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RESEARCH ARTICLE

# RP-HPLC Method for Simultaneous Quantification of Withanolides in *Withania somnifera*

**Mayuri Thumar[1*], Trupesh Pethani[2], Daya Chothani[1], Dipen Bhimani[1] and Nirav Patel[3]**

[1]B.K. Mody Government Pharmacy College, Rajkot - 360003, Gujarat, India; mayuri.thumar@gmail.com
[2]Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, - 360003, Gujarat, India
[3]Pharmaceutical and Process Technology, Patheon Inc., Ontario, Canada

## Abstract

**Background:** Withanolides are the primary bioactive components of *Withania somnifera* (L.) Dunal. Among them, withanolide A, and 12-deoxy-withastramonolide are the herb's most physiologically active ingredients. The steroidal lactones are isomers, which makes them difficult to separate. **Aim:** In the current research, a simple, precise, accurate, and decent RP-HPLC method has been developed, optimised and validated for simultaneous quantification and separation. **Methods:** Resolution was conducted on Gemini, Phenominex C18 (250mm X 4.6mm, 5 µ) column at 40°C utilising binary mobile phases of acetonitrile and ammonium acetate (10 mM) in gradient mode with 1.0 mL/min flow rate and observed at 230 nm. The total run time was twenty-five minutes. The procedure complied with ICH guidelines, covering the limit of detection, specificity, linearity, quantification, precision, accuracy, and robustness. **Results:** The standard curves of both relevant analytes displayed a linear pattern with regression values > 0.999 in the 1–10 µg/mL range. For both herbal markers, 1 µg/mL was the lowest limit of quantification. The method's accuracy was 100.38-100.93 % and 99.13-100.75 % for with withanolide A, and 12-deoxy-withastramonolide respectively. The precision of within and between days was found in the tolerable limit of >2% for both herbal markers. **Conclusion:** The suggested method worked well for simultaneously analyzing the extracted samples and identifying withanolides.

**Keywords:** HPLC, Method Validation, withanolide A, 12-deoxy-withastramonolide, *Withania somnifera*

**Abbreviation:** HPTLC: High Pressure Thin Layer Chromatography; ICH: International council of Harmonization; LC-MS/MS: Liquid Chromatography tandem mass spectrometry; PDA: Photo diode array; RP HPLC: Reversed Phase- High Pressure Liquid Chromatography; WHO: World Health Organization

## 1. Introduction

The most traditional medical system, *Ayurveda*, is the oldest and still has more value and potential than ever. *W. somnifera* known as *Ashwagandha*, is a member of the Solanaceae family and is one of the most significant and often utilized therapeutic plants in *Ayurveda*. *W. somnifera*, a little, woody evergreen shrub, is also referred to as the "Prince of Herbs" in *Ayurveda*, Indian ginseng, winter cherry, or poison gooseberry[1-3]. It has many pharmacologically significant qualities, including anti-carcinogenic[4], inflammatory inhibitors[5], anti-oxidant[6], immunomodulatory[7], Neuroprotective[8], anxiolytic effect[9], hypoglycemic, hypolipidemic[10], and adaptogenic activity[11]. It was used for centuries to treat several human ailments. *W. somnifera* is categorized as a *rasayana* herb in *Ayurveda*, which enhances longevity, health, and general resistance[12].

Various chemical classes, including steroidal lactones (withanolides), alkaloids, tannins, and flavonoids are among the constituents of *Withania* species. Chemically, withanolides that are hypothesized to contribute to the plant's bioactivity are 22-hydroxyergostan-26-oic acid-26,22-lactones, ergostane skeleton with 28 carbon atoms as its basic basis, produced a d-lactone by being oxidized at C-22 and C-26[13]. From the roots, berries,

*Author for correspondence

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and aerial portions of *W. somnifera*, more than 40 withanolides have been identified and isolated to far[14]. Evaluations of the withanolides in *W. somnifera* using HPLC[15-18], HPTLC[19,20], and LC-MS/MS[14] techniques have been reported.

*W. somnifera* has been classified as "medicinal plant species in high trade sourced largely from cultivation" by the National Medicinal Plants Board of India[21]. According to classical sources, the root is regarded as the most medicinally important portion of the plant and hence finds a position in pharmacopoeias and trade. Pharmacopoeial monographs such as the Indian Pharmacopoeia and Ayurvedic Pharmacopoeia of India use withanolides being "marker constituents" in the root. According to WHO and other relevant guidelines, the majority of marketed products derived from the roots of *W. somnifera* have been standardized according to the withanolide composition. Hence, particular analytical methods for quantitative estimation of various withanolides extracted from the roots have grown in significance.

