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Quantitative HPLC analysis of withanolides in *Withania somnifera*

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Abstract

One of the most widely used herbs in Ayurvedic medicine is Ashwaghanda, *Withania somnifera*, a shrub commonly found on the Indian subcontinent. As this plant is increasingly becoming a popular adaptogenic in the western world, analytical methods for its identification and quality control are in demand. Thus, a HPLC method for the determination of withaferin A and withanolide D was developed. The system was successfully used to investigate the presence of the markers in different *W. somnifera* plant parts as well as to analyze their content in market products.

Keywords: *Withania somnifera*; Ashwaghanda; Withanolides; HPLC

1. Introduction

Withania somnifera (L.) Dunal. (Solanaceae) is a valued herb in Ayurvedic medicine, and as such was used and cultivated for centuries in India. The up to 1.5-m high shrub with ovate leaves and greenish–yellow flowers can be found in

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Western India, and is locally known as Ashwagandha ('smells like a horse') [1]. Roots, leaves and preparations thereof are traditionally used as tonic (the plant is sometimes referred to as Indian ginseng), hypnotic, sedative and diuretic [2,3]. Claiming adaptogenic effects, Ashwagandha products are available throughout the US as dietary supplements.

Withanolides are ergostane type steroids, with atoms C-22 and C-26 bridged by a δ-lactone functionality and an oxidized C-1 position. These compounds are specific for the Solanaceae family, and in particular for the genus *Withania*, thus they are used as marker compounds [4]. The biological activities of withanolides, especially of the dominant withaferin A (**1**) and withanolide D (**2**), have been studied extensively in the past. Notable activities reported for these compounds include anti-inflammatory [5], anticonvulsive [6], antitumor [7], immunosuppressive [8] and antioxidant [9] properties. The number of analytical reports for the determination of withanolides **1** and **2** is comparatively small. Besides a TLC method for the quantification of withaferin A [10], only three HPLC methods are described in literature. All of them show major disadvantages, as either the acetylation of **1** and **2** is required prior to analysis [11], the separation time is extremely long (several hours) [12] or the compounds are not baseline separated and elute more or less with the injection peak [13]. Vitali et al. investigated a different chemotype of the plant that did not contain the compounds of interest [14].

As part of our efforts to develop analytical methods suitable for the standardization of dietary supplements, this study had several aims: to develop and validate a system for the accurate, rapid and direct determination of the major withanolides in *W. somnifera*; to investigate the distribution of these compounds in different plant parts; and finally, to analyze several commercial products to determine their composition and quality.

2. Experimental

2.1. Materials

Standard compounds **1** and **2** were purchased from Chromadex (Laguna Hills, CA, USA). Authenticated *W. somnifera* plant material was collected in the summer of 2000 near Karachi, Pakistan; the commercial samples analyzed in this study (NPC-WS-4 to NPC-WS-9) were bought from Nutrimart (Diamond Bar, CA, USA). Voucher specimens of all samples are deposited at the NCNPR, University of Mississippi.

Solvents (methanol, reagent alcohol and water) were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Reagent alcohol is denaturated ethanol and consists of ethanol, methanol and 2-propanol in the ratio of 90.6:4.5:4.9.

2.2. Sample preparation

One gram of the finely powdered plant material or commercial product was extracted three times with 3.0 ml of MeOH by sonication for 10 min. After

Table 1
Correlation coefficient (R^2), regression equation and limit of detection (LOD) for compounds (**1**) and (**2**)

Compound	R^2	Regression equation*	LOD (μg/ml)
Withaferin A (**1**)	0.9996	$y=1.25\times10^4X$	0.26
Withanolide D (**2**)	0.9996	$y=1.72\times10^4X$	0.23

* y = Peak area; X = concentration (μg/ml).

centrifugation (5 min at 3000 rev./min), the extracts were combined in a 10-ml volumetric flask and adjusted to the final volume with MeOH. The liquid samples NPC-WS-8 and NPC-WS-9 were diluted 1:1 with MeOH. Prior to use, all samples were filtered through a 0.45-μm Nylaflo membrane filter from Gelman (Ann Arbor, MI, USA). Every sample solution was injected in triplicate; R.S.D. were below 2.0% for all experiments.

