Phytochemical Analysis
Phytochem. Anal. **19**: 148–154 (2008)
Published online 18 September 2007 in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/pca.1029

Analysis of Withanolides in Root and Leaf of *Withania somnifera* by HPLC with Photodiode Array and Evaporative Light Scattering Detection

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Received 25 January 2007; Revised 17 July 2007; Accepted 17 July 2007

Abstract: A reversed-phase HPLC method for the simultaneous analysis of nine structurally similar withanolides, namely, 27-hydroxy withanone, 17-hydroxy withaferin A, 17-hydroxy-27-deoxy withaferin A, withaferin A, withanolide D, 27-hydroxy withanolide B, withanolide A, withanone and 27-deoxywithaferin A, has been developed using a linear binary gradient solvent system comprising methanol and water containing 0.1% acetic acid. Both photodiode array and evaporative light scattering detection were used to profile the extract compositions and to quantify the withanolides therein. Homogeneity and purity of each peak was ascertained by comparative evaluation of the on-line UV spectra of the eluted compounds with those of the reference compounds. The method has been validated with respect to various parameters of performance quality including computation regression analysis based on calibration curves, peak resolution factor, asymmetry factor, tailing factor, RSD (%) of retention time and peak area response, limit of quantivation, limit of detection, precision and recovery. The developed method has been applied to the analysis of leaf and root tissues of *Withania somnifera* for withanolide content. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: HPLC-PAD; HPLC-ELSD; withanolides; withaferin A; withanolide A; withanolide D; *Withania somnifera*.

INTRODUCTION

Withania somnifera Dunal (Solanaceae), known in India as Ashwagandha or winter cherry, is one of the most valuable plants of the traditional Indian systems of medicines, is used in more than 100 formulations of Ayurveda, Unani and Sidha and is therapeutically equivalent to ginseng (Sangwan *et al.*, 2004). Phytochemically, the plant is unique in possessing the largest and structurally most diversified set of withanolides (modified steroidal molecules based on an ergostane skeleton), named after the plant. The ethnopharmalogical properties of the plant include adaptogenic, anti-sedative and anti-convulsion activities, and the plant has been employed in the treatment of neurological disorders, geriatric debilities, arthritis and stress- and behaviour-related problems (Schliebs *et al.*, 1973; Ray and Gupta, 1994; Bhattacharya *et al.*, 1997; Kulkarni *et al.*, 1998; Dhuley, 2001). Several modern molecular pharmacological studies have demonstrated linkage of these therapeutic actions to one or more withanolides present in the herb (Kinghorn *et al.*, 2004; Tohda *et al.*, 2005a, b; Bargagna-Mohan *et al.*, 2006; Ichikawa *et al.*, 2006; Kaileh *et al.*, 2007).

Inevitably, varieties/experimental lines and tissues of *W. somnifera* as well as myriad of globally traded herbal preparations based on the plant are either poorly characterised from the phytochemical angle or are characterised with respect to just one or two withanolides, usually withaferin A. In fact the lack of marker-assisted quality controls for the herbal drugs and their phytoresources is the predominant reason for the vast compositional variations across the makes and batches of herbal drugs (Sangwan *et al.*, 2004). Therefore, a regular revision/development of high resolution analytical methods is necessary, not only for a better phytochemical description and quality control of the drugs but also for the authentication of raw materials and characterisation and/or development of discrete chemotypes.

So far, limited efforts have been made to develop an analytical HPLC profile of the major constituents of *W. somnifera*. The HPLC methods reported earlier (Bessalle and Lavie, 1987; Shingu *et al.*, 1989; Ganzera *et al.*, 2003; Bala *et al.*, 2004) are limited to a few markers such as the well-known withaferin A and withanolide D rather than covering a broad range of differentially functionalised withanolides. Also, the methods lack baseline resolution and/or their reported analyses times are quite high. Therefore, we have developed a reliable and efficient HPLC method involving photodiode array detection (PAD) for the analysis of the nine closely related withanolides of *W. somnifera*, viz. 27-hydroxy withanone (**1**), 17-hydroxy withaferin