This study aimed to develop a new HPLC technique for the simultaneous detection of two main withanolides, namely withanolide A (WTA) and 12-deoxy-withastramonolide (DWS) (Figure 1). Ashwagandha's neuro-regenerative benefits are attributed to its chemical component, withanolide A. *In vitro* and *in vivo* studies have reported that withanolide A can repair neuritic injury and synaptic loss[22]. A recent study found that 12-deoxy-withastramonolide had a lower elimination rate and a longer extension of absorption compared to withanolide A[14]. Some previously reported methods have longer (50-minute) analysis times. In this research, we aim to establish and validate a straightforward, streamlined, and rapid HPLC-PDA method for the concurrent analysis of WTA and DWS.

## 2. Materials and Methods

### 2.1 Plant Materials

The roots of *W. somnifera* were harvested from the Saurashtra region and taxonomically have been identified by Dr V. S. Thaker, Department of Bioscience, Saurashtra University, Rajkot, Gujarat, India. Before being analyzed, the freshly collected roots were cleaned, allowed to air dry, and carefully stored, shielded from light and moisture.

### 2.2 Reagents and Chemicals

The typical withanolides were purchased from Natural Remedies Private Ltd. (Bangalore, India) which was further confirmed by mass spectrometry. HPLC-grade water and acetonitrile were purchased from Rankem, India. HPLC grade methanol was obtained from Merck, India. Ammonium acetate and all remaining synthetic substances were of analytical grade.

### 2.3 Instrumentation and Chromatographic Conditions

A liquid chromatography system, comprised of an LC-20AD solvent delivery unit, DGU-20A5R degassing unit, CTO-20AC column oven, and SIL-20AC autosampler (Shimadzu Corporation, Japan), is coupled

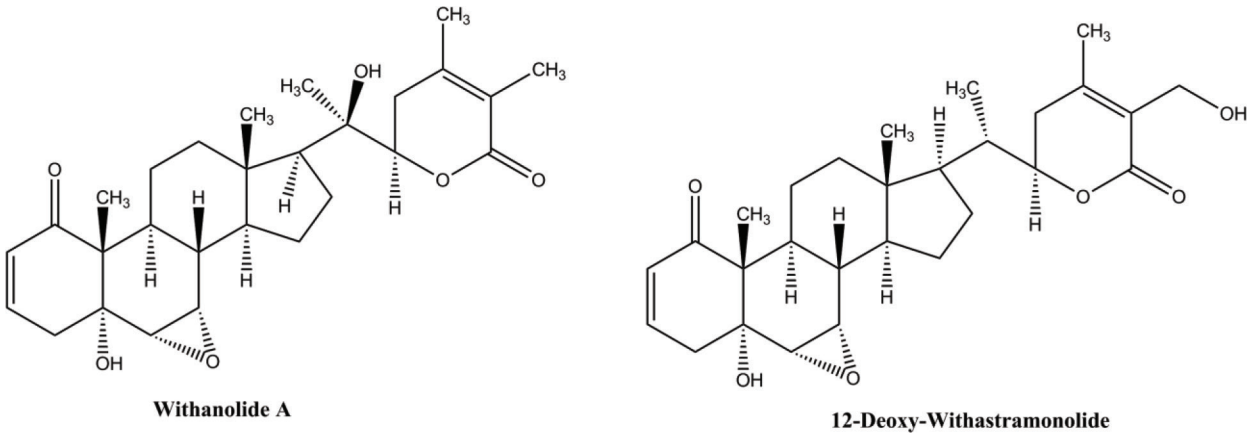


**Figure 1.** Chemical structure of WTA and DWS.

to an SPD-20A Photodiode Array Detector (PDA) for Ultraviolet (UV) absorbance detection. The HPLC-PDA system is further connected in tandem with an LCMS-80 30 triple quadrupole Mass Spectrometer (MS) for enhanced analytical capabilities. Shimadzu Corporation (version 5.99 SP3C), was utilized for data processing.

Withanolides were analyzed using Gemini's Phenomenex C18 column (250mm X 4.6mm, 5 µ) column. Separation was achieved using a gradient at 40°C. The mobile phase consists of a binary mixture of 10 mM ammonium acetate (A) and acetonitrile (B) in gradient mode with a flow rate of 1.0 mL/min. The gradient program was as follows: 0–5 min, linear gradient from 80 to 60 % B; 5–6 min, 60% B; 6–20 min, linear gradient from 60 to 20 % B; 20–23 min, linear gradient from 20 to 80% B; 23–25 min, 20% B. To separate withanolides from other biomarkers, a longer elution program was employed. The column effluent was examined using ESI-MS in positive ion mode, and mass spectra were obtained. The MS was operated using the following parameters: a heat block temperature of 450°C, a desolvation line temperature of 250°C, a nebulizing nitrogen gas flow rate of 3 L/min, and a drying gas flow rate of 15 L/min. Additionally, samples and solvents utilized in the mobile phase were properly filtered using a 0.22 µm HiMedia filter from Mumbai, India. Samples were fed into an HPLC system in 20 µL aliquots and then examined at 230 nm.

