SPECIAL GUEST EDITOR SECTION

Simultaneous Quantification of Withanolides in *Withania somnifera* by a Validated High-Performance Thin-Layer Chromatographic Method

POOJA SRIVASTAVA, NEERJA TIWARI, AKHILESH K. YADAV, VIJENDRA KUMAR, KARUNA SHANKER, RAM K. VERMA, and MADAN M. GUPTA[1]
Central Institute of Medicinal and Aromatic Plants, Analytical Chemistry Division, Lucknow–226015, India
ANIL K. GUPTA and SUMAN P.S. KHANUJA
Central Institute of Medicinal and Aromatic Plants, Genetic Resource and Biotechnology Division, Lucknow–226015, India

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

This paper describes a sensitive, selective, specific, robust, and validated densitometric high-performance thin-layer chromatographic (HPTLC) method for the simultaneous determination of 3 key withanolides, namely, withaferin-A, 12-deoxywithastramonolide, and withanolide-A, in Ashwagandha (*Withania somnifera*) plant samples. The separation was performed on aluminum-backed silica gel 60F_{254} HPTLC plates using dichloromethane–methanol–acetone–diethyl ether (15 + 1 + 1 + 1, v/v/v/v) as the mobile phase. The withanolides were quantified by densitometry in the reflection/absorption mode at 230 nm. Precise and accurate quantification could be performed in the linear working concentration range of 66–330 ng/band with good correlation (r^2 = 0.997, 0.999, and 0.996, respectively). The method was validated for recovery, precision, accuracy, robustness, limit of detection, limit of quantitation, and specificity according to International Conference on Harmonization guidelines. Specificity of quantification was confirmed using retention factor (R_f) values, UV-Vis spectral correlation, and electrospray ionization mass spectra of marker compounds in sample tracks.

Withania somnifera is well documented in Ayurvedic texts such as "Charak Samhita," "Sushrut Samhita," "Astanghirdaya," and "Bhava-prakash" for pharmacodynamics and medicinal properties and is used as an ingredient of many Ayurvedic formulations prescribed for the treatment of rheumatic pain, inflammation of joints, nervous disorders, epilepsy, all types of skin lesions, ulcers, and boils (1). Withanolides, which are polyoxygenated C_{28} steroidal lactones, are the major pharmacologically active constituents of *W. somnifera* leaves and roots (2). Withaferin-A, 12-deoxywithastramonolide, and withanolide-A are the 3 key withanolides, and the biological activities, namely, antibacterial, antiviral, antifungal, antimiotic, antitumor, anti-inflammatory, immuno-suppressant, and antistress, are mainly attributed to the major withanolide, withaferin-A (1, 3–5).

Withanolide-A elicits humoral and cell-mediated immune responses by up-regulation of Th-1-dominant polarization in BALB/c mice (6) and is an important candidate for the therapeutic treatment of neurodegenerative diseases (7). Withaferin-A potently inhibits NFκB transcription factor activation, while 12-deoxywithastramonolide and withanolide-A are far less effective; pure withanolide-A or withanolide-A enriched *W. somnifera* extracts can be considered as a novel class of NFκB inhibitors, which hold promise as anti-inflammatory agents for treatment of various inflammatory disorders and/or cancer (8).

The importance of this Indian medicinal plant is highlighted with an increasing number of research publications on various aspects of drug discovery, and its demand in the global market as a positive health promoter is growing. Many efforts were made to produce an improved variety of ashwagandha with a specific chemotype (1, 9). Although a number of column high-performance liquid chromatographic (LC) methods for the quantification of withanolides are available (10–12), only a few thin-layer chromatographic (TLC) methods, especially for withaferin-A, are reported (13–15). However, the reported TLC procedures were not found to be suitable for adequate separation and quantification of test markers. High-performance TLC (HPTLC) can be used to analyze a large number of samples with low cost and precision and accuracy comparable to that of LC methods. Our continued interest in developing rapid HPTLC methods for analysis of medicinal plants (16–20) led to the rapid, sensitive, and accurate HPTLC method for the simultaneous determination of 3 important withanolides,

Guest edited as a special report on "Modern Thin-Layer Chromatography" by Joseph Sherma.