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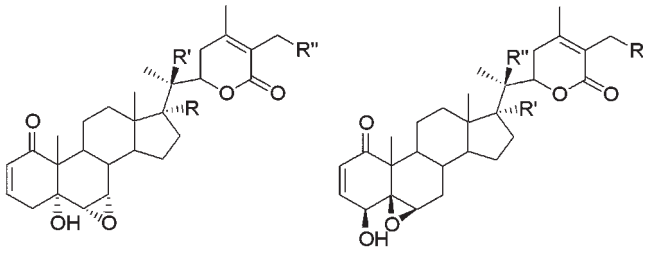
Contract/grant sponsor: New Millennium Indian Technology Leadership Initiative (Government of India).
Contract/grant sponsor: CSIR, New Delhi, India.



1, R' = H, R = R" = H
6, R = R' = H, R" = OH
7, R = R" = H, R' = OH
8, R' = R" = H, R = OH

2, R = R' = OH, R" = H
3, R' = OH, R = R" = H
4, R = OH, R' = R" = H
5, R" = OH, R = R' = H
9, R = R' = R" = H

Figure 1 Structures of the nine standard (marker) withanolides of Withania somnifera. **1**, 27-Hydroxy withanone; **2**, 17-hydroxy withaferin A; **3**, 17-hydroxy 27-deoxy withaferin A; **4**, withaferin A; **5**, withanolide D; **6**, 27-hydroxy withanolide B; **7**, withanolide A; **8**, withanone; **9**, 27-deoxy withaferin A.

A (**2**), 17-hydroxy-27-deoxy withaferin A (**3**), withaferin A (**4**), withanolide D (**5**), 27-hydroxy withanolide B (**6**), withanolide A (**7**), withanone (**8**) and 27-deoxywithaferin A (**9**) (Fig. 1). Concomitantly (on-line in series) evaporative light scattering detection (ELSD) was developed for corroborative validation of compositional homogeneity of the peaks in the PAD-based chromatograms. The analytical method has been validated with respect to the relevant parameters for better data quality and accreditation. The protocol could be usefully applied for the accurate quantification of withanolides of *Withania* and other congeners. Moreover, its utility in profiling the withanolides present in mono-herbal products may serve as a working template for developing non-interfering assays for poly-herbal combinatorial formulations.

EXPERIMENTAL

Chemicals and reagents. The standard withanolides (Fig. 1) were isolated from *W. somnifera* and structurally identified by spectral analyses (^{1}H-, ^{13}C-NMR, and MS) followed by interpretation or comparison of the spectral data (Misra *et al.*, 2005; Lal *et al.*, 2006). *n*-Hexane and chloroform used for sample preparation were of analytical grade from Merck (Darmstadt, Germany). Methanol and acetic acid used were of HPLC grade from Merck and Spectrochem (Mumbai, India), respectively. The distilled water was obtained using an all-quartz double distillation apparatus and was filtered through a 0.45 μm filter before use in HPLC.

Plant material and extraction of withanolides. The leaves and roots of *W. somnifera* were sampled from the plant germplasm at the experimental farm of the Central Institute of Medicinal and Aromatic Plants, Lucknow, India, and were processed immediately (Sangwan *et al.*, 2005). The plant material was identified by one of us (R.S.S.) and the specimen is deposited with the national medicinal and aromatic plant gene bank at CIMAP, Lucknow (India) under the reference number RS-NMITLI-II.A. In order to extract the withanolides, the fresh tissue (4.0 g) was finely powdered in liquid nitrogen and extracted overnight in 20 mL of methanol–water (25:75, v/v) at room temperature (25°C) on a rocking platform and filtered. The filtrate was collected and the residue extracted twice more at 4 h intervals with the same volume of extractant. The filtrates were pooled and extracted with *n*-hexane (3 × 60 mL). The *n*-hexane fraction was discarded and the methanol–water fraction was further extracted with chloroform (3 × 60 mL). The chloroform fractions were pooled and concentrated to a dry powder. A sample (10 mg) of the dry powder was dissolved in HPLC-grade methanol (1.0 mL), filtered through a Millipore (Bangalore, India) sample clarification kit (Millex GV; 13 mm, 0.22 μm) and subjected to HPLC analysis.