## 2.4 Preparation of Standard Solutions

One milligram per mL of the primary standard withanolides' stock solution was produced in methanol separately. Aliquots from each primary stock solution were combined to create working standard solutions, which were then diluted with a 4:6 ratio of water to methanol.

## 2.5. Sample Preparation

After being dried and finely ground, two grams of *W. somnifera* root were extracted using 100 mL of methanol: water (6:4% v/v) in a boiling water bath for thirty minutes. The supernatant was placed into a flask. Three or four iterations were conducted until the extract lost its colour. The combined extract was vacuum-concentrated, and methanol was used to adjust the volume to 40 mL. Before analysis, aliquots were passed through a 0.45 $\mu$m membrane filter.

## 2.6 Method Development and Validation

A range of buffers and organic solvents in various proportions were explored with different types of columns, pH range, and flow rates to achieve adequate separation of withanolides. Finally, a selective and sensitive technique was devised employing a Gemini Phenomenex C18 column. No internal standard was used since sample preparation was simple and a high-precision autosampler was utilized. The developed procedure underwent thorough validation in compliance with ICH criteria[23] for accuracy, linearity, specificity, precision, and robustness.

### *2.6.1 Specificity*

The method's specificity was established by examining possible disruptions at the analytes' peak area using a diluent (methanol: water 6:4). This primarily verifies the analyte's identity, and purity, and minimizes error in the result.

### *2.6.2 Linearity*

Calibration curves for 1-10 µg/mL (1, 2, 3, 4, 5, 10 µg/mL) were plotted using weighted linear regression analysis with a $1/x^2$ weighting factor against the standard concentration range.

### *2.6.3 Accuracy and Precision*

The degree to which the findings produced by an analytical approach resemble the true value is known as its accuracy, and the degree to which that likeness exists is known as its precision[24]. To achieve precision, six replicates of a single concentration (4µg/mL) were injected twice a day for intraday precision and three consecutive days for inter-day precision. The values were represented as Relative Standard Deviations (RSD). A recovery study was conducted to determine the accuracy of the suggested procedure, which involved the addition of standard markers to the samples. The samples were spiked with three different standard concentrations and assessed in triplicate. The spike recoveries were calculated using the average contents of the target compounds.

### *2.6.4 Robustness*

A robustness study was conducted by modification in optimized value for the column temperature,

wavelength detection, and strength of the mobile phase buffer. The effects of these modifications on both Retention Time (RT) and the area covered by peak were examined by calculating the %RSD.

#### *2.6.5 Limit of Detection (LoD) and Limit of Quantification (LoQ)*

According to the ICH recommendation, LoD and LoQ were calculated by determining the SD of the response (σ) and the linear equation slope (S). The LoD and LoQ were determined using this formula; LoD = 3.3 σ/S, LoQ = 10 σ/S.

## 3. Results and Discussion

The primary obstacles to the quality of herbal medicine can differ based on several aspects such as duration of harvest, plant origins, drying process, contamination with heavy metals, microbial content, and other associated variables. Therefore, identifying the vast majority of the phyto-constituents present in therapeutic plant extracts or formulations is crucial to maintaining the validity and consistency of pharmacological research and assuring the quality of the items[25]. The use of chemical markers in medicinal plants and product quality assurance is crucial. A significant issue for the quality control of herbal medications is the scant evidence of chemical markers[26]. To confirm the presence of active phyto-molecules and the repeatability of the established method, analytical method validation is required.

