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HILIC HPLC-ELSD DETERMINATION OF SIX STEROIDAL SAPONINS IN *TRIBULUS TERRESTRIS* EXTRACTS

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Abstract

A novel, cost-effective Hydrophilic Interaction Liquid Chromatography coupled with Evaporative Light Scattering Detection (HILIC HPLC-ELSD) method was developed and validated for the simultaneous quantitative determination of six key steroidal saponins (protodioscin, pseudoprotodioscin, tribulosin, prototribestin, dioscin, and tribestin) in *Tribulus terrestris* L. raw plant materials, crude extracts, and commercial end products. Serving as an economically viable alternative to expensive mass spectrometry (LC-MS) techniques, the proposed method successfully achieves baseline resolution of the targeted saponins characteristic of the Bulgarian *T. terrestris* chemotype. An extensive, optimized sample preparation procedure was established, incorporating solid-liquid extraction, liquid-liquid partitioning, and solid-phase extraction (SPE). The analytical method was validated for limits of detection, linearity, precision, and recovery. Application of the method to real samples revealed that protodioscin, dioscin, and pseudoprotodioscin were present in all tested matrices at concentrations ranging from 0.5 to 500 mg/g extract. Tribulosin and prototribestin were absent in samples of Chinese origin, whereas tribestin was detected in traces. Tribulosin was found as a major component in root extract of Bulgarian origin. The developed HILIC HPLC-ELSD approach provides a reliable and accessible tool for the routine quality control and standardization of *T. terrestris* industrial products.

Key words: spirostanol saponins, HPLC, HILIC, ELSD, solid phase extraction, *Tribulus terrestris*

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Introduction. Saponins are ubiquitous defensive secondary plant metabolites [1]. Known for their amphiphilic nature, they have been historically used as natural detergents and food additives, but their most extensive application today lies in modern phytochemistry and pharmacology [2, 3]. Among saponin-rich plants, *Tribulus terrestris* (Zygophyllaceae) is highly valued in the pharmaceutical and dietary supplement industries. Its extracts are particularly rich in bioactive steroidal saponins, which are known to support the human reproductive system and hormonal balance [3, 4]. Furthermore, these extracts are extensively utilized for muscular performance enhancement among athletes [5]. The exponential growth of this market sector makes rigorous quality assurance critical to guarantee product standardization, prevent undetected adulteration, and ensure compliance with strict regulatory frameworks [6–8]. The biological potency and standardization of *T. terrestris* extracts predominantly rely on six major steroidal saponins: protodioscin, pseudoprotodioscin, tribulosin, prototribestin, dioscin, and tribestin [4].

Recently, the global market has seen a surge in *T. terrestris* products, simultaneously accompanied by an alarming increase in extract adulteration, raising the demand for stringent and routine quality control [9]. Analytically, saponins are non-volatile compounds with molecular weights up to 2000 Da, typically determined via liquid chromatography. Most documented methods employ reversed-phase (RP) separations on various C18 stationary phases coupled with ultraviolet (UV) or tandem mass spectrometry (MS/MS) detection [10, 11]. While UV methods are widespread, they lack selectivity and are entirely blind to certain non-chromophoric saponins. Conversely, LC-MS/MS is the most powerful, selective and sensitive option for metabolomic profiling [9], but the high instrumentation and maintenance costs make it inaccessible for routine industrial quality control.

Evaporative light scattering detection (ELSD) offers a highly attractive, cost-effective compromise. It provides universal detection for non-volatile, non-UV-absorbing compounds with a relatively uniform response [12]. However, traditional RP-HPLC-ELSD methods often fail to achieve baseline separation between some characteristic saponins, such as dioscin and tribulosin, within a reasonable timeframe [12]. To overcome the limitations of RP columns in retaining highly polar compounds, Hydrophilic Interaction Liquid Chromatography (HILIC) has emerged as a powerful complementary technique [13]. HILIC presents a highly suitable separation mechanism, particularly for resolving saponins with varying sugar moieties [13, 14].