HPLC analysis. HPLC analysis was performed on a Waters (Milford, MA, USA) PAD (model 996) and separations were achieved using a Waters reversed-phase column (150 × 3.9 mm i.d.; 4 μm) subjected to binary gradient elution. The two solvents used for the analysis consisted of water containing 0.1% acetic acid (solvent A) and methanol containing 0.1% acetic acid (solvent B). Gradient programming of the solvent system was carried out at 27°C and was: initially at 60% A, changed to 40% A at 30.0 min, maintained for the next 2.0 min, changed to 25% A at 45 min, and then to 5% A at 54.0 min at a flow-rate of 0.6 mL/min and then at a flow rate of 1.0 mL/min the mobile phase was changed to 0% A at 55 min and this solvent composition was maintained until the run time reached 60 min. All the gradient segments were linear (curve type 6, Waters Empower software). The wavelength scan range of the PAD was set to 190–350 nm and the chromatograms were recorded at 227 nm. The operational conditions for ELSD were: nitrogen gas pressure, 40 psi; gain, 5; time constant, 1.0 s; drift tube temperature, 80°C; and nebuliser heater level, 80% of drift tube temperature. Calibration curves (regression from means of at least triplicate analyses) for the standards were determined from plots of peak area response data (dependent variable) against amount (up to 25 μg) of the standard per assay. All analyses were subjected to various validation parameter computations from at least five duty cycles of determinations. Also, determinations were made with the PAD and ELSD connected in series (in the order PAD–ELSD) to counter verify the chromatograms.

Validation. The limits of detection (LOD) and quantivation (LOQ) of the method for each withanolide were

DOI: 10.1002/pca

computed for both PAD and ELSD from the plots of signal (peak height) to noise, i.e. S/N, vs. amount of analyte. The LOD and LOQ values were computed as the analyte amounts corresponding to S/N values of 3.0 and 10.0, respectively. The efficiency, reliability and reproducibility of resolution in the chromatograms were evaluated by the values of relative standard deviation (RSD%) of the retention times of the peaks using data from five independent analyses covering at least three different concentrations of withanolides. The efficiency of peak resolution was measured in terms of resolution factor (R_s) with respect to the nearest neighbouring peaks (known or unknown) in the chromatogram, whereas peak types were discerned from the value of the peak asymmetry factor (A_s) and the peak tailing factor (T_f). The precision (%) of the method was evaluated by adding different concentrations of reference withanolides to the samples and comparing amounts determined from their chromatogram (by applying the respective regression equation to the increase in peak area) with the amount actually added. Similarly, recovery (%) was estimated by spiking samples by adding the marker withanolides to the tissue matrix prior to sample preparation.

RESULTS AND DISCUSSION

The present study provides an analytical HPLC system providing well-resolved and symmetrical peaks for the nine withanolides, some of which are structurally very similar and often result in peak-overlapping in the available methods of withanolide analyses. The performances/characteristics of the method have not only been compared with previously reported protocols but have also been evaluated through application of the relevant validation parameters. The HPLC chromatograms obtained with PAD and ELSD have been compared. The test set of withanolides (Fig. 1) consisted of 27-hydroxy withanone (**1**), 17-hydroxy withaferin A (**2**), 17-hydroxy-27-deoxy withaferin A (**3**), withaferin A (**4**), withanolide D (**5**), 27-hydroxy withanolide B (**6**), withanolide A (**7**), withanone (**8**) and 27-deoxywithaferin A (**9**), a range that covers not only the major bioactive components (withaferin A, withanolide A, withanolide D), but also some closely related functional variants.

