction system enabled efficient analyte recovery while limiting co-extraction of hydrophobic matrix constituents, contributing to improved baseline stability and reproducible peak shape. Under the optimised chromatographic conditions, consistent baseline resolution ($Rs \geq 1.5$) was achieved across representative potato matrices, supporting reliable and interference-free quantification in chemically complex samples. Full validation according to ICH Q2(R2) and AOAC requirements confirmed excellent linearity, low detection limits, high precision, and robust accuracy, with negligible matrix effects and demonstrated resilience to typical operational variability.

Comparative assessment with previously reported HPLC-DAD methods underscored the analytical superiority of the present approach, which overcomes longstanding limitations in selectivity, reproducibility, and validation completeness. While LC-MS/MS platforms offer higher absolute sensitivity, the present UV-based method achieves a strategically advantageous balance between analytical rigour, cost, and accessibility, making it ideally suited for breeding programmes, regulatory compliance testing, and routine food safety monitoring. The environmental evaluation (AES, GAPI, AGREE) further demonstrated that the method markedly improves the greenness profile of GA analysis relative to classical protocols, largely due to reduced solvent volumes, elimination of harsh acidic extraction conditions, and streamlined sample preparation. Although opportunities remain for further improvement, such as exploring greener organic modifiers or transitioning to UHPLC, the developed workflow already represents a substantially more sustainable alternative within the constraints of reversed-phase analytical requirements. Overall, this work establishes a robust, reproducible, environmentally conscious and broadly deployable platform for the determination of α-solanine and α-chaconine in potato. Its combination of analytical performance, methodological transparency and operational practicality positions it as a reference method for future studies in food safety control, genotype evaluation, and metabolite profiling of GAs in Solanaceous crops.

CRediT authorship contribution statement

Hajer Ben Ammar: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Peter Dolničar:** Writing – review & editing, Resources. **Barbara Pipan:** Writing – review & editing, Visualization. **Lovro Sinkovič:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2026.467165.

Data availability

Data will be made available on request.

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