### 3.1 Optimization of the Chromatographic Conditions

Initial attempts at improving the chromatography of withanolides during the method development included numerous combinations of organic solvents like methanol, and acetonitrile with aqueous solutions like water, diluted acetic acid, formic acid, as well as some specific concentrations of ammonium and formate buffer. After completing the mobile phase optimization, we chose ammonium acetate (10 mM) and acetonitrile as the final mobile phase. Acetonitrile was the solvent of choice over alcohol-based solvents because it improved separation and provided comparatively low back pressure. Many columns have experimented but the ideal chromatography, resolution, and reproducibility were noted using Gemini, Phenomenex C18 column having 25 cm length, 4.6 internal diameter and 5µ particle size. It was observed during the method development that the optimum chromatography can be achieved in a straightforward isocratic or binary mode. However, because the established approach was intended to be used for the evaluation of biomarkers in various polyherbal formulations, there were more chances for other phytoconstituents to interfere with the retention of withanolides due to the presence of numerous phytoconstituents hence we have chosen to use the gradient method with lengthy run duration to control such a probability (Figure 2). Based on the UV maximum of the withanolides, 230 nm was chosen as the detection wavelength (Figure 3). As a result, there was negligible interference from other compounds in the samples and the detector response was satisfactory. Verify the quality of the standards and whether any additional contaminants are present by obtaining the mass spectra of typical standards WTA and DWS (Figure 4).

### 3.2 Specificity

A two-dimensional chromatogram for both standards is displayed in Figure 5, which reveals no interference

