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Application Note

# HPLC Analysis of Ashwagandha Supplements Using Alliance™ iS HPLC System

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## Abstract

This study developed a High-Performance Liquid Chromatography (HPLC) method for the analysis of major withanolides in ashwagandha root and commercially available supplements. System suitability testing confirmed the method's performance. Ultrasound-assisted extraction with a 60:30:10 ethanol:methanol:water solvent for 30 minutes yielded the highest withanolide recovery from ashwagandha root. The method successfully separated and quantified the major withanolides in the USP reference root sample. However, analysis of commercially available ashwagandha supplements revealed the presence of only some of these withanolides, often in much lower quantities compared to the USP root sample.

## Benefits

- Using Alliance iS HPLC System for the separation and quantification for six active compounds in ashwagandha samples and supplements
- Successful implementation of AOAC 2015.17 for the analysis of ashwagandha on Alliance iS HPLC System
- The method successfully met the System Suitability Testing (SST) requirements

- Optimized sample preparation procedure

## Introduction

*Withania somnifera* (L.) Dunal [Solanaceae], commonly referred to as ashwagandha, is a plant native to India that gained significant scientific interest in recent years due to its potential therapeutic benefits. Withanolides, bioactive compounds found in the root of ashwagandha, have been used traditionally for medicinal purposes for thousands of years.[1,2] Several studies on ashwagandha have reported a range of pharmacological properties for these compounds, including anti-inflammatory, anti-cancer, antidepressant, neuroprotective, and hepatoprotective effects.[3–7]

Ashwagandha consists of many chemical compounds from diverse chemical classes. These include lactones, flavonoids, and tannins that can be found in different parts of the plant including, roots, berries, and aerial parts. However, the traditional Indian system of medicine specifically emphasizes the use of ashwagandha roots for therapeutic purposes. Figure 1 illustrates six common chemical constituents typically extracted from the roots: withaferin A, withanolide A, withanolide B, 12-deoxywithastramonolide, withanoside A, and withanoside B.

Ashwagandha extracts and food supplements are widely available in the market and ensuring the quality and potency of these formulations can be challenging due to the complexity and the variability of these formulations. HPLC is one of the most commonly used techniques for the analysis of these extracts due to its robustness and reliability. It plays a very crucial role in verifying the potency and purity of ashwagandha extracts.[8,9] Specifically, HPLC analysis can quantify the levels of specific withanolides to ensure therapeutic efficacy and identify and eliminate potential adulterants.[8]

This study demonstrates the use of AOAC 2015.17 for the analysis of ashwagandha root extracts and dietary supplements using the Alliance iS HPLC System.[10]

