1.0 Purpose

This document describes the analytical procedure for the determination of Withanolides in raw materials. Withanolides are calculated as the sum of Withanolide Aglycones, calculated as Withanolide A, and Withanolide Glycosides, calculated as Withanoside IV. The USP designates the following species as comprising the Withanolide Aglycones: Withaferin A, Deoxywithastramonolide, Withanolide A, Withanone and Withanolide B. The USP designates the following species as comprising the Withanolide Glycosides: Withanoside IV, Withanoside V and Withanoside VI. (Note: Withanosides V & VI coelute). Raw material samples vary, and some of the Withanolides mentioned above may be present in small quantities - or may be totally absent. The sample is considered compliant if the sum of the Withanolides *present* is NLT the stated specification.

2.0 Scope

This procedure applies to the identification and quantification of Withanolides in raw materials. This method was validated under protocol PRTCL-20-0105 and reported in RPT-20-0053.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical chemists who have verified their ability to execute this procedure to follow this procedure.

3.2 It is the responsibility of the QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of the QC Laboratory Management and AD Personnel to keep this procedure current with the associated monographs and laboratory practices.

4.0 Definitions

4.1 QC – Quality Control

4.2 AD – Analytical Development

4.3 ACN – Acetonitrile

4.4 MeOH – Methanol

4.5 DMSO – Dimethylsulfoxide

4.6 H_3PO_4 – Phosphoric Acid

4.7 KH_2PO_4 – Potassium Phosphate Monobasic

4.8 ACS – American Chemical Society

4.9 HPLC – High Performance Liquid Chromatography

4.10 UV – Ultraviolet Electromagnetic Spectra

5.0 References

5.1 PRTCL-20-0105 – Verification of an Analytical Method for the Determination of Withanolides by HPLC-UV

5.2 RPT-20-0053 – Verification of an Analytical Method for the Determination of Withanolides by HPLC-UV

5.3 USP Monograph – Ashwagandha Root Dry Extract

6.0 Supplies

6.1 Chemicals – All reagents are ACS grade or better.

6.1.1 Milli-Q Water

6.1.2 ACN

6.1.3 MeOH

6.1.4 H_3PO_4

6.1.5 KH_2PO_4

6.1.6 Withanoside IV Reference Standard

6.1.7 Withanolide A Reference Standard

6.2 Supplies and Glassware

6.2.1 HPLC vials, 12mm X 32mm with screw cap enclosures w/ septa

6.2.2 Volumetric glassware and/or adjustable pipettes and tips

6.2.3 Weigh paper and/or funnels

6.2.4 Syringes with 0.45µ Nylon Syringe Filters

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system

6.3.2 Analytical Balance

6.3.3 Sonicator Bath

7.0 Procedure

7.1 Mobile Phase & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Mobile Phase A: Add 0.14g KH_2PO_4 + 500 µL of H_3PO_4 to 1000 mL of water and mix well.

7.1.1.2 Mobile Phase B: ACN

7.1.2 Extraction Solvent = Diluent = MeOH

7.1.3 Preparations may be scaled as necessary

7.2 Standard Preparation

7.2.1 Accurately weigh and transfer about 25 mg of Withanolide A reference standard into a 25 mL volumetric flask. Add ~20 mL of Diluent and sonicate until dissolved. (Note: Withanolide A has poor solubility and must be transferred and dissolved with utmost care! When necessary, quantitatively transfer the contents of small vials with careful DMSO (1X) & MeOH (5X) rinses and calculate the mass transferred to the flask by difference.)

7.2.2 Cool to ambient then QS to volume with Diluent and mix well – this is the Withanolide A Stock.

7.2.3 Accurately weigh and transfer about 10 mg of Withanoside IV reference standard into a 100 mL volumetric flask. Add ~50 mL of Diluent and swirl to dissolve. (When necessary, quantitatively transfer the contents of small vials with careful DMSO (1X) & MeOH (5X) rinses and calculate the mass transferred to the flask by difference.)

7.2.4 Using a glass volumetric pipet, transfer 10.0 mL of the Withanolide A Stock to the 100 mL flask containing the Withanoside IV. QS to volume with Diluent and mix well – this is the Working Standard.

7.2.5 It is permissible to prepare the Working Standard using *only* Withanoside IV or *only* Withanolide A. Relative response factors are supplied in the calculation section enabling expression of one standard in terms of the other.

7.3 Sample Preparation

7.3.1 The validated range for the analytical method is 0.0595 – 0.1388 mg/mL Withanoside IV and 0.0527 – 0.1230 mg/mL Withanolide A. However, the calculation of Total Withanolides is based on several species.

7.3.2 Therefore, extract sufficient sample (based on the manufacturer assay value) with Diluent in order to generate a concentration that is 2.5 times the concentration of the Working Standard.