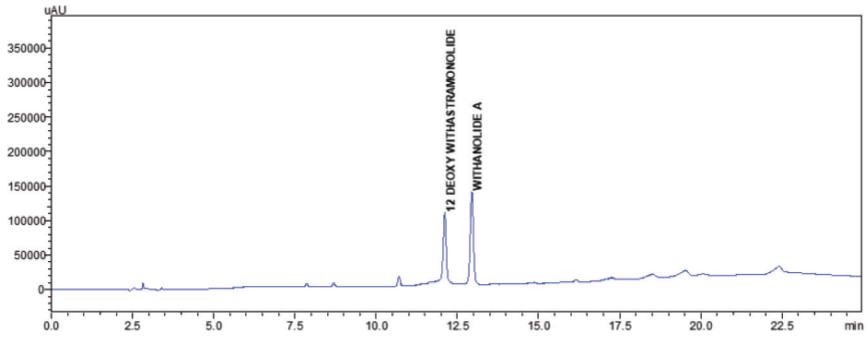


**Figure 2.** Representative HPLC chromatogram of WTA and DWS.

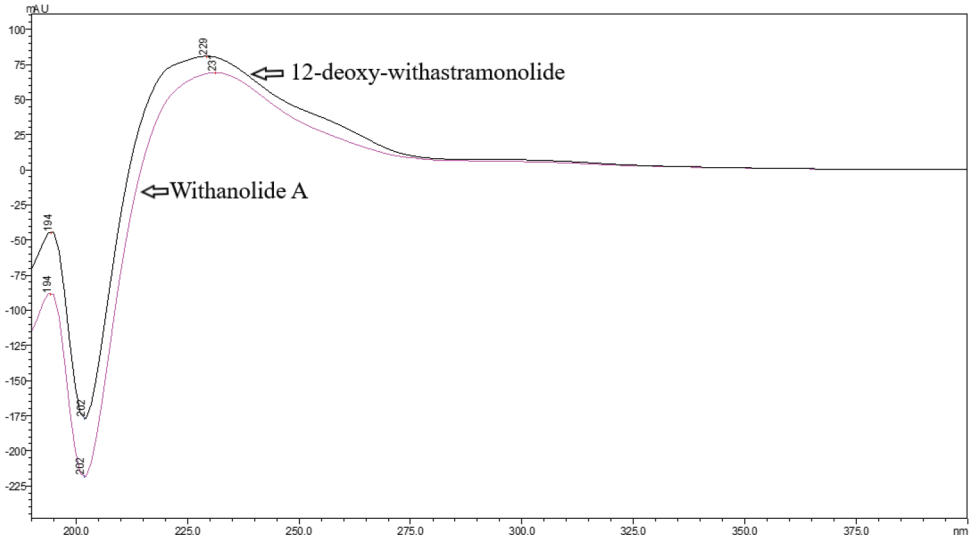


**Figure 3.** UV spectrum of WTA and DWS.

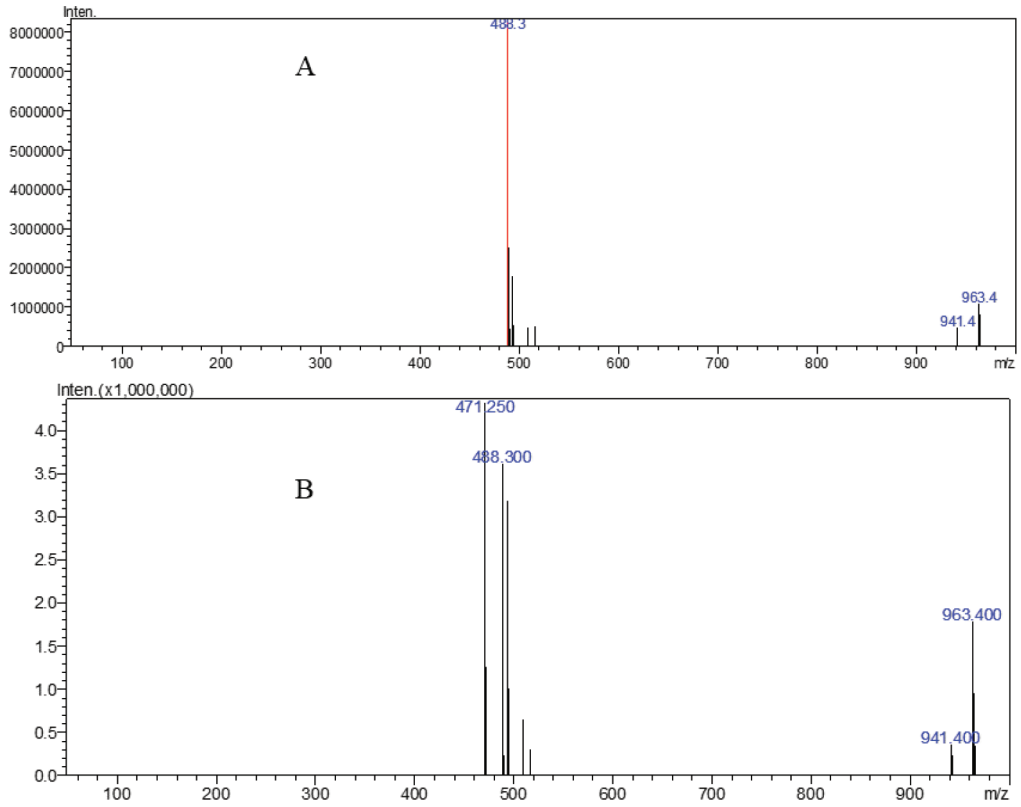


**Figure 4.** Mass spectra of **(A).** WTA and **(B).** DWS.

between the two substances at 230 nm. The retention durations of DWS and WTA, respectively, under the chromatographic conditions mentioned above, were around 12.050 and 13.150 min, indicating good separation. There were no peaks interfering with the diluent and mobile phase at the selected wavelength (Figure 6). To extend the overall chromatographic run time by 25 minutes, a late eluting peak was accommodated.

## 3.3 Linearity

The calibration curve for withanolides was plotted using peak area vs. concentration, as illustrated in