Currently, no existing method successfully covers all six major characteristic steroidal saponins (protodioscin, pseudoprotodioscin, tribulosin, prototribestin, dioscin, and tribestin) of the Bulgarian *T. terrestris* chemotype with complete baseline separation in a fast, cost-effective single run. To address these limitations, the present work proposes a validated HILIC HPLC-ELSD method for the simultaneous determination of these six key steroidal saponins in *T. terrestris* crude

extracts. The method includes an optional solid-phase extraction (SPE) sample preparation and achieves a baseline separation of all analytes on an amide-HILIC stationary phase within a 25-minute gradient run, serving as a robust alternative to "gold standard" LC-MS methods.

Materials and methods. ***Instrumentation.*** Chromatographic analysis was performed using a Series 200 quaternary HPLC pump, vacuum degasser and autosampler (PerkinElmer Inc., USA) coupled to an Alltech Model 500 evaporative light scattering detector (ELSD) (Alltech Inc., USA). Data acquisition and processing were performed on Totalchrom software connected to the detector via NCI901 analog to digital converter (PerkinElmer Inc., USA). Separations were carried out on an XBridge BEH Amide column (130 Å, 3.5 µm, 4.6 × 150 mm; Waters, UK) at ambient temperature. Sample preparation utilized a 5510DTH ultrasonic bath (Branson, USA), Buchi rotary vacuum evaporator, and a SpeedVac concentrator. Solid-phase extraction (SPE) was performed using Strata® C18-T cartridges (55 µm, 140 Å, 1 g / 6 mL; Phenomenex Inc., USA) on a vacuum manifold (Merck Millipore, Germany).

Chemicals and reagents. HPLC-gradient grade acetonitrile (ACN) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Suprapur® formic acid was supplied by Merck (Darmstadt, Germany). HPLC-grade water (18 MΩ·cm) purified by an ELGA system was used throughout. All solvents were filtered through a 0.45 µm nylon membrane (Nylaflo; Pall Corp., USA) prior to use. All other chemicals were of analytical grade. Reference standards of protodioscin and pseudoprotodioscin (> 98% purity) and tribulosin (> 98% purity) were obtained from ChromaDex (USA). Prototribestin, dioscin, and tribestin were previously isolated [4, 14], with purities > 98% determined by HPLC-UV internal normalization, and their structures firmly confirmed via NMR, MS, and IR spectroscopy. Tribestan® tablets were purchased from a local pharmacy.

Plant material. Plant materials used to demonstrate method capability included aerial parts (yielding crude saponin extract CSE1) and roots (yielding CSE4) of *T. terrestris* of Bulgarian origin collected in 2025. The plant material was purchased from herbal pharmacy (Herb commerce LTD). For extraction, 10 g of dried plant material were ground and extracted according to a modified procedure based on [4], yielding 1.2 g and 0.89 g of dry extract, respectively. Commercial crude saponin extracts included a Chinese fruit extract marked as 45% butanol TT extract (CSE2) and a Chinese aerial parts extract marked as 16% TT extract (CSE3).

Extraction of plants and sample preparation. Ten grams of dried, powdered plant material were initially defatted by extraction with chloroform on a laboratory shaker at room temperature (3 × 90 mL, 1 h each), following a modified procedure based on [4]. The chloroform layers were discarded, and the remaining plant residue was subsequently extracted with 70% (v/v) ethanol under reflux at 80 °C (3 × 150 mL, 2 h each). The combined ethanol extracts were

concentrated under vacuum at 70 °C to a volume of approximately 50 mL. This concentrated aqueous-ethanol solution was then partitioned in a separatory funnel with n-butanol (3 × 20, 15, and 15 mL) to extract the saponins. The remaining aqueous layer was discarded, and the combined n-butanol layers were evaporated to dryness under vacuum to yield the crude saponin extract (CSE). CSE and solid dosage forms were further processed as follows: Ten tablets Tribestan® (containing 250 mg *T. terrestris* dry extract) were finely ground in a porcelain mortar. Twenty milligrams CSE or tablet powder were dissolved in 5 mL of 10% ACN in water (stock sample solution) and sonicated for 10 min at 50 °C. Sample solutions were subjected to SPE or diluted 10 times with ACN and directly injected into the HPLC column.