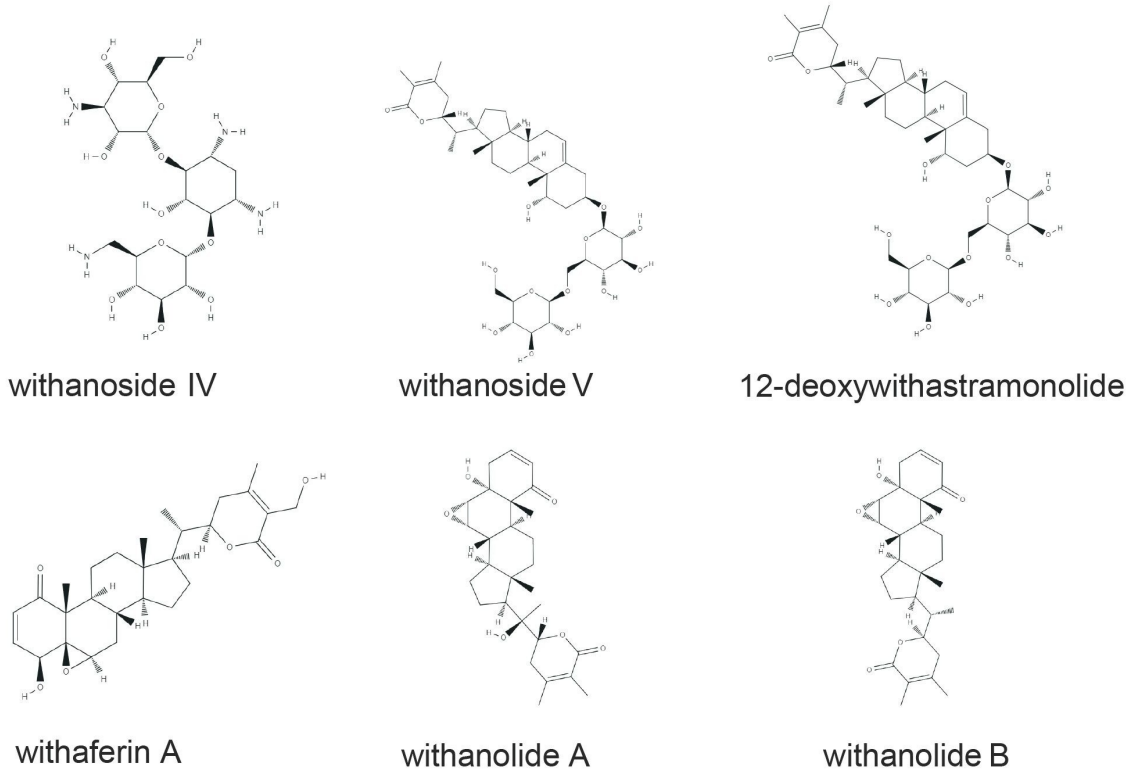


*Figure 1. Chemical structures of six common bioactive compounds normally found in ashwagandha root samples.*

# Experimental

## Standard Preparations

Withanolide standard solutions were prepared by accurately weighing 10 mg each of the reference standards: withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A, and withanolide B. These were then transferred to a 50 mL volumetric flask and dissolved in 10 mL of methanol using gentle heat. After cooling, the solution was diluted to the mark with methanol, yielding a stock solution with a concentration of 200 µg/mL for each standard. Further dilutions were made from this stock solution using appropriate volumes of methanol to obtain working concentrations of 150, 100, and 50 µg/mL.

Mobile Phase Preparation

Solvent A was prepared by dissolving 0.136 g of anhydrous potassium dihydrogen phosphate ($KH_2PO_4$) in 900 mL of HPLC grade water using the Milli-Q Water purification system from Millipore. 0.5 mL of orthophosphoric acid was then added. The solution was diluted to a final volume of 1000 mL with water, filtered through a 0.45 µm membrane, and de-gassed in a sonicator for three minutes (referred to as mobile phase A).

## Sample Preparations

In this study, various samples containing ashwagandha root extracts were explored. These samples included a powdered ashwagandha root extract (reference standard) purchased from the United States Pharmacopeia (USP) in Rockville, MD, as well as several other herbal supplements obtained from online drug stores. The goal was to ensure complete extractions of ashwagandha extracts.

Sample Preparation (a)

A certain amount of sample, equivalent to the recommended serving size, was obtained. This sample was then transferred to a 50 mL volumetric flask, which was filled to the mark with the extraction solvent (described in the text). The flask was placed in a hot water bath at 50 °C for an hour. After cooling down, the contents of the flask were agitated for five minutes. Next, the flask contents were transferred into a plastic centrifugation tube, which was then centrifuged at 10,000 rpm for five minutes. The resulting supernatant was filtered through a 0.2 µm PVDF disk filter before being injected onto the HPLC system.

Sample Preparation (b)

Another specific amount of sample, also equivalent to the recommended serving size, was obtained. This sample was transferred to a 50 mL volumetric flask, which was filled with the extraction solvent. The flask was then sonicated for the desired period of time instead of heating in a water bath. The remaining steps were exactly the same as in sample preparation a.

## LC Method conditions

| | | | |
|---|---|---|---|
| System name | Alliance iS HPLC System | | |
| Column details | XBridge™ BEH $C_{18}$, 4.6 mm × 250 mm, 5 µm<br>p/n 186003117 | | |
| Column temperature | 30 °C | | |
| Mobile phase A | $KH_2PO_4/H_3PO_4$ in water (described in the text) | | |
| Mobile phase B | Acetonitrile | | |
| Flow rate | 1.5 mL per minute | | |
| Gradient profile | Time (mins) | % Mobile Phase A | % Mobile Phase B |
| | 0 | 95 | 5 |
| | 18 | 55 | 45 |
| | 25 | 20 | 80 |
| | 28 | 20 | 80 |
| | 35 | 55 | 45 |
| | 40 | 95 | 5 |
| | 45 | 95 | 5 |
| Detector type | TUV<br>Filter: Normal Data Rate: 10Hz | | |
| Detector wavelength | 227 nm | | |
| Injection volume | 10 µL | | |
| Autosampler needle wash solvent | 2:8 ACN/$H_2O$ | | |
| Wash solvent | 2:8 ACN/$H_2O$ | | |
| Run time | 45 minutes | | |

Data management

| | |
|---|---|
| Chromatography software | Empower 3.8 |

# Results and Discussion

## System Suitability

To evaluate the performance of the chromatographic system, System Suitability Testing (SST) was carried out.

