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Research article

# High-Performance Liquid Chromatography: Validation of bioactive compounds present in Ashwagandha extract

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**Abstract:** The phytochemical constituents of the Ashwagandha include 40 types of Withanolides, and 12 types of alkaloids. Glycowithanolides and Withaferin are the compounds have a wide range of medicinal properties such as anticancer, anti-inflammatory, antiviral and angiogenic effects. In this present investigation, suitable extraction method of Withanolides was seized and In-House Performance Liquid Chromatography (RP-HPLC) analysis method for targeted eight Withanolides of *Withania somnifera* was developed. The mobile phase consisted of 0.1% ortho-phosphoric acid in water and acetonitrile with gradient elution. Photodiode array detection was used to profile the composition and quantification of Withanolides. The method described good resolution and easily applied to the separation and identification of Withanolides.

**Keywords:** Indian Ginseng - RP-HPLC - Bio-Active Compounds - Secondary Metabolites - Withanolides - Withaferin.

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## INTRODUCTION

*Withania somnifera* (L.) Dunal (Solanaceae), commonly known as Ashwagandha or Indian Winter Cherry or Indian Ginseng is a well-known medicinal plant used for long time in Ayurveda medicine (Kumar *et al*. 2015). Traditional plant remedies are generally a preferential starting point for projects of drug discovery from natural sources it is widely found in India, Iran, Sri Lanka, Syria, Turkey, Iraq and Africa (Mehra *et al*. 2014, Nasir 2017, Kassa *et al*. 2021). The search for novel bioactive metabolites used in pharmaceutical and cosmetic products is to a wide extent directed towards natural resources and India holds rich plant diversity, and therefore it is attracting much interest for the discovery of new bioactive compounds (Doughari 2012, Kumaran & Citarasu 2015, Shrestha *et al*. 2020). However, Indian traditional medicine to meet people's health needs has been recorded for thousands of years in herbal pharmacopoeia in the form of scriptures and herbal medicinal books like Unani, Ayurveda and Sushruta & Charaka Samhita (Bajpai *et al*. 2016, Nile *et al*. 2019).

*Withania somnifera* is used as an emetic, sedative, diuretic and for the treatment of asthma, atherosclerosis, cognitive behavior, potency issues, and aging; as it has bioactive compound specifically for anti-cancer, immunomodulatory, anti-oxidative, neurological effects (Lal *et al*. 2006). Withanolides are 22-hydroxyergostan-26-oic acid-26, 22-lactones, structurally based on an ergostane skeleton of 28 carbon atoms, oxidized at C-22 and C-26 to form a d-lactone (Saleem *et al*. 2020). Till now 35 Withanolides have been identified and isolated from *Withania somnifera* (Sharma 2013). In dry regions of South Asia, Africa, and Central Asia Ashwagandha is cultivated. More than 50 chemical constituents have been isolated from different parts of the Ashwagandha plant (Sinha & Rosenberg 2013). The metabolic constituents, particularly secondary metabolites vary with variety of *W. somnifera* and its tissue use and growth condition. This leads in inconsistency in composition and standardization for herbal composition as this plant has multi-component therapeutic system and this negatively impact on commercialization (Chaurasiya *et al*. 2009, Kumar *et al*. 2015).

The number of analytical reports for the determination of Withanolides is comparatively less. Besides a TLC method for the quantification of Withanolides, very few HPLC methods are described in the literature. Different parts of the plant have been studied and found over 12 alkaloids, 35 Withanolides and other phytochemicals (Qamar *et al*. 2012, Deepak *et al*. 2014).


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*W. sominifera* are withanolides with C28 steroidal nucleus with C9 side chain, they are highly oxygenated phytochemicals, and the oxidation at various sites of the skeleton is responsible for the structural variation in different classes of withanolides (Abdu *et al*. 2018). All of them show major disadvantages, as the separation time is extremely long (several hours) or the compounds are not baseline separated and elute more or less with the injection peak (Qamar *et al*. 2012). In the literature, there is no report for the combined method of extraction and identification of Withaferine-A and Withanolides-A and B and other Withanolides in roots and herbal formulations of *Withania somnifera*. Therefore, in the present investigation, we have reported a simple, rapid and reproducible method for the extraction and identification of major Withanolides from Ashwagandha roots.