[1]Author to whom correspondence should be addressed; e-mail: guptammg@rediffmail.com

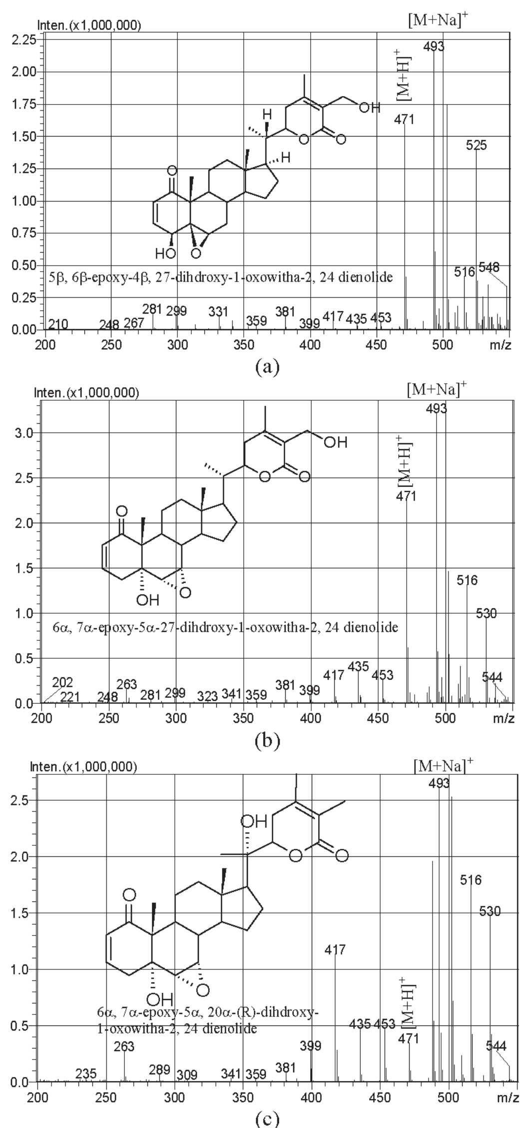

Figure 1. Electrospray ionization-mass spectra in +ve mode and structures of (a) withaferin-A, (b) 12-deoxy-withastramonolide, and (c) withanolide-A.

withaferin-A, 12-deoxywithanolide, and withanolide-A, in *W. somnifera* plant samples that is reported in this paper.

Experimental

Plant Materials

Various genotypes of ashwagandha (vouchers are deposited in the Gene Bank of the Central Institute of Medicinal and Aromatic Plants) were collected from the experimental farm of the Institute.

Apparatus

(**a**) *HPTLC system*.—Linomat 4 semiautomatic sample applicator, TLC Scanner 3, winCATS software, viewing cabinet with dual wavelength UV lamps, Reprostar 3 illumination unit, Vario system, twin-trough chambers, Chromatogram Immersion Device, and TLC plate heater (CAMAG, Muttenz, Switzerland); Hamilton 100 μL syringe (Anchrom Enterprises Pvt Ltd, Mumbai, India).

(**b**) *Precoated HPTLC plates*.—Silica gel $60F_{254}$, 10 × 10 cm, and 20 × 10 cm, layer thickness 0.2 mm, with aluminum backing (E. Merck, Darmstadt, Germany).

(**c**) *Liquid chromatography-photodiode array detector/mass spectrometry (LC-PDA/MS) system*.—Pumps LC-20AD, column oven CTO-20A, autosampler SIL-10AF, controller CBM-20A, photodiode array detector SPD-M20A, mass detector LCMS-2010EV (Shimadzu, Columbia, MD).

(**d**) *Filtration assembly*.—0.45 μm membrane (Millipore, Billerica, MA).

(**e**) *Ultrasonicator*.—Oscar (Mumbai, India).

Reagents and Materials

All solvents and reagents used were either of analytical or LC grade (E. Merck Ltd, Mumbai, India) unless otherwise specified. Standard withaferin-A, 12-deoxywithastramonolide, and withanolide-A (Figure 1) were purchased from ChromaDex (Irvine, CA). Water (Chromosolv® LC/MS grade; Sigma, St. Louis, MO) was used for LC/MS analyses.

Chromatographic Conditions

Samples were applied to silica gel $60F_{254}$ HPTLC plates (10 × 10 cm) by means of a Linomat 4 equipped with 100 μL syringe and operated with settings of band length, 6 mm; distance between bands, 15 mm; distance from the plate edge, 15 mm; distance from the bottom of the plate, 15 mm; and speed, 8 μL/min. The plates were developed in the linear ascending mode for a distance of 9 cm in a vertical twin-trough chamber previously saturated for 2 min with the mobile phase dichloromethane–methanol–acetone–diethyl ether (15 + 1 + 1 + 1, v/v/v/v) under laboratory conditions (temperature, 25 ± 3°C and relative humidity, 35–40%). The bands on the air-dried plates were scanned at 230 nm in the reflection/absorption mode. The plates were also immersed (dipping time 2 s, dipping speed 5 cm/s) in freshly prepared vanillin–sulfuric acid derivatizing reagent [vanillin–ethanol–H_2SO_4–(1 g + 95 mL + 5 mL)] followed by heating at 110°C for 10 min. The densitometric digital scanning was performed in the reflectance/absorbance mode; slit width, 6.00 × 0.40 mm; scanning speed, 20 mm/s; and data resolution, 10 μm/step. Savitsky-Golay-7 was used for data filtering and the lowest slope for baseline correction in order to integrate the area. For recording of characteristic UV absorption spectra (200–400 nm) of a sample track, the deuterium lamp was used, while for derivatized spots of reference withanolides and sample tracks in the range of 400–800 nm, the tungsten lamp was used. Reprostar 3 with cabinet cover and mounted digital camera (PowerShot G5 with Neck Strap NS-DC2; Canon, Tokyo, Japan) was used for imaging and archiving the thin-layer chromatograms. Quantification was performed using linear regression of peak area vs amount (ng/band).