7.3.3 Samples can be extracted in Diluent at any volume starting from 10mL. The volume chosen must be in the solubility range (validated at ~0.25 mg/ml). To manage large volumes, the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion further diluted to bring the concentration into the linear range.

7.3.4 Fill the flask to about 70% of the chosen volume with Diluent then sonicate for 25 minutes. Cool to ambient, perform further dilutions as required using Diluent then filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.3.5 For raw materials being analyzed for the first time using this method, an in process validation is required to demonstrate spectral purity, baseline separation of peaks, and extraction efficiency as a part of system suitability before data can be reported using this method.

7.4 HPLC Parameters

7.4.1 Column: Restek Raptor ARC-18, 4.6 x 150mm, 2.7µm (Or Equivalent)

7.4.2 Column Temperature: 45°C

7.4.3 Flow rate: 1.2 mL/min

7.4.4 Mobile Phase: Gradient

7.4.4.1

Time, min	%B (ACN)
0	5
18	45
25	80
28	80
30	5
40	5

7.4.5 Wavelength: 227 nm

7.4.6 Injection Volume: 5 µL

7.4.7 Run Time: 40 minutes

7.4.8 3-D Spectral Range (for Identification) - 210nm to 350nm

7.4.9 Retention Times: Withanoside IV ~ 14.0 min, Withanolide A ~ 19.7 min

7.4.10 Assign the Withanolide Aglycone / Glycoside peaks using the approximate relative retention times provided in the table below:

Analyte	Relative Retention Time
Withanoside IV	0.71
Withanoside V & VI	0.90
Withaferin A	0.93
Withastramonolide	0.96

Withanolide A	1.00
Withanone	1.01
Withanolide B	1.10

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the Diluent.

7.5.2 Make at least five (5) injections of Working Standard.

7.5.3 Make a single injection of each Sample Preparation.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive standard injections is NMT 5.0%.

7.6.2 The average asymmetry of the peak(s) present in the standard injections is NMT 1.5.

7.6.3 If present, any interference in the diluent should be subtracted out of the sample and standard peak areas.

7.7 Example calculations for determining raw material % assay:

7.7.1 $$\%\ \text{Withanolide Glycosides} = \frac{R_{Glycosides}}{R_{Withanoside\ IV}} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{1}{SA} \times \frac{V_{spl}}{1} \times 100$$

$R_{Glycosides}$ Sum of sample peak areas for Withanolide Glycoside peaks

$R_{Withanoside\ IV}$ Average peak area for Withanoside IV reference standard injections

Wt_{std} Weight of the Withanoside IV reference standard

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount

V_{spl} Volume of the sample preparation accounting for dilutions in mL

7.7.2 $$\%\ \text{Withanolide Aglycones} = \frac{R_{Aglycones}}{R_{Withanolide\ A}} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{1}{SA} \times \frac{V_{spl}}{1} \times 100$$

$R_{Aglycones}$ Sum of sample peak areas for Withanolide Aglycone peaks

$R_{Withanolide\ A}$ Average peak area for Withanolide A reference standard injections

Wt_{std} Weight of the Withanolide A reference standard

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount

V_{spl} Volume of the sample preparation accounting for dilutions in mL

7.7.3 % Withanolides = % Withanolide Glycosides + % Withanolide Aglycones

7.7.3.1 When standardizing the quantitation using Withanoside IV reference standard alone, divide the % Withanolide Aglycones term in the sum in 7.7.3 by 3.1126.

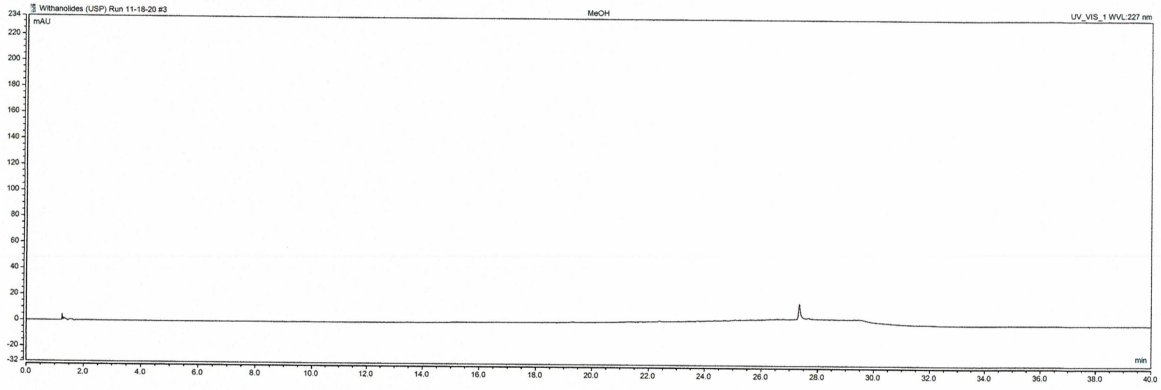
7.7.3.2 When standardizing the quantitation using Withanolide A reference standard alone, divide the % Withanolide Glycosides term in the sum in 7.7.3 by 0.3213.