The HPLC parameters for the analysis were selected after screening the previously reported solvent systems, gradient profiles and adsorbents. The isocratic methods reported for withanolides were not considered suitable for the analysis, as the sample matrix was too complex and could not be reliably analysed by isocratic systems. The binary gradient method reported by Ganzera *et al.* (2003) when applied to this set of withanolides resulted in co-elution of **1** and **2**, and also of **6** and **7**. Similarly, other methods such as those used by Vitali *et al.* (1996) and Bala *et al.* (2004) resulted in the co-elution of **6** and **7**. Therefore, in order to enable their discrete resolution and unambiguous quantification, we have developed an alternative composition and gradient profile of the HPLC solvent system that could preclude overlapping of peak of the withanolides that differ by position-specific hydroxyl groups or that have an otherwise identically oriented (β-oriented side chain) ergostane skeleton.

After trials of different compositions of methanol–water as mobile phase and use of different gradient shapes of mobile phase solvents to resolve the withanolides in the standard mixture as well as in the leaf and root extracts, the complete resolution (Figs 2 and 3) could be achieved using methanol–water as the solvent system under time-programmed gradient conditions starting (0 min) with solvent A (water containing 0.1% acetic acid) at 60% and solvent B (methanol containing 0.1% acetic acid) at 40%, changing to 40% A at 30.0 min, holding the mobile phase composition for the

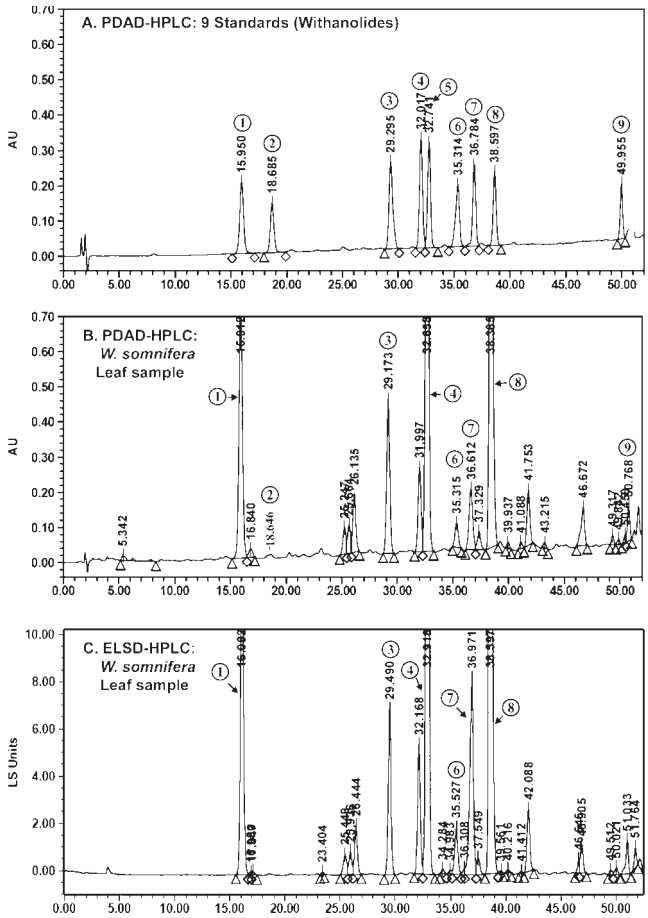


Figure 2 HPLC chromatogram of standard (photodiode array detection, PDAD) and leaf (PDAD and evaporative light scattering detection, ELSD) withanolides of *Withania somnifera*. **1**, 27-Hydroxy withanone; **2**, 17-hydroxy withaferin A; **3**, 17-hydroxy 27-deoxy withaferin A; **4**, withaferin A; **5**, withanolide D; **6**, 27-hydroxy withanolide B; **7**, withanolide A; **8**, withanone; **9**, 27-deoxy withaferin A.