Solid-phase extraction (SPE). Orthogonal SPE was applied for sample clean-up and concentration of low abundant analytes if needed. Cartridges were conditioned with 6 mL ACN and 6 mL water. Stock sample solutions (5 mL) were loaded at a flow rate of 2 mL/min, followed by a 5 mL wash with 35% ACN. Elution was performed in three distinct steps, each followed by a 2-min vacuum application to prevent analyte loss: Fraction 1 (containing protodioscin, prototribestin, pseudoprotodioscin) with 3 × 0.5 mL of 65% ACN; Fraction 2 (containing dioscin and tribulosin) with 3 × 0.5 mL of 74% ACN; and Fraction 3 (containing tribestin) with 3 × 0.5 mL of 83% ACN.

When lower limits of detection were needed each fraction was evaporated to dryness (via SpeedVac or nitrogen stream at 65 °C), reconstituted in 100 µL of 90% ACN in water, sonicated for 5 min, and injected into the HPLC column in 10–100 µl volume. In this way 125 times lower limits of detection could be achieved for lower abundant analytes as tribestin and tribulosin compared to directly injecting the CSE solution.

Standard solutions preparation. Five milligrams of each saponin were dissolved to 2 ml stock solutions with 80% aqueous ACN. A working solution was prepared from the stock solutions with concentration 230, 490, 470, 245, 235 and 735 µg/mL for tribestin, dioscin, prototribestin, pseudoprotodioscin, tribulosin, and protodioscin, respectively. Calibration levels were prepared by serial dilution ranging from 700 to 2 µg/mL.

HPLC instrumental conditions. The ELSD drift tube temperature was set to 88 °C and the nitrogen gas flow rate was maintained at 1.8 L/min. HILIC separation was performed using filtered (0.2 µm) mobile phases: (A) 95% acetonitrile (ACN), (B) 50% ACN, and (C) water. All mobile phases contained 6 mM ammonium formate and 0.1% formic acid (pH 4.0). The gradient elution program started with 90% A for 0.5 min, followed by a linear gradient to 70% A and 30% B over 28 min. The mobile phase composition was then changed to 100% B for 3 min, followed by washing with 100% C for 10 min and re-equilibration with 90% A for 10 min. The flow rate was maintained at 0.8 mL/min throughout the entire chromatographic program, including the washing and re-equilibration steps. For

direct analysis, stock sample solutions were diluted with ACN before injection, and an injection volume of 10 µL was used. SPE-concentrated fractions 2 and 3, containing lower-abundance saponins, including tribestin and tribulosin, were injected at a volume of 50 µL.

Results and discussion. ***Method development.*** Protodioscin, pseudoprotodioscin, tribulosin, prototribestin, dioscin, and tribestin are characteristic steroidal saponins of Bulgarian *Tribulus terrestris* extracts. This study aimed to establish optimal chromatographic conditions for their baseline separation from both each other and the matrix components using Hydrophilic Interaction Liquid Chromatography (HILIC) coupled with evaporative light scattering detector (ELSD). Initial tests with reversed-phase (RP) C8 and C18 stationary phases provided adequate separation for five of the compounds but consistently failed to resolve the critical pair of dioscin and tribulosin, regardless of the applied gradient. Conventional RP-HPLC methods, such as those reported by Ganzera et al. [12] and Mulinacci et al. [10], frequently struggle to achieve adequate selectivity for closely related steroidal saponins and fail to baseline-resolve critical pairs within a reasonable timeframe. To overcome these limitations, the proposed amide-HILIC stationary phase was evaluated. An amide-HILIC column (4.6 × 150 mm) successfully baseline-resolved all six analytes within 10 min. However, a 25-min gradient at standard backpressure was adopted to ensure complete separation from complex matrix interferences following sample preparation. Since ELSD is a universal, non-selective detector for non-volatile compounds and is generally less sensitive than MS or UV detectors, achieving high chromatographic resolution and eliminating matrix interferences is crucial. To address the sensitivity limitations, an orthogonal solid-phase extraction (SPE) pre-concentration step was implemented. Furthermore, the typically narrow linear range of the ELSD (covering 2.0–2.5 orders of magnitude) required the construction of either multiple linear calibration curves for distinct narrow ranges or a nonlinear polynomial curve. In our approach, the detector response demonstrated excellent linearity across two distinct ranges: 0.1 to 10 µg and 1 to 30 µg on column. During routine analysis, stock sample solutions were initially injected directly after dilution. Each analyte was quantified using the specific calibration curve corresponding to its detected concentration range. If a specific minor component fell below the limit of detection during this direct analysis, the sample was subjected to SPE. The targeted SPE fraction was then concentrated and re-analyzed. This pre-concentration strategy is essential for trace components such as tribulosin and tribestin, and this exact approach was successfully applied for their precise determination in the analyzed real samples.