7.8 Column Wash and Storage

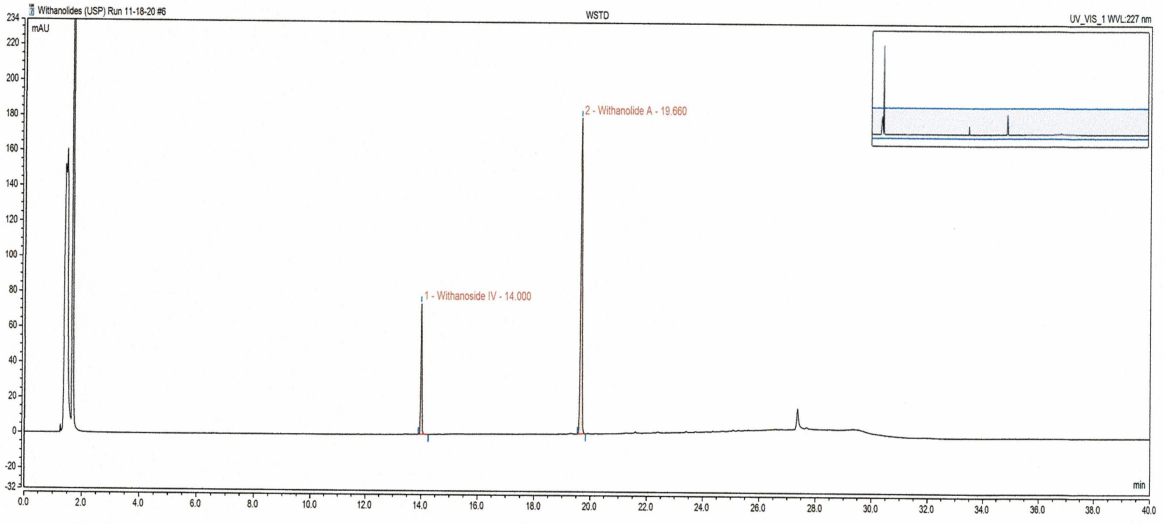
7.8.1 Wash and store the column with 90% ACN.

8.0 Chromatograms

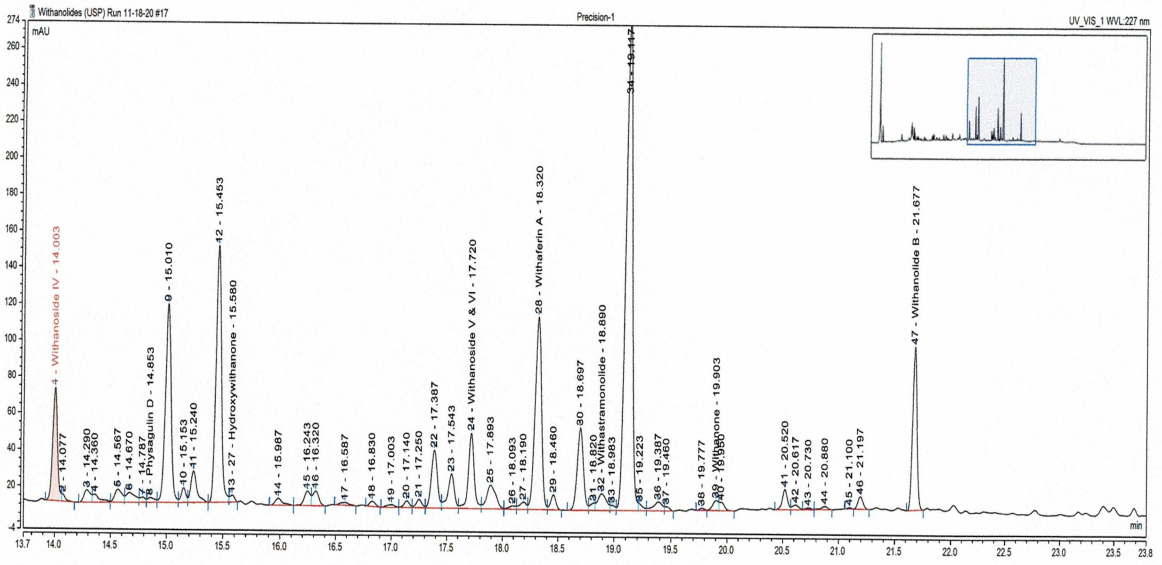
8.1 Typical Diluent Chromatogram



8.2 Typical Working Standard Chromatogram



8.3 Typical Raw Material Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	04/14/21	New	N/A	C. Perry