DOI: 10.1002.pca

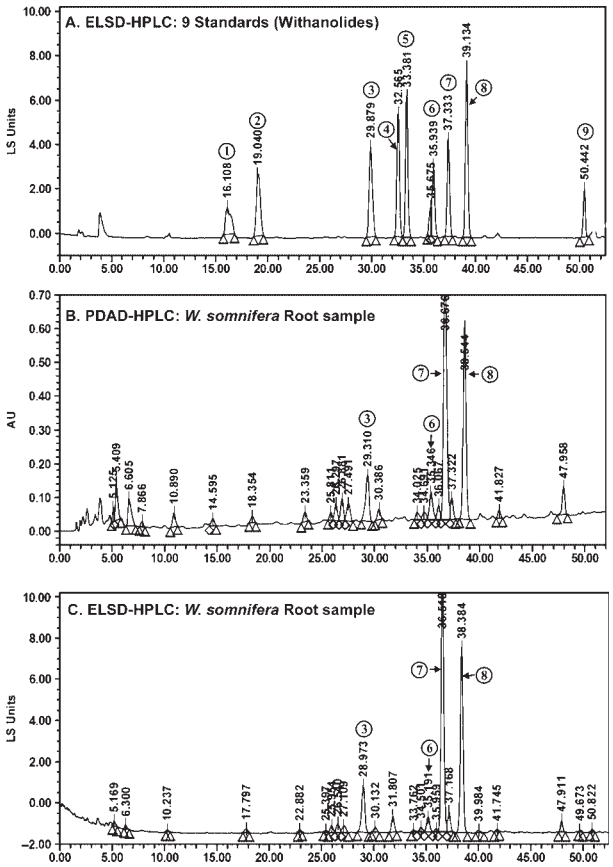


Figure 3 HPLC chromatogram of standard (evaporative light scattering detection, ELSD) and root (photodiode array detection-PDAD and ELSD) withanolides of *Withania somnifera*. **1**, 27-Hydroxy withanone; **2**, 17-hydroxy withaferin A; **3**, 17-hydroxy 27-deoxy withaferin A; **4**, withaferin A; **5**, withanolide D; **6**, 27-hydroxy withanolide B; **7**, withanolide A; **8**, withanone; **9**, 27-deoxy withaferin A.

next 2.0 min followed by changing to 25% A at 45 min, and then to 5% A at 54.0 min with a flow rate of 0.6 mL/min, and finally with a flow-rate of 1.0 mL/min changing to 0% A at 55 min and maintaining this composition until the run-time reached 60 min. Under these HPLC conditions, the mean retention times (R_t) for withanolides **1**–**9**, respectively, were 15.99, 18.69, 29.34, 32.09, 32.78, 35.39, 36.79, 38.65 and 49.99 min.

The validity of the method with reference to these compounds was confirmed by comparing their on-line UV spectra (Fig. 4) with those of the reference compounds using library matching. Also, the full identity of the HPLC-PAD chromatograms with those obtained in series using ELSD was taken as corroborative evidence for the compositional homogeneity of the peaks resolved by the HPLC method.

Calibration curves were constructed for PAD and ELSD data by regression analysis of plots of peak area response (units) from each detector against amount of each withanolide per assay (Table 1). The values of the correlation coefficients (r^2) of the regression curves of withanolides were greater than 0.95 and 0.96, respectively, with PAD and ELSD. With PAD, the LOD varied from 0.12 to 1.71 μg and the LOQ from 0.41 to 5.71 μg (Table 2). With ELSD, the LOD and LOQ values ranged from 0.38 to 2.94 μg and from 1.27 to 9.80 μg, respectively (Table 2). The higher LOQ and LOD values with ELSD compared with PAD are as expected because of the lower sensitivity of the former. As such, ELSD may not be preferred for estimation of withanolides since they absorb at 220–230 nm. Thus, the context of comparison of the two detectors in this study is only emphatic as a gross status/specificity of withanolide sample preparation and as a collateral validation of peak purities (i.e. lack of contamination with UV non-absorbing phytochemicals).