2.3. Calibration

Two milligrams of each standard compound were placed in one 5-ml volumetric flask and dissolved in MeOH (stock solution). Five additional calibration levels were prepared by diluting this solution with MeOH. Standard solutions were stored at 4 °C and were stable for at least 30 days (confirmed by re-assaying the solution). Within the range of concentrations injected (400.0–1.6 μg/ml) the detector response was linear; see Table 1 for calibration data. All data were recorded and processed by Millennium 32 software from Waters (Milford, MA, USA).

2.4. Analytical method

HPLC analysis was performed on a Waters Alliance 2690 HPLC system, equipped with a 996 photodiode array detector (Waters, Milford, MA, USA). For all separations a Synergi MAX-RP 80 Å column (150×4.6 mm, 4 μm particle size) from Phenomenex (Torrance, CA, USA) was used. The mobile phase consisted of water (A) and a mixture of MeOH and reagent alcohol in the ratio of 1:1 (B), which were applied in the following gradient elution: from 65 A/35 B in 25 min to 55 A/45 B. Each run was followed by a 5-min wash with 100 B and an equilibration period of 10 min. The separation temperature was kept constant at 50 °C, flow rate and sample volume were set to 1.0 ml/min and 10 μl, respectively. All separations were monitored at 230 nm. Peaks were assigned by spiking the samples with authentic samples of **1** and **2**, and comparison of the UV-spectra and retention times.

For peak confirmation an LC-MS experiment, using an AQA mass spectrometer from Finnigan (San Jose, CA, USA), together with a Finnigan HPLC (AS3000 autosampler, P4000 pump and UV6000LP detector) was performed. Best results were obtained in positive ESI mode, with ionization voltage set to 50 V, source voltage to 3.0 kV and probe temperature to 350 °C. With this instrumentation the

Table 2
Intra- and inter-day precision of sample NPC-WS-7 assayed under optimized conditions

Compound	Intra-day*			Inter-day**
	Day 1	Day 2	Day 3	
1	353.81 (4.74)	358.96 (2.85)	346.54 (4.86)	353.10 (1.76)
2	186.76 (2.69)	190.90 (1.00)	183.49 (2.97)	187.05 (1.98)

Samples in μg/g; R.S.D.'s are given in parentheses; *($n=5$); **($n=3$).

separations had to be performed at ambient temperature. Thus, in order to obtain similar retention times and good detection sensitivity the flow rate was reduced to 0.5 ml/min and the solvent gradient modified (55 A/45 B to 45 A/55 B in 25 min).

2.5. Accuracy

A recovery experiment was performed to confirm the accuracy of the method. Sample NPC-WS-7 (1.0 g) was spiked with 1.00 ml of the standard stock solution, and then extracted and analyzed under optimized conditions. The recovery rates obtained were 97.59% for **1** and 100.0% for **2**.

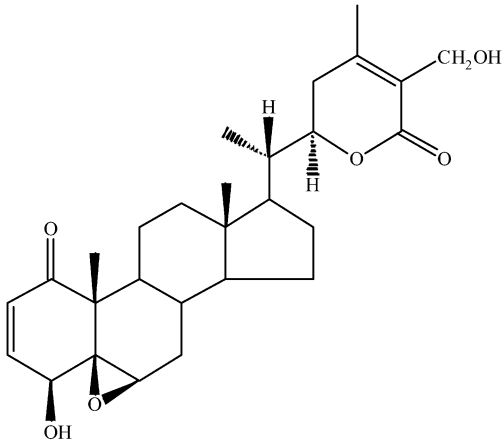
2.6. Ruggedness

Intra- and inter-day assay precision of the method was determined by analyzing five individual samples of one specimen (sample NPC-WS-7) on 3 consecutive days. The samples were extracted and assayed under optimized conditions; for detailed results see Table 2.