SST serves as a standard procedure to verify the performance of a chromatographic system, ensuring its suitability for specific analyses. In this study, the SST followed the guidelines outlined in AOAC 2015.17, which encompasses several measurements, including repeatability, relative retention time, resolution, peak tailing, and coefficient of determination.[10] The results of our experiment demonstrated excellent agreement with all the system suitability criteria outlined in the AOAC 2015.17.[10] These findings are summarized in Table 1 and the chromatograms for the different concentration levels of the standard are visually represented in Figure 2.

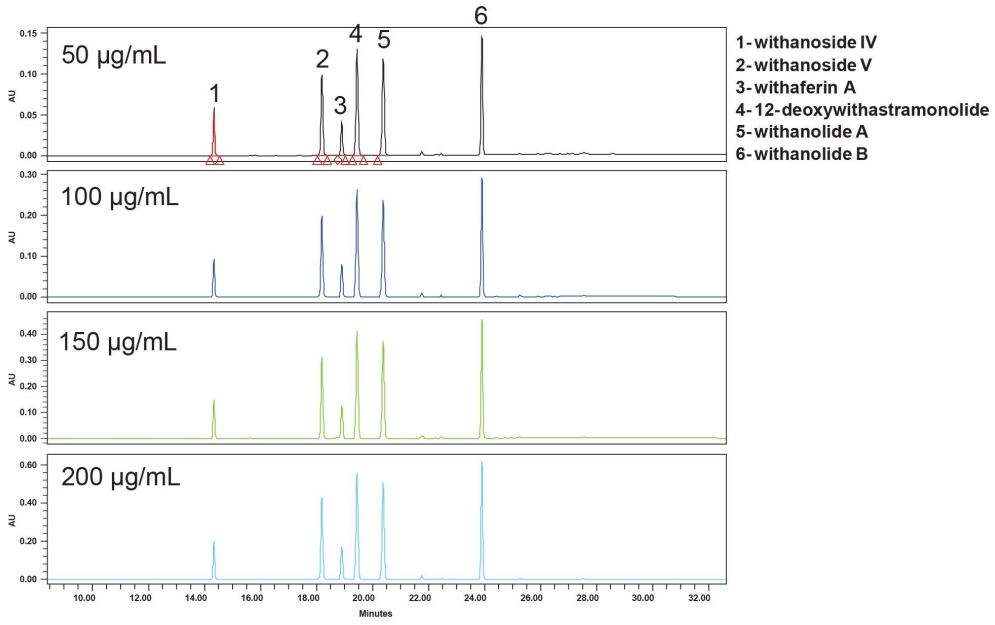


*Figure 2. Representative separations of ashwagandha extracts in standard solutions at four concentration levels, 50, 100, 150, and 200 µg/mL. HPLC conditions are the same as in the experimental section.*

| Criteria | | Results |
|---|---|---|
| Resolution | Not less than (NLT) 3 between withanoside V and withaferin A peaks in the 50 µg/mL mixed standard preparation | 5.7 |
| Tailing factor | The tailing factor must be ≤1.5 for all individual withanolides in the standard solution | All withanolides have tailing factor of around 1.0 |
| Relative retention time | The relative retention times of the standards should be 0.70 for withanoside IV, 0.89 for withanoside V, 0.92 for withaferin A, 0.96 for 12-deoxywithastramonolide, 1.0 for withanolide A, and 1.15 for withanolide B. | Relative retention times matches the system suitability criteria for all analytes |
| Repeatability | The RSD of the peak areas from the triplicate injections of the 50 µg/mL mixed standard preparation must be ≤2.0% for each withanolide | %RSD for peak areas of all withanolides ≤0.55% |
| Coefficient of determination ($R^2$) | $R^2$ for the regression line of peak areas vs concentration for each withanolide must be ≥0.998 | All withanolides have $R^2$ ≥0.999 |

*Table 1. System Suitability criteria and actual results generated in this study of the analysis of a standard solution containing common ashwagandha extracts.*