## MATERIAL AND METHODS

### *Plant material*

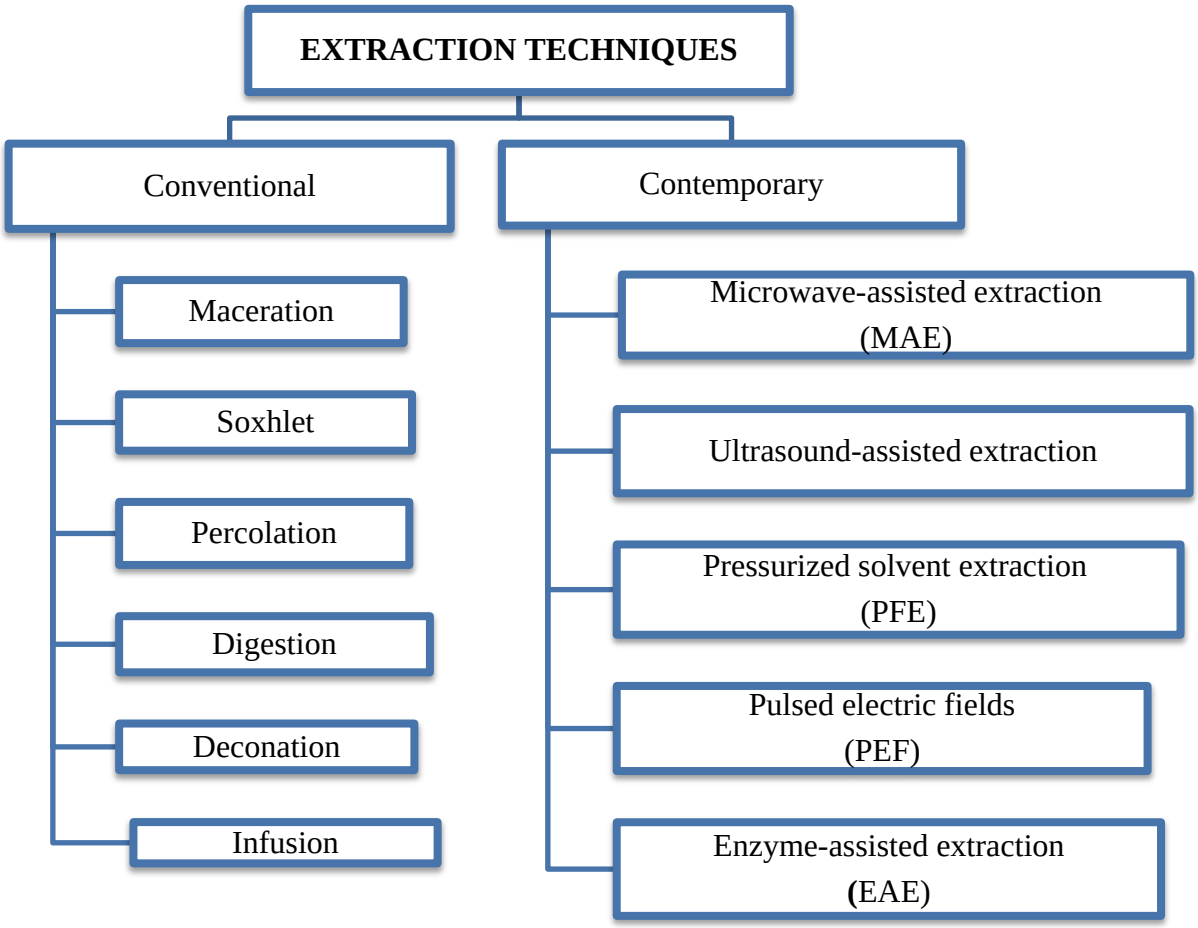
Roots of *Withania sominifera* were collected from the local market, in house botanist verified the physiological quality of root as Ashwagandha roots are habitat of molds. To avoid any contamination material then was air dried at room temperature for 4–5 days in a contaminant free area, then using mixture-grinder root was powdered mechanically in 40–60 mesh size rang (Sharma 2013, Kaul *et al*. 2016). As this particle size gives good extraction results. This root powder was used as plant material for the extraction of withanolides, withanifera and other physiochemical and analytical analysis (Kharel *et al*. 2011).