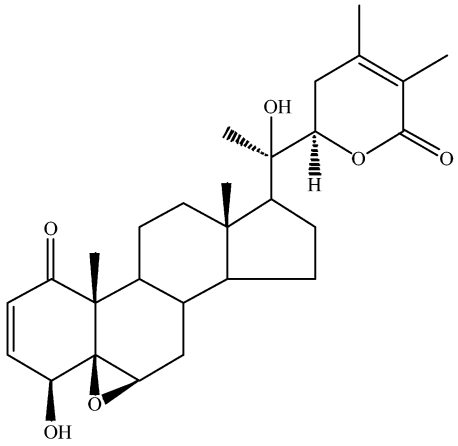
3. Results and discussion

The separation of three methanolic *W. somnifera* extracts (root, stem and leaf) under optimized conditions is shown in Fig. 2 (for corresponding structures see Fig. 1). Withaferin A (**1**) and withanolide D (**2**), two biologically active withanolides which are also commercially available, were chosen as marker compounds for standardization purposes. Both could be baseline separated from compounds of similar polarity in less than 25 min after all separation parameters were carefully assessed.

Stationary phases of several manufacturers, with different polarity (C-8, C-12, C-18, phenyl-hexyl), particle size (3–5 μm) and column length (10–25 cm) were initially screened for their applicability. A Synergi MAX-RP 80 Å column (C-12 phase, 4 μm particle size, 150×4.6 mm) from Phenomenex showed the best results regarding peak symmetry and separation selectivity. The right mobile phase was crucial for a satisfactory result as well. Only by using methanol, compound **2** could be well resolved from the signal of the unidentified substance (a) (Fig. 2). In combination with reagent alcohol the separation was further improved, whereas the



Withaferin A (**1**)



Withanolide D (**2**)

Fig. 1. Structures of compounds **1** and **2**.

addition of acid, a buffer or a modifier (MBE, THF) to the mobile phase was not advantageous. Performing the separation at 50 °C significantly reduced the separation time and the column backpressure, without any decrease in peak resolution.

Additionally, an LC-MS experiment was performed to confirm the identity of the peaks of interest (Fig. 3). The HPLC conditions were slightly changed as the separation had to be performed at room temperature and a flow rate of 1.0 ml/min did not allow a sensitive detection of the compounds. With modifications in the solvent composition and flow rate, the MS signals were readily assignable. In positive ESI mode, the spectra of **1** showed signals at m/z of 488.3 $[M+NH_4]^+$

and 493.3 $[M+Na]^+$; a signal at m/z 488.4 $[M+NH_4]^+$ was observed for compound **2**.

The method was validated in accordance to USP by determining several analytical and statistical parameters. Linearity of the detector response for the two standard compounds was confirmed between 400.0 and 1.6 μg/ml, with a detection limit of at least 0.26 μg/ml (see Table 1 for the exact data). Peak purity and identity were verified by studying the PDA- and MS-data, as well as by spiking samples with