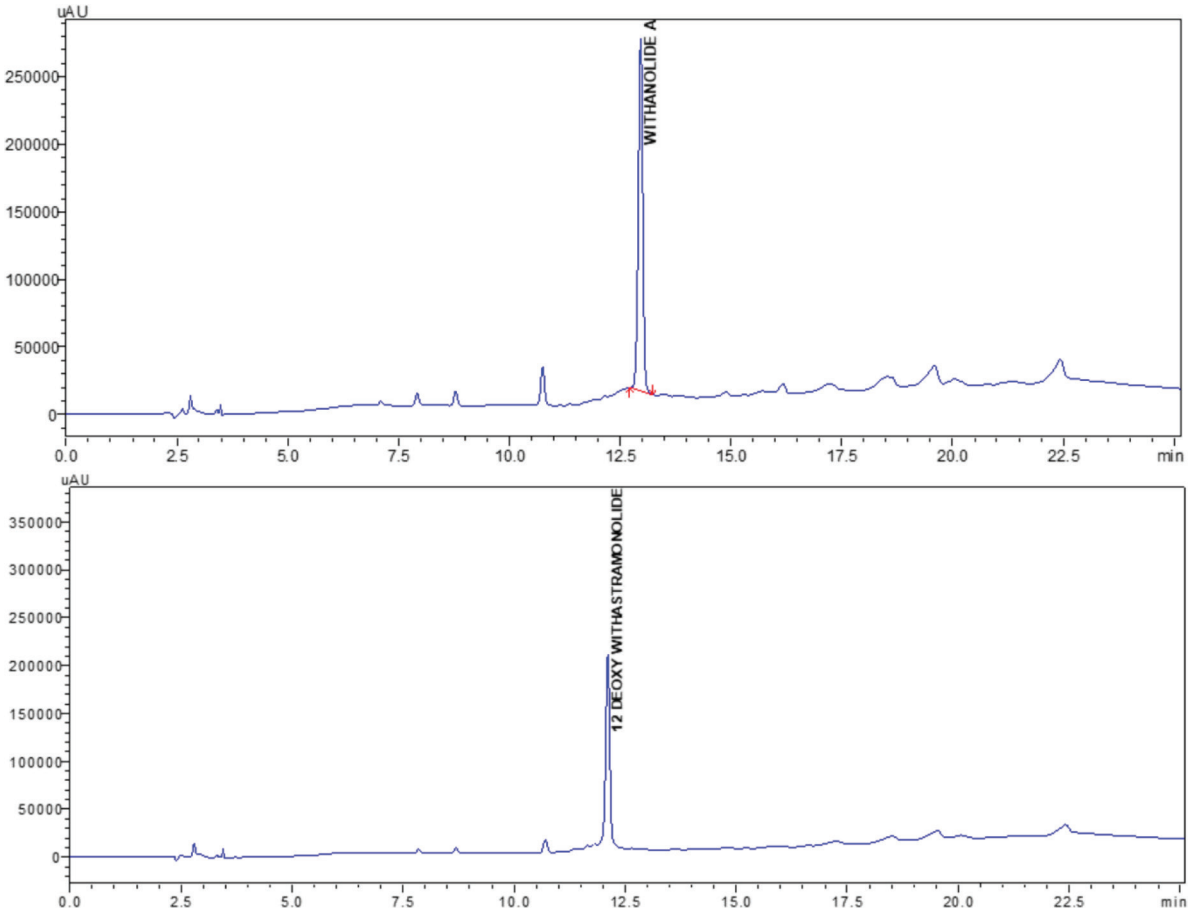


**Figure 5.** Representative HPLC chromatogram of reference standards WTA and DWS.

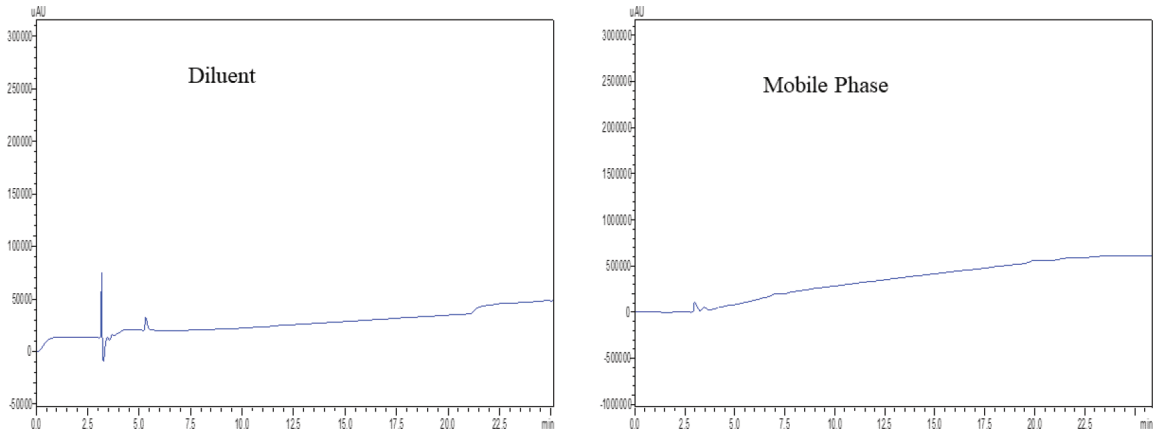


**Figure 6.** Representative HPLC chromatogram of Diluent and Mobile phase.

Figure 7. The experiment was conducted thrice (n = 3). Retention time, intercept, slope, and regression coefficient values of the withanolides calibration curve are represented in Table 1. For both WTA and DWS, this technique has demonstrated improved linearity by spanning the actual concentration range of 1–10 µg/mL (r2 = 0.999). As the regression coefficient's % RSD was determined to be extremely small (<2%), the response of the method was demonstrated to be reproducible.

**Table 1.** Linearity range, Regression parameter, LOD and LOQ of the proposed HPLC method

| Biomarker | Concentration Range (µg/mL) | Retention time (min)[a] | Regression equation | $R^2$ ± RSD[b] | LOD | LOQ |
|---|---|---|---|---|---|---|
| 12-deoxy-withastramonolide | 1-10 | 12.05 ± 0.07 | Y= 134436X+ 470.83 | 0.9992 ± 0.005 | 0.32 | 0.90 |
| Withanolide A | 1-10 | 13.12 ± 0.16 | Y= 184320X- 5762.12 | 0.9999 ±0.003 | 0.23 | 0.82 |

[a] Mean ± SD (n=6), [b] Mean ± RSD (n=3)