### *Sample preparation/isolation & extraction*

Isolation by using conventional and contemporary methods. Initially, 20g *W. somnifera* root powder (40 mesh) with food grade absolute organic solvent extracted by Soxhlet extraction method at 65–70 °C temperature, allowed continues cyclical repetitions of the extraction during a control period for complete extraction of *withanolides* and *withaferine* (Altemimi *et al*. 2017).

### *Extraction method*

Extraction is method to separate the desire natural products from raw materials. According to the extraction principle popular extraction methods includes solvent extraction, distillation method, pressing and sublimation (Dev *et al*. 2011, Doughari *et al*. 2012, Cao *et al*. 2015).



The objective of this study was to develop an RP-HPLC method for separation and identification of eight targeted Withanolides like Withanoside IV, physagulin D, 27-Hydroxywithanone, Withanoside VI, Withaferin A, Withastramonolide, Withanolide A and B, Withanone from the roots of Ashwagandha.

*Instrument/Apparatus*

The HPLC system consisted of an Agilent series 1260 infinity II equipped with a quaternary pump, an auto sampler, and a thermostatic column oven and diode array detector with open Lab CDS software (Srivastava *et al*. 2008).

*Chemicals and solvents*

Mobile phases for the development of chromatographic methods were prepared with Ortho-phosphoric acid (88% HPLC grade), Potassium dihydrogen orthophosphate (AR grade), Acetic acid (HPLC grade >88%), acetonitrile (HPLC grade), organic solvent (HPLC grade) solvent were purchased from Merck Life science private limited, Mumbai, and purified water used from in-house Milli-Q purification system (Merck-IQ 7003/05/10/15).

*Preparation of stock solutions and samples*

Stock solutions of reference compounds were prepared in HPLC grade organic solvent refrigerated. Working solutions of these reference compounds, concentration range of 20µg to 100 µg $ml^{-1}$ were prepared by diluting stock solution with HPLC grade organic solvent. For standard curve graph 5 µl final concentration of seven withanolides combined mixture was prepared.

*Standard reference material*

Pure stock solution Withaferin-A (5119-48-2, ≥95%), Withanoside-IV (1719532, ≥95%), Withanoside-V/VI (256520-908, ≥95%), 12-deoxywithastramonolide (60124-17-6, ≥95%), Withanolide-A (32911-62-9, ≥95%), Withanone (27570-38-3, ≥95%), Withanolide-B (56973-41-2, ≥95%) were purchased from sigma-Aldrich and Merck life science private limited, Mumbai, India.

*HPLC method development*

The aim of the optimization was the separation of minor and major Withanolides. An HPLC condition was performed using the mix reference compound solution (Lal *et al*. 2014, Girme *et al*. 2020). Many HPLC methods had been reported to identify the characteristic of Withanolides in the literatures (Cao *et al*. 2015) and by taking them as reference methods were developed. For method development mobile phase, column type, column temperature, detector wavelength's variation was studied.