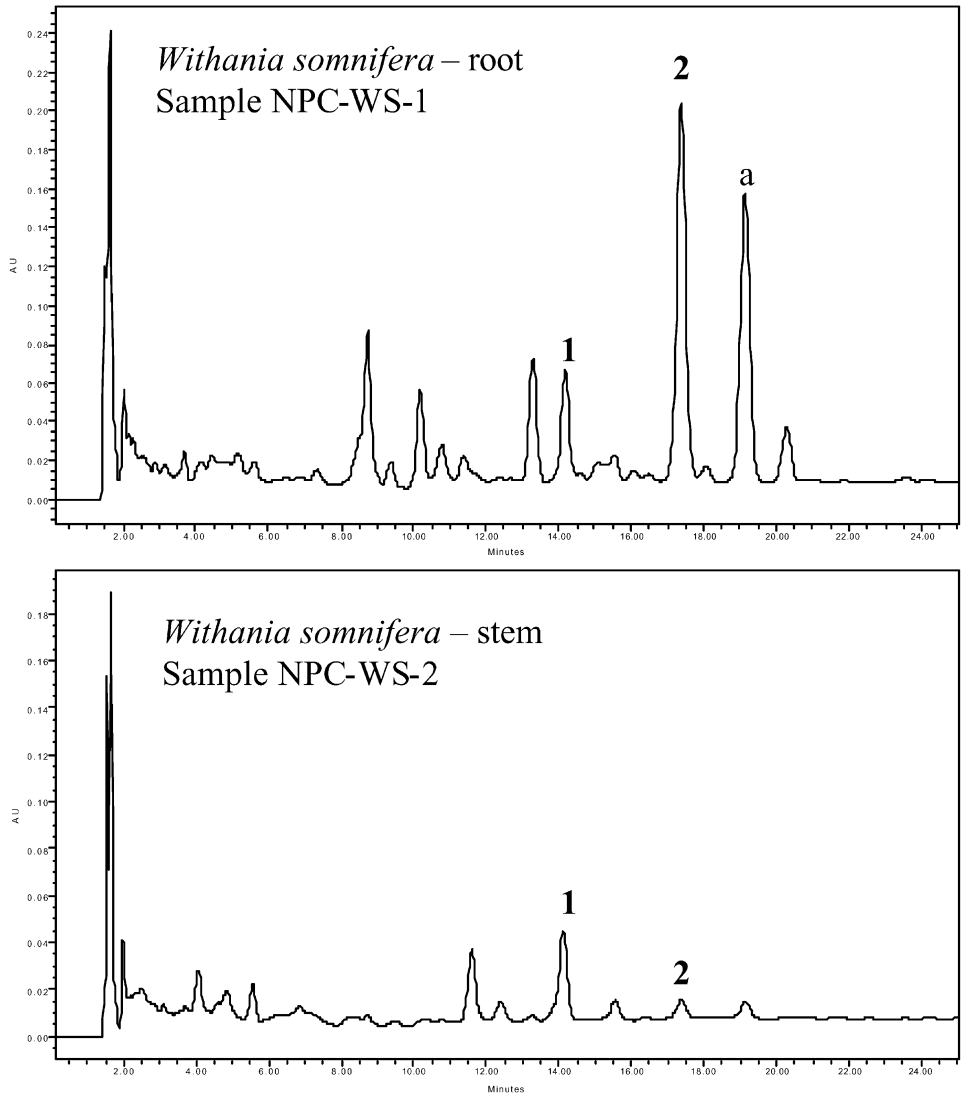


Fig. 2. HPLC profiles of *W. somnifera* root, stem and leaf extracts.