Sample preparation and validation. Following an initial water-ethanolic extraction and butanolic liquid-liquid partitioning, an orthogonal solid-phase extraction (SPE) was implemented aligning with recent trends in the purification of complex botanical matrices [15]. Since tribestin and tribulosin are naturally

present at lower concentrations than the other saponins, the role of the solid phase extraction was also to concentrate them ensuring that all analytes concentrations are detectable and remain within the linear range of the detector. Three distinct SPE fractions were collected: one enriched in tribestin (Fraction 3), one in dioscin/tribulosin (Fraction 2), and one containing the remaining analytes (Fraction 1). Upon need fractions are concentrated (by evaporation on a speedvac concentrator) reconstituted in a desired volume and run separately or combined, reconstituted in 90% ACN before injection. By the end of the SPE procedure, minor saponins such as tribestin and tribulosin are concentrated 50 times in the final solution. Combined with a larger injection volume (50 µL), this achieves a total analytical sensitivity increase of over 100 times. The effectiveness of this preconcentration is illustrated by comparing the chromatogram of an extract (CSE1) analyzed directly without SPE (Fig. 1B) to the chromatogram of the concentrated SPE Fraction 2 (Fig. 1C), which clearly enables the detection of trace amounts (0.04 mg/g) of tribulosin.

The developed HILIC HPLC-ELSD method was validated for linearity, precision, limits of detection (LOD), limits of quantification (LOQ), and recovery. The validation data are summarized in Table 1. The limits of detection for the analytes were determined by serially diluting and analyzing a standard solution until a signal-to-noise (S/N) ratio of approximately 3 was reached. Detection limits of 0.04–0.06 µg on column (equivalent to a concentration of 0.8–1.2 µg/mL in the injected solution) were achieved for all analytes. For the crude saponin extract (CSE) samples, this corresponds to a LOD of 0.5 mg/g when analyzed directly without pre-concentration. Furthermore, when the solid-phase extraction (SPE) pre-concentration step is applied, the detection limit in the sample is effectively lowered to 0.004 mg/g. The limits of quantification were calculated as three times the LOD, varying between 0.13 and 0.17 µg on column (or 2.5–3.5 µg/mL in solution with a 50 µL injection volume). According to the proposed sample preparation procedure incorporating SPE, the minimal quantifiable amount starts from 16 µg/g of sample (representing a more than 100-fold increase in sensitivity). The limits for the different analytes did not differ substantially due to the relatively universal response of the ELSD detector. These values are presented in Table 1 as the injected amount on column.