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

Table 1. Regression analysis of the calibration graphs for withanolides

Parameter	Withaferin-A	12-Deoxywithastramonolide	Withanolide-A
	Densitometric relationship[a]		
Digital scanning at 230 nm			
Concentration range, ng/band	66–330	66–330	66–330
Linear fit	$Y = 3.537X - 115.2$	$Y = 3.831X - 167.2$	$Y = 7.395X - 137.1$
Goodness of fit ($S_{y.x}$)	23.13	17.45	57.01
Correlation coefficient (r^2)	0.997	0.999	0.996
Digital scanning at 675 nm			
Concentration range, ng/band	66–330	66–330	66–330
Linear fit	$Y = 17.89X - 246.6$	$Y = 23.6X - 254.5$	$Y = 10.34X - 188.1$
Goodness of fit ($S_{y.x}$)	198.6	225.5	75.23
Correlation coefficient (r^2)	0.992	0.994	0.996
	Specificity		
Peak purity			
R (s,m)[b]			
Standard track	0.9999	0.9999	0.9999
Sample track	0.9998	0.9998	0.9998
R (m,e)[c]			
Standard track	0.9999	0.9999	0.9999
Sample track	0.9999	0.9995	0.9999

[a] Digital scanning at 230 or 675 nm with postderivatization with vanillin–sulfuric acid reagent; statistical relationships were established considering 5 data points each in triplicate; X = amount of compound (ng/band); Y = peak area counts.

[b] Correlation of spectrum at start of peak with spectrum at the center of peak for 230 nm scanning.

[c] Correlation of spectrum at center of peak with spectrum at the end of peak for 230 nm scanning.

Standard Solution and Sample Preparation

Five mg of each standard compound was placed in a 5 mL volumetric flask and dissolved in methanol (stock solution). Working standard solutions for calibration studies were prepared by dilution. Dried and finely milled plant tissues (0.1 g) of *W. somnifera* were sonicated in 10 mL methanol for 30 min followed by centrifugation for 15 min at 10 000 rpm. The supernatant was transferred to a flask. The procedure was repeated 3 times. The pooled extract was concentrated under vacuum and redissolved in 1.0 mL methanol for TLC or LC analysis.

Method Validation

Validation of quantitative TLC method involves the performance evaluation of various parameters following the guidelines of the International Conference on Harmonization (ICH; 21) and the International Union of Pure and Applied Chemistry (IUPAC; 22).

(**a**) *Calibration graph and linearity*.—The calibration graphs were constructed by plotting the peak areas vs concentrations of withanolides, and the regression equations were calculated using the least-squares method in Graph PAD Prism 3.0 (Table 1). The calibration graphs were plotted over the concentration range of 66–330 ng/band of withanolides. Aliquots of the standard working solutions of withanolides (2.0, 4.0, 6.0, 8.0, and 10.0 μL) were applied in triplicate to the plates.

(**b**) *Accuracy (recovery)*.—The accuracy of the method was determined by calculating recoveries of withanolides by the standard addition method. Known amounts of standard solutions of the withanolides in 3 concentration ranges (66, 132, and 198 μg/mL) were added to prequantified extract of plant samples. The amounts of withanolides were estimated by applying these values to the regression equations of the calibration graphs.

(**c**) *Method precision (repeatability)*.—The instrumental precision of the HPTLC method was checked by repeated spotting and densitometric scanning [number of replicates (n = 5)] of standard solutions of withanolides at 2 concentration levels, i.e., 66 and 198 ng/band. The relative standard deviation (RSD) values are summarized in Table 2.

(**d**) *Intermediate precision (reproducibility)*.—The intraday and interday variations of the proposed method were evaluated by estimating the corresponding responses in triplicate on the same day and on 3 different days over a period of 1 week at 2 concentration levels, i.e., 66 and 198 ng/band. The results were reported as RSD.