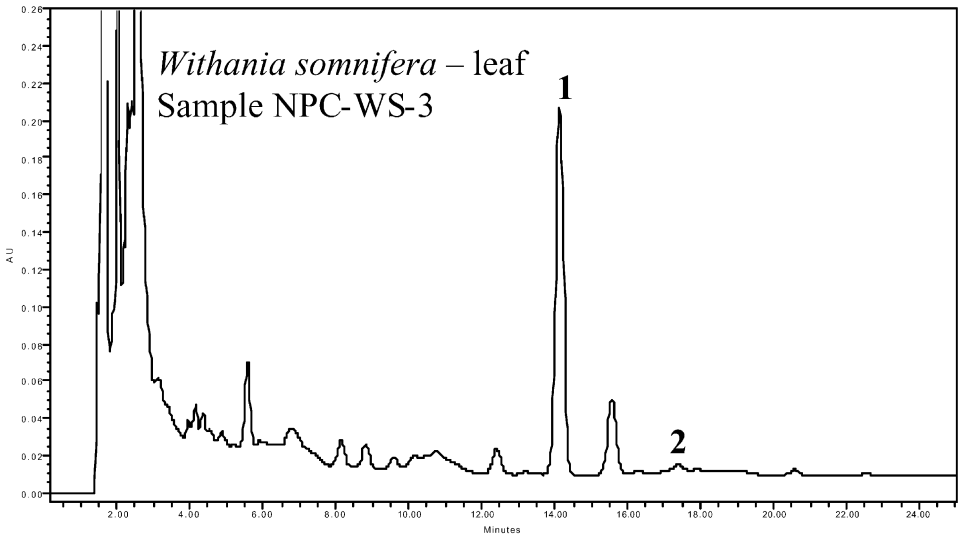


Fig. 2 (Continued).

reference compounds; no indications of impurities could be found. Accuracy of the method was confirmed by performing a recovery experiment. Sample NPC-WS-7 was spiked with a known amount of the authentic compounds, extracted and analyzed. Compared to the theoretical amount, recoveries of 97.59% for **1** and 100.0% for **2** were obtained. All standards and samples were injected in triplicate. The resulting R.S.D. of less than 2.0% confirmed the precision of the method. Intra- and inter-day variation of the assay was determined and showed to be lower than 5.0%, with a maximum R.S.D. of 4.86% reached at day 3 for compound **1**. The inter-day precision was better than 2.0% for all compounds (Table 2).

Prior to the analysis of Ashwaghanda samples and products, the efficiency of the extraction procedure was verified. Thus, one sample (NPC-WS-7) was repeatedly extracted with 3 ml of methanol, and each extract analyzed separately. Compounds **1** and **2** were not detectable after the fifth repetition of the procedure. A minimum of 97.8% of the compounds was in solution after a 3-fold extraction, therefore, this was already considered as an exhaustive procedure.

The analysis of *W. somnifera* root, stem and leaf confirmed the presence of **1** and **2** in all parts of the plant, but with significant differences in their ratio (Table 3). In the roots, compound **2** was most dominant (0.193%). This compound was only minor in leaves, where a rather high amount of **1** was found (0.238%). Stems contained the lowest percentage of total withanolides **1** and **2** (0.055%). Finally, six market products (capsules, tablets and liquid extracts containing *W. somnifera*) were analyzed. In all of these, even in multi component preparations, (e.g. NPC-WS-6), the two marker compounds were assignable. Not all of the samples followed the previously described pattern with **1** being major in leaves and **2** being dominant in