To determine the linear range of the method standard solutions ranging from 2 to 600 µg/ml (0.1 to 30 µg on column) were analyzed and the corresponding calibration curves plotted. Within this concentration range determination coefficients (R^2) for all analytes were observed between 0.95–0.97. Because of the typically narrow linear range of the ELSD detector, two calibration curves were constructed for each analyte (0.1–10 µg and 1–30 µg on column) with determination coefficients (R^2) above 0.99. For sample quantification, the lower range curve (illustrated for tribestin and tribulosin in Fig. 1E) was applied for analyte amounts below 1 µg on column, while the broader range curve (1–30 µg, shown

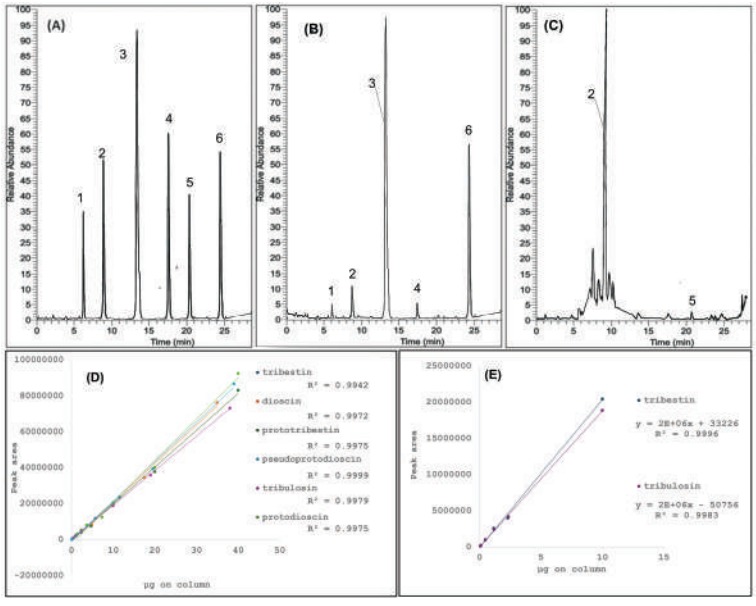

Fig. 1. HILIC HPLC-ELSD chromatograms and calibration curves: (A) Baseline separation of the six steroidal saponin reference standards (1. tribestin; 2. dioscin; 3. prototribestin; 4. pseudoprotodioscin; 5. tribulosin; 6. protodioscin); (B) Chromatogram of a crude saponin extract (CSE1) analyzed directly without SPE (sample diluted 10-fold with ACN, 10 µL injected); (C) Chromatogram of SPE Fraction 2, showcasing the pre-concentration step (5 mL initial solution eluted with 1.5 mL, concentrated under vacuum and reconstituted in 100 µL mobile phase; 50 µL injected) to detect minor component tribulosin; (D) Calibration curves for all analytes within the broad range (1 to 30 µg on column); (E) Calibration curve demonstrating the low concentration range (0.1 to 10 µg on column) specifically for minor components tribestin and tribulosin

in Fig. 1D) was used for all higher concentrations. To test the precision of the method including SPE procedure Tribestan solid dosage form (ground tablets) were analyzed 3 times on 3 consecutive days. Each fraction was analyzed separately. The relative standard deviations of the peak areas for each analyte are presented in Table 1. As no tribulosin was detected in this sample, it was spiked additionally.

Due to the unavailability of a matrix-matched standard, the recovery of the SPE procedure was evaluated by performing a spike recovery study on the tablet sample. Briefly, 20 mg of ground tablet powder was dissolved in 5 mL of solvent, as described in the Materials and methods section. Three independent 0.5 mL aliquots of this solution were each fortified with 50 µL of a mixed standard stock solution containing all targeted analytes. These spiked solutions were subjected to the SPE procedure, and Fractions 1, 2, and 3 were collected for each sample. The