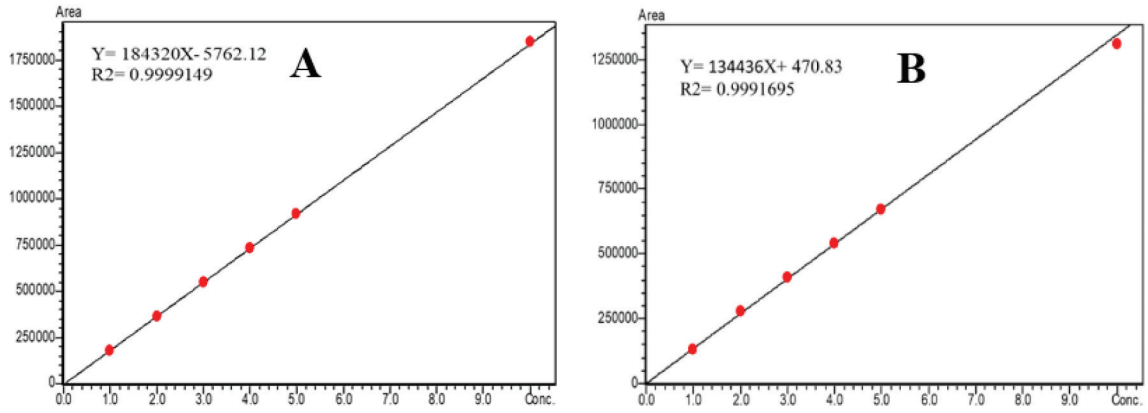


**Figure 7.** Linearity response for **(A).** withanolide A; **(B).** 12-deoxy-withastramonlide across the whole concentration range by linear regression.

## 3.4 Accuracy and Precision

The % RSD of within and between day precision were 0.23 and 0.30 for DWS and 0.50 and 0.27 for WTA, respectively, meeting the ICH acceptance criteria of <2%. Less value of % RSD revealed that the method is precise (Table 2). In terms of recovery, accuracy was performed at three levels (50%, 100%, 150%) of standard markers. Due to the unavailability of an herbal formulation with a known concentration of withanolides, the prepared sample was used as a test. Percentage mean accuracy and recovery were found 100.38 to 100.93 with % RSD 0.49-1.26 for WTA and 99.13 to 100.75 with % RSD 0.53-1.33 for DWS. The results of the recovery experiment for withanolides demonstrated that the method was accurate (Table 3). The developed method performed satisfactorily in terms of baseline separation and sensitivity of each marker component without influence from other phytoconstituents.

## 3.5 Robustness

By examining (n = 6) the mixed working standard solution of withanolides (4 µg/mL) under minor

**Table 2.** Precision of within and between-day HPLC measurement for withanolides

| Biomarker | Intraday (n=6) | | Interday (n=9) | |
|---|---|---|---|---|
| | Mean Response | % RSD | Mean Response | % RSD |
| 12-deoxy-withastramonolide | 547586.8 | 0.23 | 550326.2 | 0.30 |
| Withanolide A | 734362.8 | 0.50 | 733978.1 | 0.27 |

**Table 3.** Recovery study for withanolides

| Biomarker | Amount added (µg/mg) | Recorded amount (µg/mg)[a] | % Mean Recovery | % RSD |
|---|---|---|---|---|
| 12-deoxy-withastra monolide | 1 | 1.006 ± 0.015 | 100.75 | 1.33 |
| | 2 | 1.980 ± 0.010 | 99.13 | 0.53 |
| | 3 | 2.993 ± 0.032 | 99.87 | 1.10 |
| Witha nolide A | 1 | 1.005 ± 0.006 | 100.93 | 0.49 |
| | 2 | 2.006 ± 0.025 | 100.38 | 1.20 |
| | 3 | 3.017 ± 0.035 | 100.58 | 1.26 |

[a] Mean ± SD (n=3)

variations (±2) in the ideal circumstances, such as column set temperature, the strength of mobile phase buffer and detection wavelength, the robustness was assessed. There were no discernible alterations found in the peak area or retention time. RSD of retention time and response was determined to be within 2% for each of the optimal and changing circumstances which reveals that the method is robust (Table 4). As a result, modest variations in column temperature, detection wavelength, and mobile phase buffer strength will not affect the method for analyzing withanolides in herbal extracts and formulations.

## 3.6. LOQ and LOD

A method's sensitivity is determined by its LOD and LOQ values. The study indicated that the LOQ values for WTA and DWS were 0.23 and 0.32 µg/mL, with detection thresholds of 0.82 and 0.90 µg/mL, respectively (Table 1).