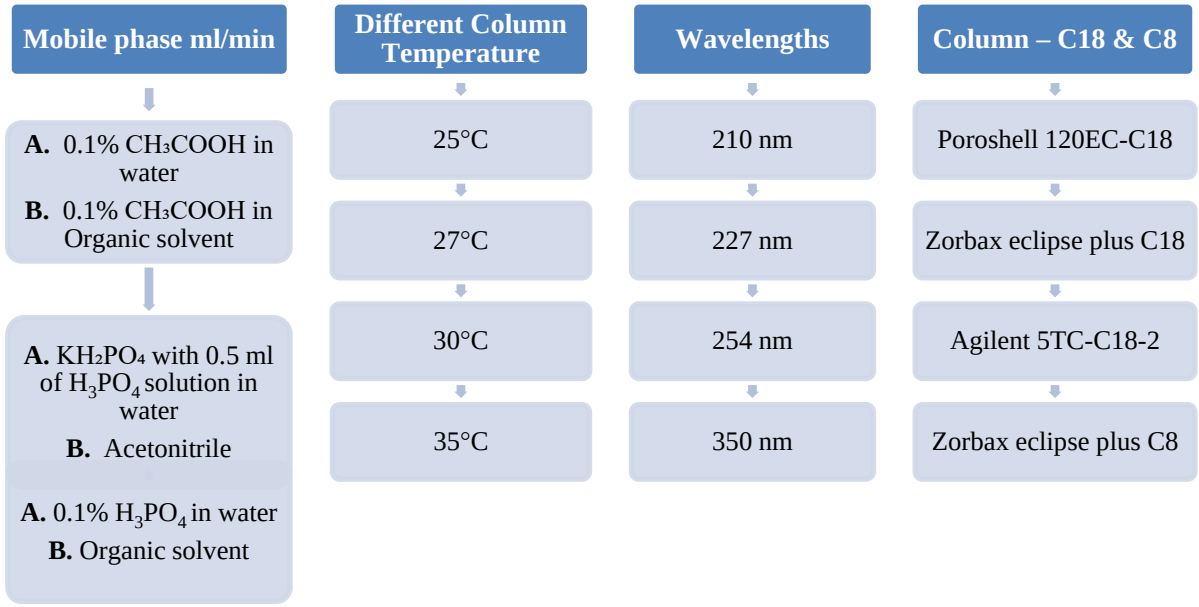



**Figure 1.** Variation in HPLC condition.

For mobile phase three different combinations of organic solvent with water and organic solvent with organic solvent combination was used, for column type four different columns were used apart from this column temperature and wavelength was also varied which are pointed out in figure 1 (Nadia *et al*. 2011, Kumar *et al*. 2018).

## RESULTS AND DISCUSSION

This study aimed to separate and identify 08 Withanolides and optimize system suitability parameters. For the mobile phase buffer selection experiment conducted with mobile phase A: 2% Acetic acid in water and B:2% acetic acid in organic solvent was given in Kharel *et al*. (2011) but it is observed that this mobile phase is suitable for the separation of Withaferine A only. Further, experiment different concentration of potassium dihydrogen orthophosphate with 0.5 ml of ortho-phosphoric acid in water as mobile phase A and organic solvent as a buffer B was used but results were not satisfactory. Good resolution isolated peak obtained by using 0.1% Ortho-Phosphoric Acid in water and Acetonitrile as mobile phase A and B respectively. This method designed by using elution gradients of mobile phase A and B. After many combination of mobile phase A & B like 0- 15 min, buffer B was increased from 5–30 %; from 16–28 min buffer B was increased 45%; and 29–45 min buffer was increased to 95%., during this method Poroshell 120 C-18,150 cm long column was used for high-resolution separation of withanolides, and it observed that chromatograph of this method gave merged peaks between 12–18 min retention as showed in figure 2.