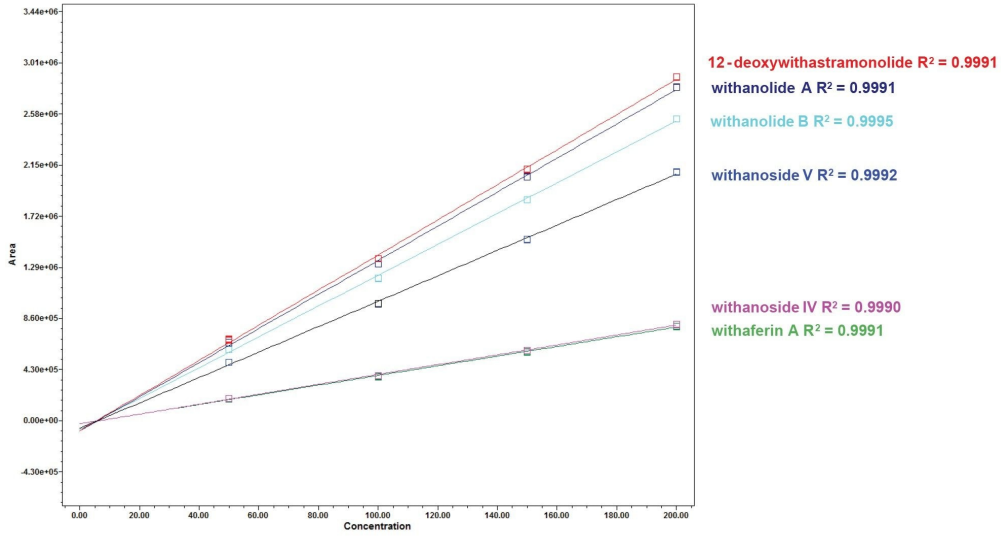


*Figure 3. Calibration curves for six common bioactive compounds in ashwagandha at four concentration levels (50, 100, 150, and 200 µg/mL) in standard solutions.*

## Optimizing Extraction Efficiency in Ashwagandha Root

Various extraction techniques have been implemented to isolate the active compounds in ashwagandha from

plant material and dietary supplements. Some of the most frequently used methods for extracting these active compounds include reflux extraction, Soxhlet extraction, ultrasound-assisted solvent extraction (UASE), and microwave-assisted solvent extraction (MASE).[11,12]

In our study, two approaches were investigated: ultrasound-assisted extraction using an ultrasonic bath and conventional solid-liquid extraction with heated organic solvent, as previously outlined in the experimental section. The objective of this experiment was to determine which sample preparation method yields the highest extraction recovery of active compounds from ashwagandha root samples. To achieve this, a root sample was obtained from the USP and prepared as previously described in the text prior to analyzing it using the Alliance iS HPLC System. The amount of root sample that was used in these experiments was 0.5 g (equivalent to 5 mg of each active compound) as recommended by AOAC 2015.17 and multiple other references.[10,13]

1. Solid-Liquid Extraction Using Heated Organic Solvent

In this experiment, samples were extracted to assess the extraction efficiency of solvents heated in a water bath at 50 °C according to "sample preparation a" procedure described earlier in the text. All samples were then analyzed using the Alliance iS HPLC System. Three different solvent compositions were explored here and these included methanol, ethanol:water 90:10, and ethanol:methanol:water 60:30:10. Results of this experiment were not very successful as the extracted amounts of each compound fell below the lowest concentration point on the calibration curve. Consequently, it was decided not to pursue this extraction method further.

2. Extraction Using Ultrasonic Bath

A. The Impact of the Extraction Solvent

The goal of this experiment was to establish the efficiency with which ashwagandha extracts could be extracted. To examine this, USP ashwagandha root reference standard were sonicated for the same period of time using different extraction solvents that have been previously reported.[13] The results of these experiments are shown in Figure 4. The ethanol:methanol:water (60:30:10) mixture yielded the highest extraction recovery for most of these compounds in ashwagandha, surpassing other extraction solvents. For instance, the extracted amount of each compound ranged from 1.0 to 4.7 mg, whereas with ethanol-water it was 1.0–3.5 mg, and with methanol it was 1.0–3.7 mg. As a result, this extraction solvent was then employed for all subsequent extractions across all samples.