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

Table 2. Summary of the validation parameters for the proposed HPTLC method

Parameter	Withaferin-A	12-Deoxywithastramonolide	Withanolide-A
Retention factor (R_f) value	0.61 ± 0.04	0.72 ± 0.03	0.86 ± 0.03
Peak area[a] counts (average)	1100.2 ± 0.18	1375.2 ± 0.56	2429.8 ± 0.48
Limit of detection			
Scanning at 230 nm	19.62	13.66	23.13
Scanning at 675 nm	33.30	28.67	21.83
Limit of quantitation			
Scanning at 230 nm	65.39	45.55	77.09
Scanning at 675 nm	111.01	95.55	72.76
Accuracy[b], %	98.44–100.54	98.00–101.25	95.83–98.95
Repeatability, RSD, %, *n* = 5 at 66 and 198 ng/band	0.20, 0.18	0.66, 0.56	0.54, 0.48
Precision[b]			
Intraday RSD, % (*n* = 3)	0.22–0.86	0.18–0.74	0.18–0.85
Interday RSD, % (*n* = 3)	0.42–0.86	0.21–0.89	0.22–0.80

[a] Densitometric scanning at 230 nm of 198 ng/band.
[b] Densitometric scanning at 230 nm.

(**e**) *Sensitivity of the HPTLC method [limits of detection (LOD) and quantitation (LOQ)]*.—The LOD and the LOQ were calculated for withanolides using the linear regression equation (Table 2). The following equations were applied:

$$LOD = 3S_{y,x}/b \text{ and } LOQ = 10S_{y,x}/b$$

where $S_{y,x}$ is the standard deviation of the *Y*-value distribution around the regression line and b is the slope of the calibration graph.

(**f**) *Specificity*.—The specificity of the HPTLC analysis of withanolides was ascertained by coanalyzing standard and sample. The band for reference withanolides in a sample was confirmed by comparing the R_f, peak purity, and absorption spectra of the band to that of withaferin-A, 12-deoxywithastramonolide, and withanolide-A. The peak purity of each withanolide peak in sample track was assessed by comparing the spectra at peak start, peak apex, and peak end positions of the band. Further, to confirm the specificity using electrospray ionization-MS (ESI-MS), silica was scraped from the center of band corresponding to the respective withanolides in both the standard track and sample track and extracted with methanol. The solution was introduced into the LC-PDA/MS system [mobile phase H_2O–methanol (20 + 80, v/v), flow rate 0.8 mL/min] to obtain the ESI mass spectrum through a 1 m long restricted coil protected with a guard column (no analytical column). ESI in the positive mode was performed on a Shimadzu 2010EV LC/MS system with interface voltage, +1.6 kV; entrance lens voltage, –70 V; mass-to-charge ratio (*m/z*) range, 200–550; heating block temperature, 250°C; N_2 flow, 1.5 mL/min; and curved desolvation line temperature, 250°C. Mass spectra of the withanolides from the standard track were stored in the library of LCsolution software (Shimadzu) to match the respective compound in the sample track.

(**g**) *Robustness*.—To test the robustness of the method, deliberately small changes were made in the chromatographic parameters that may affect the performance of the method, i.e., mobile phase composition, delay between spotting and plate development, plate heating time, and delay in digital scanning after derivatization. The RSD of the peak areas was calculated for each parameter.

Results and Discussion

The mobile phase selection was performed using the Vario System wherein mobile phase compositions consisting of different ratios of solvents of varying polarity with the stationary phase silica gel $60F_{254}$ were tried. Finally, a mobile phase consisting of dichloromethane–methanol–acetone–diethyl ether (15 + 1 + 1 + 1, v/v/v/v) was found suitable for satisfactory separation and simultaneous quantification of withaferin-A, 12-deoxywithastramonolide, and withanolide-A in the sample matrix. Good sensitivity, precision, and accuracy were obtained for densitometric quantification of the target withanolides at 230 nm. Satisfactory resolution of the components (Figure 2a) with pure scan peaks (Table 1) were obtained. The response, i.e., peak area/ng (Figure 2b) and LOD and LOQ (Table 2) of individual withanolides, after derivatization with vanillin–sulfuric acid reagent, were comparatively higher. The robustness (Table 3) was found to be within ICH limits when zones were scanned at 230 nm but was compromised (higher RSD value) after derivatization.