Table 1 Calibration curve regression equations for the HPLC analysis of nine withanolides using a photodiode array detector (PAD, $\lambda = 227$ nm) and an evaporative light scattering detector (ELSD)

	Regression equation[a]	
Withanolide	PAD	ELSD
27-Hydroxy withanone	$Y = (0.83 \times 10^6)X + (-2.12 \times 10^6)$	$Y = (0.12 \times 10^4)X + (-0.02 \times 10^4)$
17-Hydroxy withaferin A	$Y = (0.42 \times 10^6)X + (-0.90 \times 10^6)$	$Y = (0.80 \times 10^4)X + (-5.42 \times 10^4)$
17-Hydroxy, 27-deoxy withaferin A	$Y = (0.86 \times 10^6)X + (-1.86 \times 10^6)$	$Y = (1.59 \times 10^4)X + (-14.33 \times 10^4)$
Withaferin A	$Y = (0.71 \times 10^6)\ X + (-1.71 \times 10^6)$	$Y = (1.75 \times 10^4)X + (-14.95 \times 10^4)$
Withanolide D	$Y = (0.68 \times 10^6)X + (-1.49 \times 10^6)$	$Y = (1.82 \times 10^4)X + (-15.33 \times 10^4)$
27-Hydroxy withanolide B	$Y = (0.59 \times 10^6)X + (-1.36 \times 10^6)$	$Y = (1.01 \times 10^4)X + (-8.80 \times 10^4)$
Withanolide A	$Y = (0.61 \times 10^6)X + (-1.28 \times 10^6)$	$Y = (1.39 \times 10^4)X + (-12.87 \times 10^4)$
Withanone	$Y = (0.57 \times 10^6)X + (-1.25 \times 10^6)$	$Y = (1.23 \times 10^4)X + (-9.66 \times 10^4)$
27-Deoxy withaferin A	$Y = (0.32 \times 10^6)X + (-6.87 \times 10^6)$	$Y = (0.36 \times 10^4)X + (-2.11 \times 10^4)$

[a] Regression equation in the form $Y = bX + a$ where X = amount (μg)/withanolide assay and Y = peak area (au); r^2 values for PAD- and ELSD-based calibration curves were greater than 0.95 and 0.96, respectively.