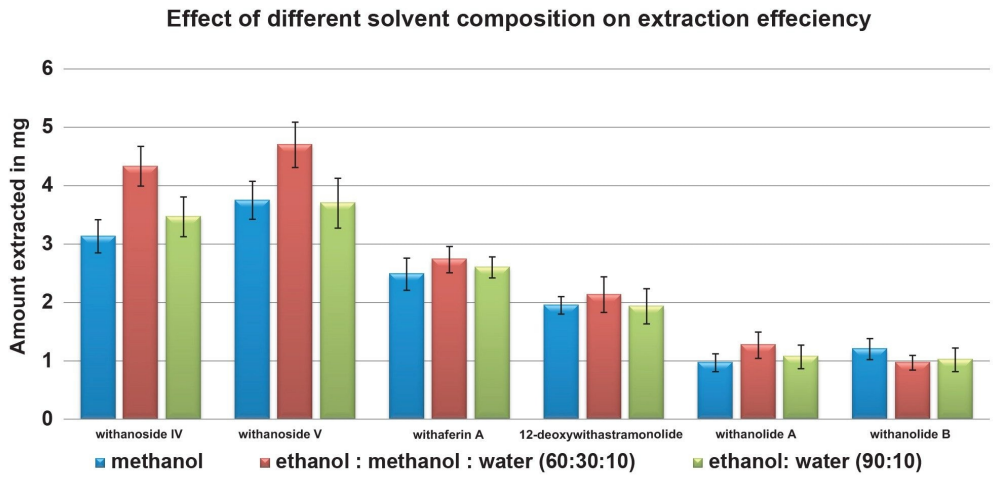


*Figure 4. The effect of the extraction solvent composition on extraction efficiency of active compounds from ashwagandha root sample. Each data point is based on three replicate measurements, where the error bar denotes the standard deviation.*

B. The Impact of Sonication Time

In this experiment, the effect of sonication time on the extraction recovery was investigated. To do this, multiple durations were tested to determine the influence of this variable. As depicted in Figure 5, longer sonication times led to improved extraction efficiency. For instance, a greater amount of material was extracted from ashwagandha root at 20 and 30 minutes, compared to ten minutes. Interestingly, extending the sonication time to 40 minutes did not yield any additional improvement. This suggests that 30 minutes is sufficient for achieving complete extractions. Consequently, we chose a combination of 30 minutes of extraction and the use of ethanol:methanol:water (60:30:10) as the extraction solvent for all subsequent extractions in ashwagandha supplements during the next phase of this study.

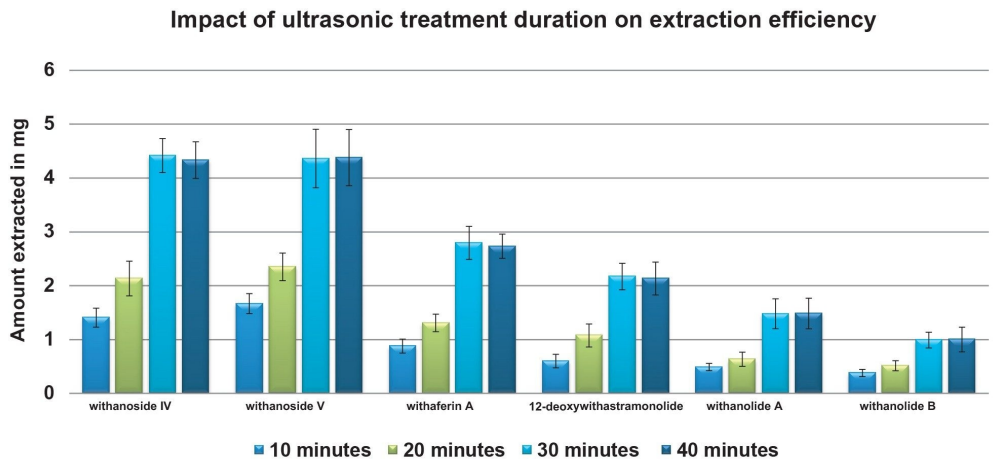


*Figure 5. The effect of sonication time on the extraction efficiency of compounds from ashwagandha root sample. Each data point is based on three replicate measurements, where the error bar denotes the standard deviation.*

## Analysis of Ashwagandha Supplements

In this part of the study, the goal was to utilize the HPLC method in conjunction with the developed extraction method for analyzing both the USP ashwagandha root and commercially available tablets and liquids supplements. To do this, the samples were prepared using an ethanol:methanol:water (60:30:10) extraction solvent and sonicated for 30 minutes, based on our findings from the previous experiment. The prepared samples were subsequently injected onto the Alliance iS HPLC System using the conditions listed in Figure 2. The results, presented in Figure 6, demonstrate that the major compounds present in the ashwagandha USP root sample were successfully separated and quantified by the method. For instance, these extracts exhibited concentrations ranging from 0.2% to 0.8% (wt/wt), as detailed in Table 2.