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

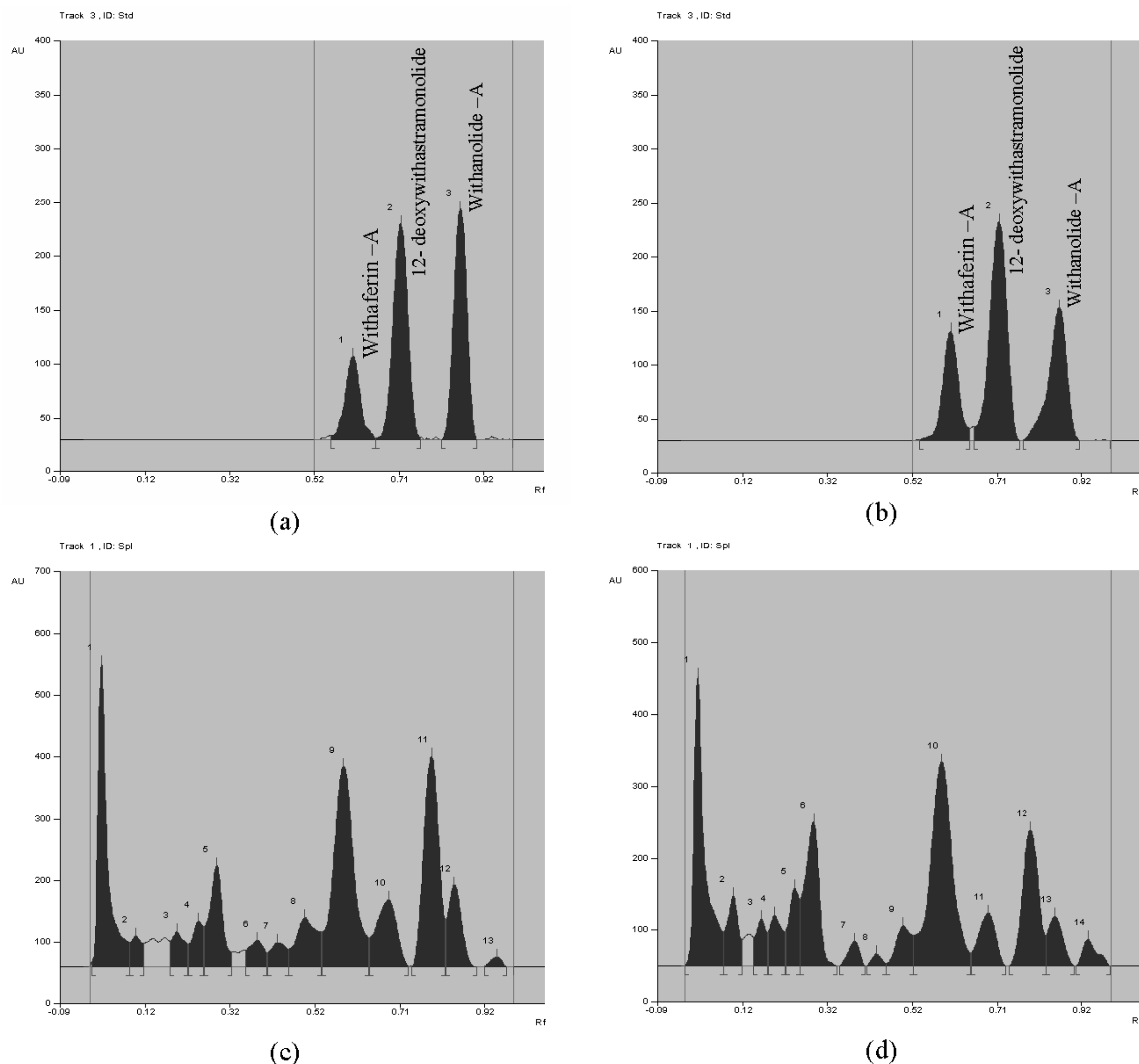

Figure 2. Representative HPTLC densitograms of withanolides in standard (a, b) and sample (c, d) tracks of *W. somnifera* with scanning at 230 and at 675 nm after derivatization with vanillin–sulfuric acid reagent, respectively.

Validation of the Proposed Method

Linearity.—A linear correlation was obtained in between peak areas and concentrations of withanolides in the working range of 66–330 ng/band. Correlation coefficients (r^2) values of regression equations ranged between 0.996–0.999 (Table 1).

Accuracy.—The recoveries obtained were 98.44–100.54, 98.00–101.25, and 95.83–98.95% for withaferin-A, 12-deoxywithastramonolide, and withanolide-A, respectively. These good recoveries indicate the accuracy of the method (Table 2).

Method precision.—The RSD values for withaferin-A, 12-deoxywithastramonolide, and withanolide-A in the plant samples were found to be in the range of 0.18–0.66% (Table 2).

Intermediate precision.—The intraday and interday RSD values for withaferin-A, 12-deoxywithastramonolide, and withanolide-A were 0.22–0.86, 0.18–0.74, 0.18–0.85%, and 0.42–0.86, 0.21–0.89, 0.22–0.80%, respectively. The low values reveal that the proposed method is reproducible (Table 2).