DOI: 10.1002/pca

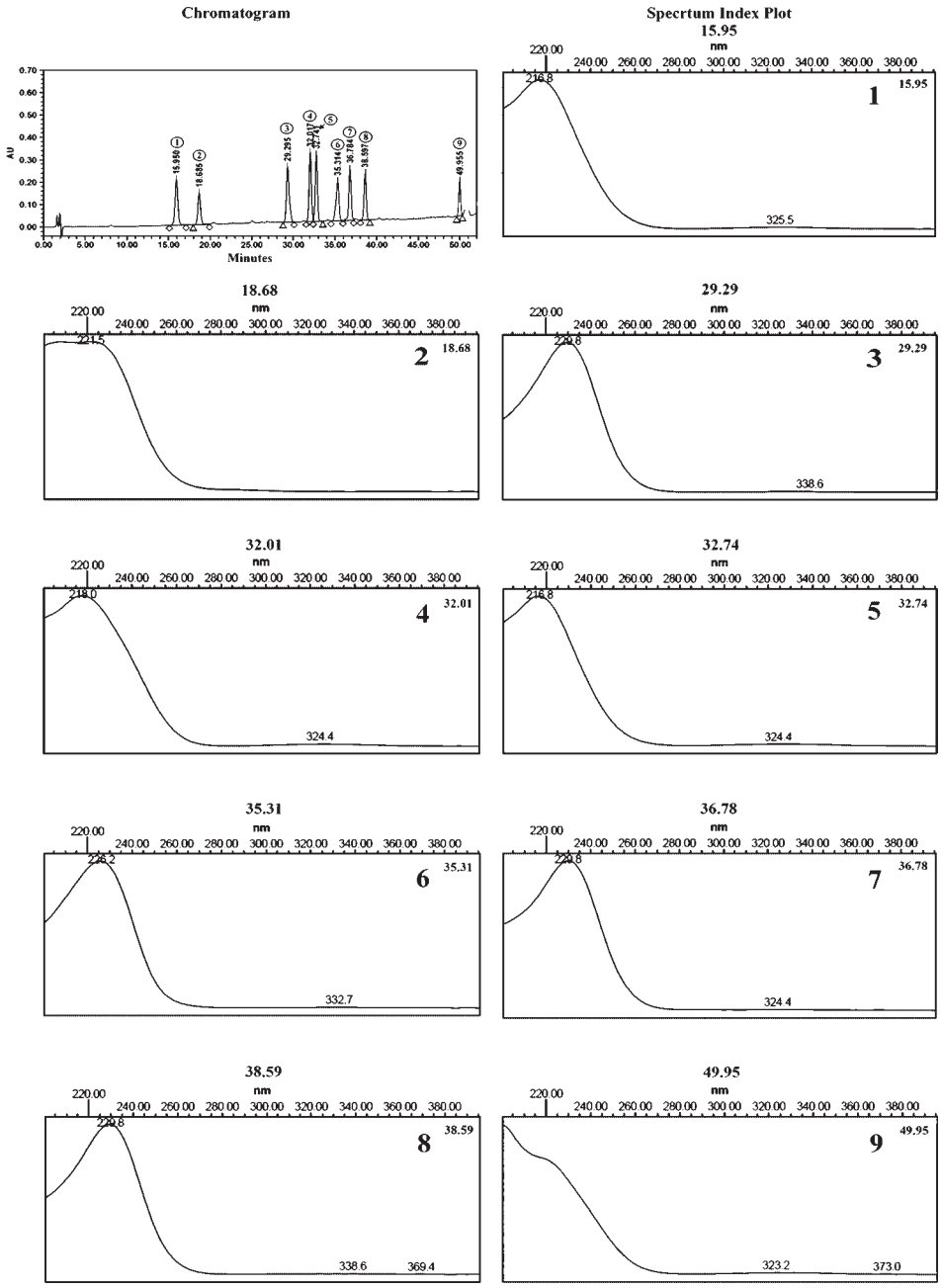


Figure 4 Photodiode array detection (PDAD)-HPLC generated online UV spectra (λ 205–395 nm) of the nine standard (marker) withanolides of *Withania somnifera*. **1**, 27-Hydroxy withanone; **2**, 17-hydroxy withaferin A; **3**, 17-hydroxy 27-deoxy withaferin A; **4**, withaferin A; **5**, withanolide D; **6**, 27-hydroxy withanolide B; **7**, withanolide A; **8**, withanone; **9**, 27-deoxy withaferin A.

The method was subjected to analysis of quality and validation parameter estimations. The peaks were nearly Gaussian (symmetrical) in shape with negligible peak tailing showing A_s and T_f values for the chromatograms close to 1.0 for almost all the withanolides (Table 2). The efficiency, reliability and reproducibility of resolution were validated by the observed low values of RSD% of the retention times of the peaks (Table 2). R_s values for most of the peaks met the typical requirement of ≥1.5 for routine samples. Although slightly lower R_s values were noted for a few peaks, their near-ideal (Guassian) peak shapes and stable (very low RSD%) R_t suggested their clear discrimination for quantitative determinations. Some of the lower values in the samples were also due to the presence of rudimentary peaks of unknown withanolides of very low abundance in the immediate neighbourhood of the peak. The precision and recovery of the method were estimated to be better than 95 and 85%, respectively. The RSD% of intra- and inter-day variations of the peak area responses of the nine withanolides were in the ranges 1.1–2.1 and 0.8–8.3%, respectively. The

DOI: 10.1002/pca

Table 2 Performance quality and validation data of the HPLC method described: LOD and LOQ with PAD (λ = 227 nm) and ELSD