Table 1

Analytical parameters of the method and sample concentrations

Analytes (numbers correspond to chromatogram in Fig. 1)	Retention time [min]	LOD, LOQ [µg on column]	Linear range [µg on column]	CSE1 [mg/g]	CSE2 [mg/g]	CSE3 [mg/g]	CSE4 [mg/g]	Tribestan tablets [mg/g]	Intra-day precision (RSD) after SPE procedure	Before Spike [µg] (average of 3 replicates)	Spiked with [µg] (3 samples)	After spike [µg] (average of 3 replicates)	Recovery (%)
1. Tribestin	6.15	0.055, 0.165	0.1-10 1-30	19±1	0.03±0.01	0.3±0.05	0.6±0.02	8±0.4	5.1	16.3±0.3	11.5	27±3	92
2. Dioscin	8.82	0.048, 0.144	0.1-10 1-30	40±3	165±11	500±30	2±0.1	21±1.5	7.7	41.5±0.4	24.5	66±7	99
3. Prototribestin	13.56	0.050, 0.150	0.1-10 1-30	369±22	<LOD	< LOD	0.9±0.04	87±5	5.9	173.4±2.2	23.5	195±5	94
4. Pseudoprotodioscine	17.54	0.051, 0.153	0.1-10 1-30	22±2	141±9	184±13	0.5±0.02	71±5	6.8	142.2±0.9	12.3	153±3	89
5. Tribulosin	20.45	0.058, 0.174	0.1-10 1-30	0.04±0.02	<LOD	<LOD	146±7	< LOD	7.9	<LOD	11.8	11±3	91
6. Protodioscin	24.19	0.042, 0.126	0.1-10 1-30	201±3	351±21	272±16	0.6±0.03	48±3	6.1	47.6±2	36.75	82±7	94

fractions were subsequently evaporated to dryness using a SpeedVac concentrator and reconstituted in 90% aqueous ACN. Each fraction was analyzed separately. Quantification was performed against the broad-range calibration curve (1–30 µg on column), as the final analyte amounts in the fortified samples fell well within this upper linear range. Unspiked aliquots of the same sample were analyzed in triplicate to determine the basal concentrations of the saponins. Percentage recovery was calculated as the difference in analyte amount between the spiked and unspiked samples, divided by the known added amount, and multiplied by 100. As summarized in Table 1, the SPE procedure demonstrated excellent efficiency, yielding recoveries above 90% for all evaluated analytes.

Sample analysis. The applicability of the method was demonstrated by analyzing four crude saponin extracts (CSE1–CSE4) and one commercial solid dosage form (Tribestan® tablets). All extracts and tablets were prepared and analyzed as described in the Materials and methods section. Samples were analyzed directly, and with SPE for the minor components tribestin and tribulosin. Quantitative results are presented as mg/g extract/tablet in Table 1. Protodioscin, dioscin, and pseudoprotodioscin were detected in all samples, with concentrations varying from 0.6 to 351, 2 to 500, and 0.5 to 184 mg/g extract respectively. Notably, tribulosin and prototribestin were below limits of detection in the sample derived from *T. terrestris* of Chinese origin. Tribulosin was detected in high concentration (146 mg/g) in the root-derived sample (CSE 4) and in the aerial parts from the Bulgarian origin in traces (0.04 mg/g). The highest concentration of dioscin was found in the aerial plant part sample from China and prototribestin was highest in the Bulgarian aerial plant parts extract. The highest protodioscin concentration of 350 mg/g was found in the commercial fruit extract from China (CSE2). All analytes except tribulosin were present in the Tribestan tablets ranging from 8 to 80 mg/g. These significant quantitative and qualitative variations confirm that the structural architecture and phytochemical density of *T. terrestris* saponins are highly dependent on geographical origin and the specific plant morphological part utilized [4].

Conclusions. A novel, cost-effective HILIC HPLC-ELSD method was successfully developed and validated for the simultaneous quantitation of six key steroidal saponins in *Tribulus terrestris* raw plant materials, crude extracts, and commercial end products. Following the evaluation of various chromatographic regimes, an amide-HILIC stationary phase was selected as the optimal approach, achieving complete baseline resolution of the targeted saponins characteristic of the Bulgarian *T. terrestris* chemotype. To overcome matrix complexities and the inherent lower sensitivity of ELSD, an extensive sample preparation procedure was established, incorporating sequential solid-liquid, liquid-liquid, and orthogonal solid-phase extractions (SPE). The method demonstrated excellent linearity, precision, recovery, and adequate limits of detection. Its practical applicability was proven through the successful analysis of four different plant extracts (two of

which started from raw plant material) and one solid dosage form. Ultimately, this validated approach serves as a robust, fit-for-purpose and economically viable alternative to expensive MS-based methods for the routine quality control and standardization of *T. terrestris* products.

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