LOD and LOQ.—The values of LOD and LOQ are summarized in Table 2. These data show that densitometric scanning at 230 nm is sensitive for the quantification of the tested withanolides.

System suitability parameters.—The RSD data of R_f values and peak areas are summarized in Table 2.

Specificity.—The bands of reference withanolides in sample tracks were confirmed by comparing the respective R_f values (0.61, 0.72, 0.86) and absorption spectra of the bands to those of standard withaferin-A, 12-deoxywithastramonolide, and withanolide-A, respectively. Interference of co-eluting peaks was checked by monitoring the peak purity of withanolides peaks in sample tracks. The spectra at peak start,

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

Table 3. Robustness testing of the HPTLC method

Parameter	RSD of peak area[a], %		
	Withaferin-A	12-Deoxywithastramonolide	Withanolide-A
Mobile phase composition	0.46	0.86	0.78
Time gap between spotting and plate development	0.21	0.26	0.28
Derivatization time (plate heating time)	2.87	2.47	2.26
Time gap between derivatization and scanning	2.88	2.66	2.66

[a] $n = 5$.

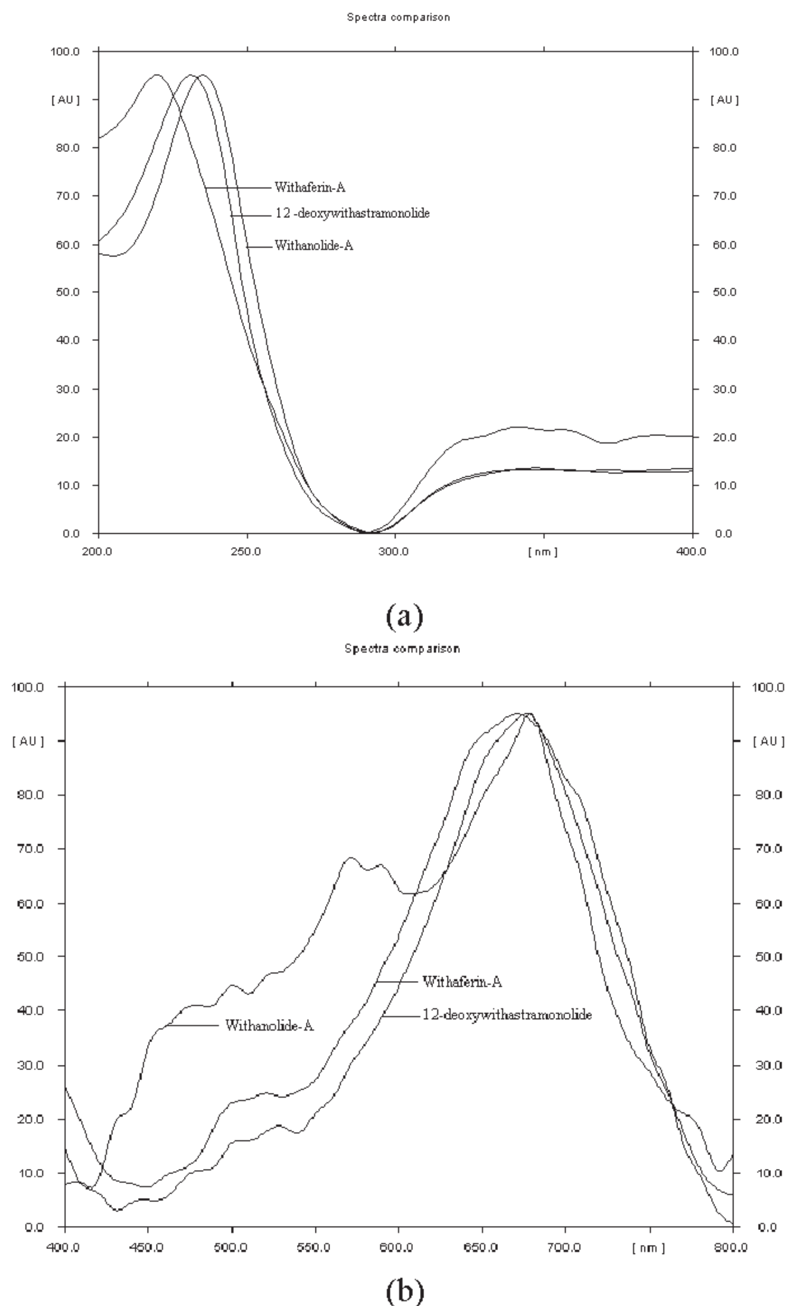

Figure 3. Absorbance spectra of withaferin-A, 12-deoxywithastramonolide, and withanolide-A obtained by scanning (a) in the range of 200–400 nm prederivatization and (b) in the range of 400–800 nm postderivatization with vanillin–sulfuric acid reagent.