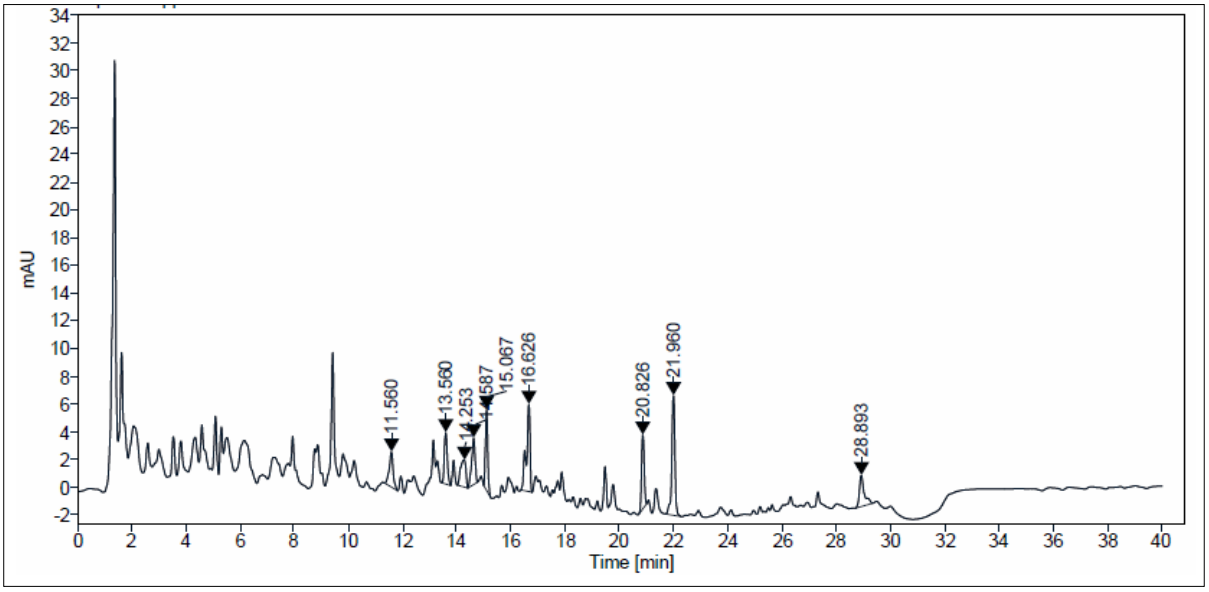



**Figure 2.** Gradient flow and column suitability peak separation.

From the above chromatograph it was concluded that gradient flow and column type is not suitable for withanolides separation for this study. Finally, by keeping gradient condition 0–18 min %B was increased 5–45%; at 19–25 min %B was increased 80%; and for 26–28 min flow was constant; from 29–35 min it was again varied 5% and equilibrated for 10 min., by using this mobile phase condition and column eclipsed C-18, 250 cm maximum peak was separated.

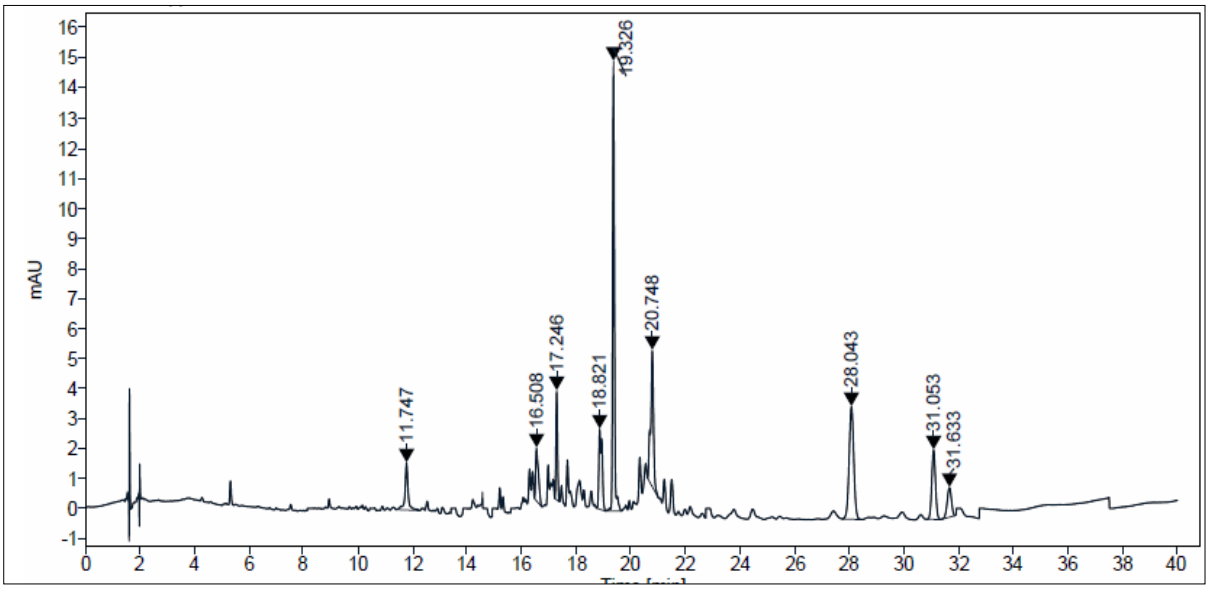



**Figure 3.** Peak separation with tailing of mid polar compound.

As wavelength is an important parameter during hplc analysis, in this study for the separation of 08 withanolides four wavelengths from 210 to 350 nm were tested for the maximum number of peak responses and relatively high sensitivity. It was tried one by one to optimize proper wavelength for the major withanolides detection. With 210 nm wavelength only two minor peaks were detected, with 254 nm wavelength three peaks were detected, so 227 nm wavelength was used in method development and maximum withanolides peaks were detected. By this we concluded that for our study of 08 withanolides, 05 withanolides were separated at 227 nm wavelength. However, it is observed during separation of compounds that Withanoside-V/VI and Withaferine-A were hard to separate on 227nm wavelength as retention time of both compound is almost nearby (Girme *et al*. 2020).

During this study we also changed column temperature (25–35 °C) to observe the effect on compound peak separation of polar and nonpolar compounds and we found that column temperature could be problematic for the chromatographic behavior (Cao *et al*. 2015). And so we opt for 25°C column temperature as, at this temperature all six compounds were well separated.