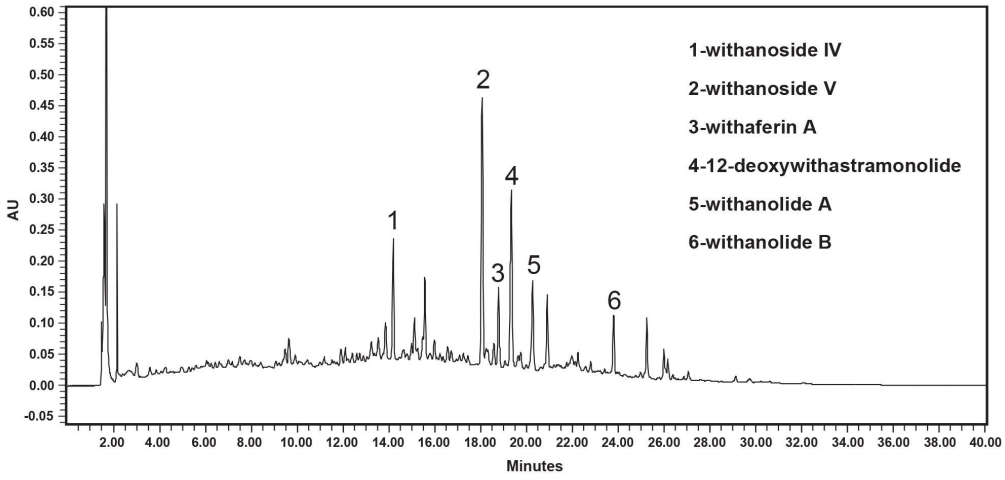


*Figure 6. Representative separations of active compounds in ashwagandha root sample under the same conditions as those in Figure 2.*

For the analysis of commercially available ashwagandha dietary supplements (tablets and drops), the results demonstrated successful separation and quantification of the major ashwagandha extracts. However, only some of these active compounds were detected and quantified in the samples. These findings emphasize the importance of having a robust HPLC method and extraction procedure to accurately quantify the major peaks in these supplements. Notably, many of the analyzed samples lacked several of these peaks, and the ones that did contain them had very small quantities compared to the USP root sample, as illustrated in Table 2.

| Sample | withanoside IV | | withanoside V | | withaferin A | | 12-deoxywithastramonolide | | withanolide A | | withanolide B | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amount | S/N | Amount | S/N | Amount | S/N | Amount | S/N | Amount | S/N | Amount | S/N |
| USP Ashwagandha root | 0.88% wt/wt | 163 | 0.78% wt/wt | 361 | 0.59% wt/wt | 107 | 0.43% wt/wt | 240 | 0.30% wt/wt | 119 | 0.20% wt/wt | 82 |
| Formula 1 (Capsule) | 2.68 mg/dose | 122 | 1.3 mg/dose | 157 | 1.33 mg/dose | 157 | 0.87 mg/dose | 134 | 1.24 mg/dose | 190 | BCR* | 64 |
| Formula 2 (Capsule) | Missing | – | BCR | 25 | 0.62 mg/dose | 55 | Missing | – | BCR | 48 | Missing | – |
| Formula 3 (Capsule) | Missing | – | BCR | 52 | missing | – | BCR | 54 | BCR | 70 | BCR | 52 |
| Formula 4 (Drops ) | Missing | – | Missing | – | Missing | – | Missing | – | BCR | 17 | Missing | – |
| Formula 5 (Drops ) | Missing | – | Missing | – | Missing | – | Missing | – | Missing | - | Missing | – |
| Formula 6 (Drops ) | Missing | – | Missing | – | 1.13 m/dose | 79 | BCR | 28 | 0.53 mg/dose | 109 | Missing | – |

*Below the calibration range (calibration range is 10–200 µg/mL)

*Table 2. Quantification of ashwagandha extracts in ashwagandha root reference standard and supplements.*

## Conclusion

- This application note demonstrates employing a robust HPLC method for analyzing ashwagandha samples using the Alliance iS HPLC System
- This study established a robust HPLC method for analyzing the major active compounds in ashwagandha
- The optimized extraction method using ultrasound and ethanol/methanol/water solvent mixture improved the recovery of ashwagandha extracts
- The analysis of commercially available dietary supplements highlights potential variability in ashwagandha bioactive compounds content, suggesting the importance of robust analytical methods, and reliable HPLC systems for quality control

## References

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