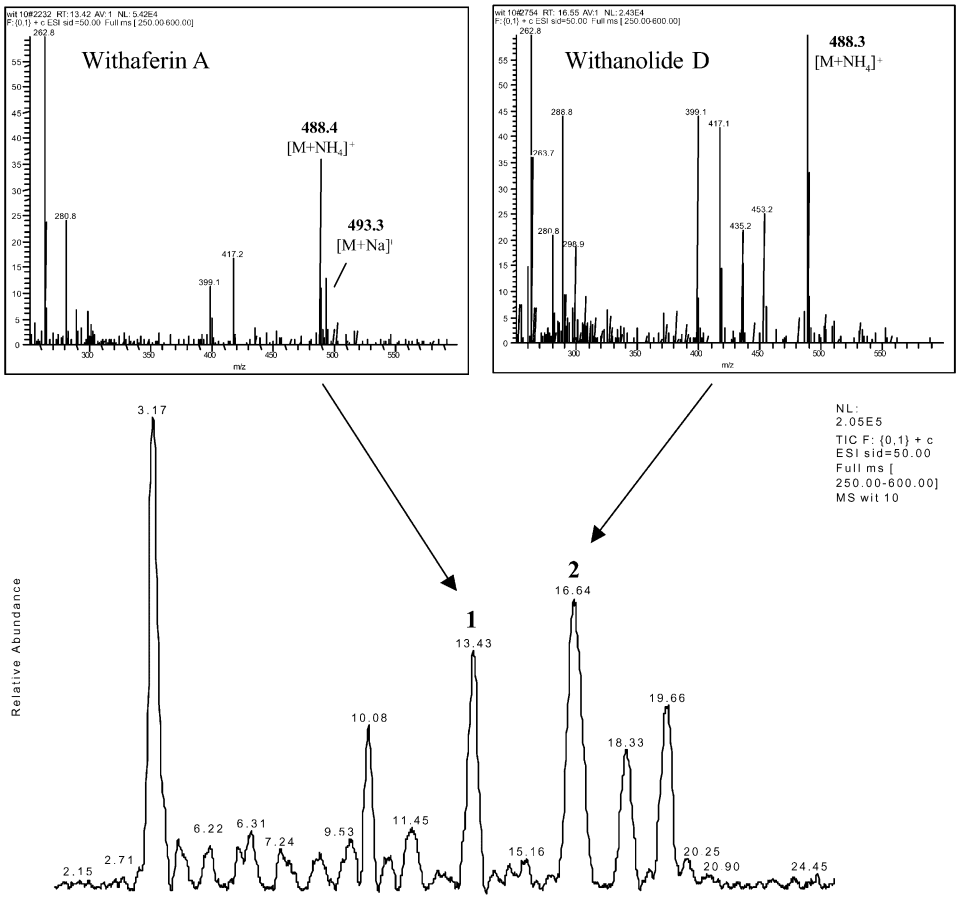


Fig. 3. LC-MS analysis of a methanolic *W. somnifera* root extract.

roots. The higher percentages of **1** in samples NPC-WS-4 and NPC-WS-7 (both containing the root) could be explained by seasonal variations, a different extraction procedure applied by the manufacturer or different chemotypes of the plant (which have been reported in the literature [11,14]). The content of **1** and **2** in solid samples (NPC-WS-4 to NPC-WS-7) varied from 0.009% to 0.100%. Comparing the total daily uptake, based on the manufacturers suggestions, permits a better estimation of the overall product quality. As it is evident from Table 3, values from 0.66 to 2.57 mg/day were obtained, which is nearly a 4-fold variation.

In conclusion, the method described herein represents a significant improvement in the analysis of withanolides in *W. somnifera*. It not only allows the direct, rapid and accurate determination of withanolide A and withaferin D in plant material, but it also fulfills all criteria of a validated method. Thus, it should be helpful for scientific as well as commercial applications.

Table 3
Percentage of withanolides in different *W. somnifera* samples

Sample	Description	**1**	**2**	**1–2**§
NPC-WS-1	Root	0.066 (1.91)*	0.193 (1.96)*	–
NPC-WS-2	Stem	0.048 (1.78)*	0.007 (1.97)*	–
NPC-WS-3	Leaf	0.238 (1.16)*	0.003 (0.54)*	–
NPC-WS-4	Root extract	0.051 (0.88)*	0.049 (1.14)*	1.02
NPC-WS-5	Root and leaf extract	0.012 (0.75)*	0.040 (1.66)*	0.66
NPC-WS-6	Root extract	0.003 (1.37)*	0.006 (0.32)*	0.73
NPC-WS-7	Root	0.035 (1.04)*	0.019 (1.61)*	2.51
NPC-WS-8	Root extract (liquid)	0.027 (1.04)**	0.238 (1.52)**	1.32
NPC-WS-9	Root extract (liquid)	0.065 (1.02)**	0.364 (1.06)**	2.57

*Values in g/100 g; **values in mg/ml; $n=3$, R.S.D.'s are given in parentheses; §, daily uptake of withanolides in commercial samples (mg/day).

Acknowledgments

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