## 3.7 System Suitability

Table 5 displays the results of the evaluation of system suitability parameters. The resolution factors for WTA and DWS were 3.718 and 7.556, respectively. These values exceeded the minimal values specified in the ICH recommendations, indicating that the method provided good resolution of withanolides. The tailing factors of WTA and DWS were 0.933 and 0.923, respectively which complied with ICH criteria (<2%). Thus, the approach provides well-resolved sharp peaks for the withanolides with good symmetry. The capacity factor is commonly used to define an analyte's migration rate on a column, and according to ICH recommendations, it should be between 1 and 10. The method determined capacity factors for WTA and DWS of 3.564 and 3.274, respectively, which were judged to be within the limits of ICH standards. It was demonstrated by all the parameters that the chromatographic condition employed was appropriate for the analysis of withanolides in herbal extracts and formulations.

**Table 4.** Results of Robustness study for withanolides

| Parameters | Deliberate changes | Withanolide A | | | | 12-deoxy-withastramonolide | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Response (AU) | | RT (min) | | Response (AU) | | RT (min) | |
| | | Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| Column Temperature | 40 ± 2°C | 733258 | 0.72 | 13.21 | 0.19 | 549452 | 1.28 | 12.15 | 0.53 |
| wavelength | 230 ± 2 nm | 734698 | 0.85 | 13.14 | 0.40 | 551200 | 1.59 | 12.09 | 0.46 |
| Mobile phase strength | 10 ± 2 mM | 735483 | 1.20 | 13.08 | 0.26 | 548068 | 1.59 | 12.04 | 0.13 |

**Table 5.** Results of system suitability parameters

| Parameters | Withanolide A | 12-deoxy-withastramonolide |
|---|---|---|
| Tailing factor | 0.933 | 0.923 |
| Theoretical Plates | 69884 | 64713 |
| Resolution | 3.718 | 7.556 |
| Capacity factor | 3.564 | 3.274 |

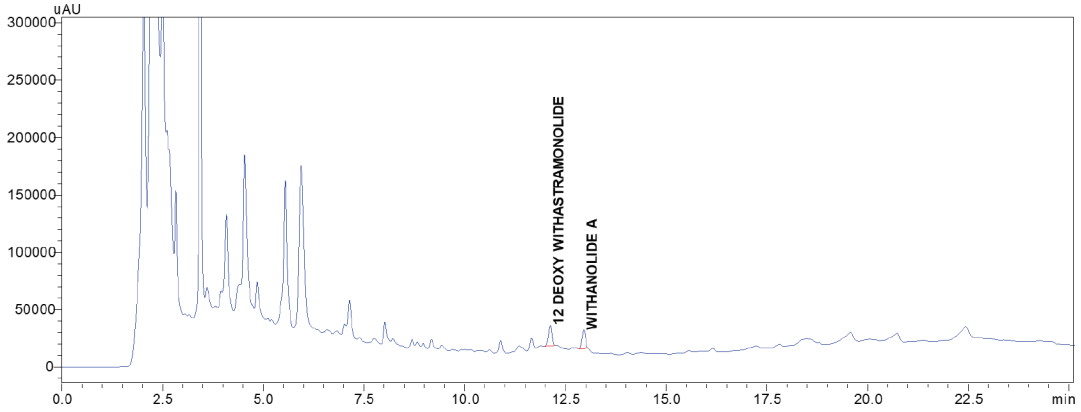


**Figure 8.** Representative HPLC chromatogram of *W. somnifera* root extract.

### 3.8 Content of Withanolides in *W. somnifera* Root

When the standard (Figure 1) and extract chromatograms (Figure 8) were compared, the mean retention times of withanolides were found to be 12.04 ± 0.06 min for WTA and 13.14 ± 0.10 min for DWS, respectively. The content of WTA and DWS in a hydroalcoholic extract of *W. somnifera* root was 1.15 (µg/mg) and 0.46 (µg/mg) respectively. The values of total withanolides were near the standard extract value[27,28].

## 4. Conclusion

The results suggest that several phytoconstituents present in *W. somnifera* root extract may be the cause of its potential therapeutic benefits. The HPLC method that was developed and validated is accurate, precise, specific, reproducible, and repeatable for estimating WTA and DWS. Aside from the standards mentioned, *W. somnifera* root extract and its formulation contain various additional compounds that are now being investigated further. The approach is suitable for detecting WTA and DWS in hydroalcoholic extracts from the plant's roots. With an increasing demand for herbal pharmaceuticals and a growing trust in the efficacy of herbal medicine, the development of a reliable standardization tool would aid in the quality of such vital herbal formulations.

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