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

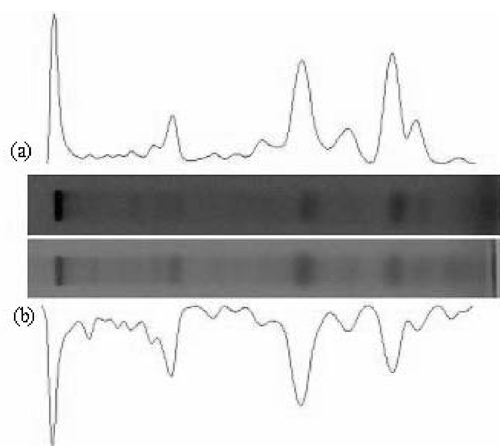

Figure 4. HPTLC image and digital scanning profile of a methanol extract of *W. somnifera* at (a) 230 nm and (b) 675 nm after derivatization with vanillin–sulfuric acid reagent.

peak apex, and peak end positions of the band were compared. Good correlation was also obtained between standard and sample overlay spectra ($r^2 > 0.999$; Table 1). Representative absorbance spectra of withanolide are presented in Figure 3. ESI-MS spectra of the respective withanolides from a standard track were obtained and stored in the library of LCsolution software and matched with that of a sample track. The software matching is allowed when the 2 mass spectra are harmonized more than 80%, which further confirms the specificity of withanolide analysis by the proposed HPTLC method. The ESI-MS spectra of the withanolides are presented in Figure 1.

Robustness.—Results of the robustness study are given in Table 3.

Quantitative and Qualitative Evaluation of Withanolides in W. somnifera

The proposed validated method was successfully applied to determine withaferin-A, 12-deoxywithastramonolide, and withanolide-A in different accessions of *W. somnifera* leaves and roots. Digital scanning of the HPTLC plate, popularly known as HPTLC fingerprint analysis, also provides the peak profile of the methanolic extract, which may be used for quality assurance of herbal crude drugs as well as derived herbal products. The HPTLC fingerprint of *W. somnifera* is presented in Figure 4. The selected accessions of *W. somnifera* were also analyzed by a reported LC method (23) with slight modification using the column Waters (Milford, MA) Spherisorbs® C_{18} (4.6 × 250 mm, 10 μm ODS2); mobile phase acetonitrile–0.1% trifluoroacetic acid in water (40 + 60); flow rate 1.0 mL/min; and detection wavelength 220 nm. The retention times were 13.5 ± 0.1, 15.0 ± 0.1, and 18.0 ± 0.1 min for withaferin-A, 12-deoxywithastramonolide, and withanolide-A, respectively. The results of withanolide analysis using both LC and TLC are presented in Table 4. The analysis results for withanolides in *W. somnifera* were compared by applying the paired *t*-test. The calculated

Table 4. Content of withanolides in *W. somnifera* calculated on a plant dry weight basis

	Withanolides content, %					
	Withaferin-A		12-Deoxywithastramonolide		Withanolide-A	
Sample ID	LC	HPTLC	LC	HPTLC	LC	HPTLC
LW-7[a]	0.104	0.103	0.019	0.020	0.124	0.123
LW-19	0.129	0.130	0.004	0.004	0.136	0.137
LW-88	1.026	1.024	0.223	0.221	1.041	1.045
LW-89	1.074	1.078	0.250	0.252	1.014	1.013
LW-102	0.593	0.596	0.028	0.025	1.008	1.008
LW-104	0.883	0.880	0.044	0.045	0.925	0.927
LW-105	0.650	0.652	0.017	0.016	1.257	1.256
RW-27[b]	0.002	0.004	0.012	0.012	0.108	0.109
RW-43	0.037	0.038	0.094	0.094	0.164	0.163
RW-84	0.006	0.005	0.011	0.015	0.058	0.059
RW-99	0.005	0.003	0.020	0.021	0.152	0.153
RW-104	0.003	0.005	0.018	0.017	0.155	0.154
RW-109	0.003	0.005	0.012	0.013	0.109	0.108
RW-110	0.004	0.002	0.020	0.023	0.147	0.149

[a] LW Series = leaf samples.
[b] RW Series = root samples.

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022

t-values of 0.7260 for withaferin-A, 0.8578 for 12-deoxywithastramonolide, and 1.0310 for withanolide-A were less than the tabulated *t*-value, 1.943, at the 95% confidence level. Therefore, there was no significant difference in the determined content of withanolides by the LC and HPTLC methods.