	PAD Limit (µg/assay)		ELSD Limit (µg/assay)				R_t variation-RSD (%)[c]			R_s[d] value for peak in the chromatogram[e] of					
										Standard		Leaf sample		Root sample	
Withanolide	LOD	LOQ	LOD	LOQ	A_s[a]	T_f[b]	Intraday	Interday	Sample load	LS[f]	RS[g]	LS[f]	RS[g]	LS[f]	RS[g]
27-Hydroxywithanone	0.76	2.53	2.94	9.80	0.96	1.02	0.26	0.49	0.33	NA[h]	2.38	11.19	0.92	(—)[i]	(—)[i]
17-Hydroxywithaferin A	0.36	1.19	1.34	4.54	1.00	1.00	0.22	0.40	0.27	2.38	8.27	2.78	7.54	(—)[i]	(—)[i]
17-Hydroxy, 27-deoxy withaferin A	0.88	2.95	0.65	2.17	1.13	1.11	0.16	0.24	0.29	8.27	2.50	4.22	2.19	2.04	0.93
Withaferin A	0.19	0.64	0.43	1.42	1.00	1.00	0.03	0.05	0.15	2.50	0.80	0.32	2.43	(—)[i]	(—)[i]
Withanolide D	0.36	1.19	0.38	1.27	1.00	1.00	0.10	0.20	0.17	0.80	1.99	(—)[i]	(—)[i]	(—)[i]	(—)[i]
27 Hydroxy withanolide B	0.22	0.72	0.85	2.85	0.95	1.03	0.34	0.26	0.17	1.99	1.20	1.51	1.21	5.55	1.21
Withanolide A	0.12	0.41	0.56	1.86	1.00	1.00	0.12	0.18	0.21	1.20	1.45	1.21	1.79	1.02	0.42
Withanone	0.17	0.58	0.59	1.98	1.00	1.02	0.09	0.25	0.14	1.45	12.76	1.27	3.24	1.76	1.52
27-Deoxy withaferin A	1.71	5.71	1.10	3.65	1.12	1.01	0.05	0.10	0.05	12.76	NA[h]	4.82	NA[h]	1.52	NA[h]

[a] Peak asymmetry factor (A_s); [b] peak tailing factor (T_f); and [c] retention time (R_t) variation as percentage relative standard deviation (RSD) for the HPLC peaks of the nine withanolides; [d] resolution factor (R_s) of HPLC-PAD chromatogram of standards and leaf and root samples of *Withania somnifera*; [e] R_s equals ratio of twice of difference of migration distance to the half of the sum of the vertical dimensions of spots under reference; [f] LS, with respect to nearest left-side peak; [g] RS, with respect to nearest right-side peak; [h] NA, not applicable; [i](—), not detectable in the sample.

DOI: 10.1002/pca

values of coefficients of variation for precision and accuracy were much lower than the general threshold of 15%, thus confirming the suitability of the method for quantitative analyses.

In the leaf extract, **6** and **9** were detected by PAD and ELSD as minor constituents, while all other withanolides occurred substantially [Fig. 2(B, C)]. Withanolide D was a rare withanolide present only in the leaf of an experimentally developed chemotype and in the hybrids developed through crossing with other chemotypes (data not shown). In roots, the sample matrix was more complex in the early (polar) region of the chromatogram as many minor polar constituents were present in the tissue. Compounds **3**, **7** and **8** were detected as the major constituents whilst **1**, **2**, **4**, **6** and **9** were detected as minor constituents in the tissue [Fig. 3(B, C)]. The closely similar pattern of peaks in the PAD and ELSD chromatograms [Fig. 2(B, vs. C) and Fig. 3(B vs. D)] endorses the specificity of the sample preparation procedures for withanolides and the lack of significant extraneous phytochemical contaminants as the ELSD senses constituents by their physical presence *per se*. The method described offers better resolution than previous protocols, and is simple and can be easily and reliably applied to the quantitative analyses of withanolides.

Acknowledgements

The authors are thankful to New Millennium Indian Technology Leadership Initiative (Government of India) and CSIR, New Delhi, India, for granting financial support in the form of an NMITLI Project on Ashwagandha, and to the Director of CIMAP for providing logistic support for carrying out the study.

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