After studying all major factors, a method was optimized for separation of six withanolides which is shown below in table 1.

**Table 1.** HPLC optimized method.

| | |
|---|---|
| Mobile phase buffer A | 0.1% Ortho-phosphoric acid in water |
| Mobile phase buffer B | Acetonitrile |
| Elution | Gradient (A-95 to 25 in –25 min, reverser after 28–35 min; B-5 to 75 in 0–25 min, reverse after 28–35 min) |
| Column | Eclipsed C-18, 250 mm* 4.5 µ, Agilent |
| Flow rate | 1 ml $min^{1}$ |
| Injection volume | 20 µl |
| wavelength | 227 nm |

After several trials, the polar solvent system of water with 1% Acetic acid and acetonitrile with pH 2.5 was found effective in the separation of nonpolar and mid polar compounds, to achieve a better resolution and an acceptable tailing factor mobile phase gradient composition given in table 1 and peak separation shown in chromatogram figure 3. With this method we got effective separation of nonpolar and mid polar compounds, and achieved high resolute with acceptable tailing factor chromatogram which is shown in figure 4.

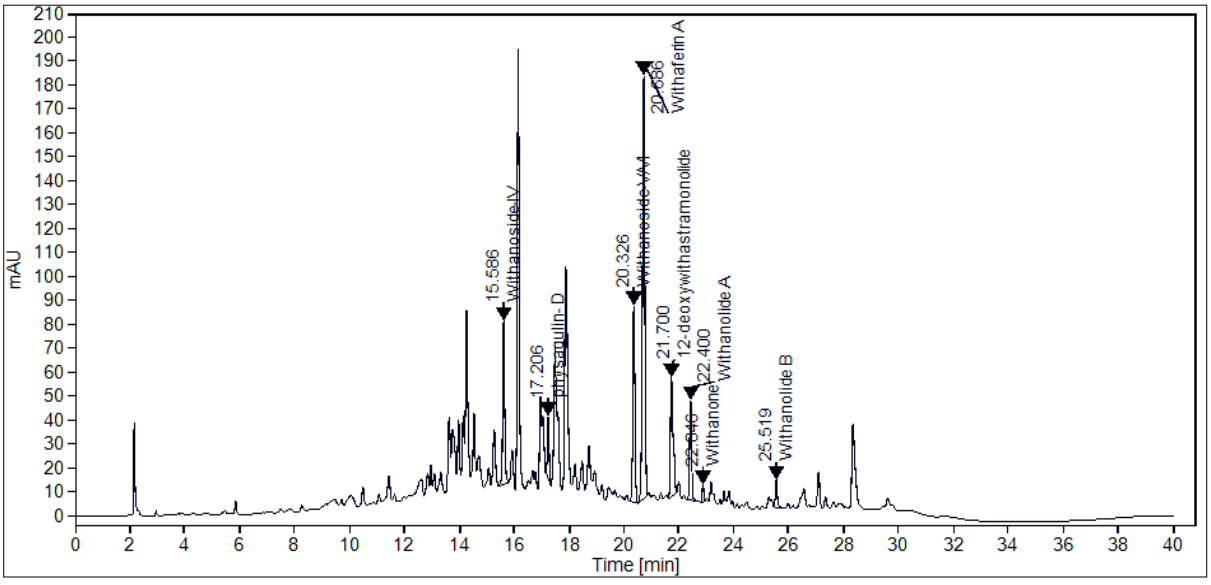



**Figure 4.** Separated peaks of Withanolides.

During our study it is also observed and noted, that Withanolides-V/VI and Withaferine-A could be separated only on a Zorbax Eclipse plus C18, (250×4.6 mm, 5-micron) column at 25°C among those tested columns and column temperature. By using condition mentioned in table-1, 08 withanolides like 1.Withanoside-IV, 2.Physagulin-D, 3.Withanoside-V/VI, 4.Withaferin-A, 5.12-deoxywithastramonolide, 6.Withanolide-A, 7.Withanone, 8.Withanolide-B with peak area of 354.91, 110.64, 430.11, 1037.38, 343.75, 217.03, 28.94, 59.70 mAu and retention time of 15.586, 17.206, 20.326, 20.686, 21.700, 22.400, 22.846, 25.519 respectively was successfully separated.

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