Conclusions

The results of analysis of *W. somnifera* by the proposed HPTLC method are reproducible and in good agreement with the LC-PDA analysis data. The HPTLC method on silica gel $60F_{254}$ with dichloromethane–methanol–acetone–diethyl ether (15 + 1 + 1 + 1, v/v/v/v) and densitometric evaluation at 230 nm is a simple, specific, precise, accurate, and robust method for the determination of key withanolides withaferin-A, 12-deoxywithastramonolide, and withanolide-A.

Acknowledgments

We are thankful to the Council of Scientific and Industrial Research and Department of Biotechnology, New Delhi, India, for providing financial support.

References

(1) Singh, S., & Kumar, S. (1998) *Withania somnifera the Indian ginseng Ashwangandha*, Central Institute of Medicinal and Aromatic Plants, Lucknow, India

(2) Matsuda, H., Murakami, T., Kishi, A., & Yoshikawa, M. (2001) *Bioorg. Med. Chem.* **9**, 1499–1507

(3) Pucheault, M. (2008) *Org. Biomol. Chem*. **6**, 424–432

(4) Owais, M., Sharad, K.S., Shehbaz, A., & Saleemuddin, M. (2005) *Phytomedicine* **12**, 229–235

(5) Kambizi, L., & Afolayan, A.J. (2008) *Afr. J. Biotechnol.* **7**, 12–15

(6) Malik, F., Singh, J., Khajuria, A., Suri, K.A., Satti, N.K., Singh, S., Kaul, M.K., Kumar, A., Bhatia, A., & Qazi, G.N. (2007) *Life Sci.* **80**, 1525–1538

(7) Kuboyama, T., Tohda, C., & Komatsu, K. (2005) *Br. J. Pharmacol.* **144**, 961–971

(8) Kaileh, M., Berghe, W.V., Heyerick, A., Horion, J., Piette, J., Libert, C., De Keukeleire, D., Essawi, T., & Haegeman, G. (2007) *J. Biol. Chem.* **282**, 4253–4264

(9) Dhar, R.S., Verma, V., Suri, K.A., Sangwan, R.S., Satti, N.K., Kumar, A., Tuli, R., & Qazi, G.N. (2006) *Phytochemistry* **67**, 2269–2276

(10) Chaurasiya, N.D., Uniyal, G.C., Lal, P., Misra, L., Sangwan, N.S., Tuli, R., & Sangwan, R.S. (2007) *Phytochem. Anal.* **19**, 148–154

(11) Ganzera, M., Choudhary, M.I., & Khan, I.A. (2003) *Fitoterapia* **74**, 68–76

(12) Khajuria, R.K., Suri, K.A., Gupta, R.K., Satti, N.K., Amina, M., Suri, O.P., & Qazi, G.N. (2004) *J. Sep. Sci.* **27**, 541–546

(13) Bhattacharya, S.K., Kumar, A., & Ghosal, S. (1995) *Phytother. Res.* **9**, 110–113

(14) Mahadevan, N., Rahul, P.K., Subburaju, T., & Suresh, B. (2003) *J. Sep. Sci.* **26**, 1707–1709

(15) Sharma, V., Gupta, A.P., Bhandari, P., Gupta, R.C., & Singh, B. (2007) *Chromatographia* **66**, 801–804

(16) Singh, D.V., Verma, R.K., Gupta, M.M., & Kumar, S. (2002) *Phytochem. Anal.* **13**, 207–210

(17) Tripathi, A.K., Verma, R.K., Gupta, A.K., Gupta, M.M., & Khanuja, S.P.S. (2006) *Phytochem. Anal.* **17**, 394–397

(18) Srivastava, A., Misra, H., Verma, R.K., & Gupta, M.M. (2004) *Phytochem. Anal.* **15**, 280–285

(19) Srivastava, V., Singh, M., Malasoni, R., Shanker, K., Verma, R.K., Gupta, M.M., Gupta, A.K., & Khanuja, S.P.S. (2008) *J. Sep. Sci.* **31**, 47–55

(20) Shanker, K., Singh, S.C., Pant, S., Srivastava, P., Yadav, A.K., Pandey, R., Verma, R.K., & Gupta, M.M. (2008) *Chromatographia* **67**, 269–274

(21) *ICH, Q2 (R1) Validation of Analytical Procedures: Methodology* (2005) in Proceedings of the International Conference on Harmonization, Geneva, Switzerland

(22) IUPAC Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis (2002) *Pure Appl. Chem.* **74**, 835–855

(23) Scartezzini, P., Antognoni, F., Conte, L., Maxia, A., Troìa, A., & Poli, F. (2007) *Nat. Prod. Res.* **21**, 923–932

Downloaded from https://academic.oup.com/jaoac/article/91/5/1154/5656147 by guest on 21 August 2022