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Article

Analysis of Major Withanolides in *Physalis longifolia* Nutt. by HPLC-PDA

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An analytical method based on high-performance liquid chromatography-photodiode array detection was developed for the simultaneous determination of three anti-proliferative withanolides [withalongolide A (1), withaferin A (2) and withalongolide B (3)] present in the aboveground biomass of the long-leaf groundcherry, *Physalis longifolia*. This method was achieved by biomass extraction followed by chromatographic separation on C18 column eluted with a gradient acetonitrile-water mobile phase. Calibration curves produced satisfactory linear regression (r^2 > 0.9995) for each examined sample. The method was also validated for accuracy, precision and limits of detection and quantification. Such an approach is applicable for the rapid detection and quantitative assessment of withanolides in various *P. longifolia* accessions.

Introduction

Withanolides are a group of natural products that are structurally identifiable with an ergostane-type steroidal nucleus adhered to a δ-lactone side chain. These compounds are primarily reported in Solanaceous plants, such as the extensively studied *Datura*, *Jaborosa*, *Physalis* and *Withania* genera (1). The field of withanolide chemistry began with the discovering of the promising chemotherapeutic agent withaferin A (**2**) that was first isolated from *Withania somnifera* (2, 3). Preliminary screening of our North American plant extract library collection revealed the presence of **2** in the wild tomatillo *Physalis longifolia* Nutt., a native species commonly referred to as long-leaf groundcherry and distributed throughout continental North America, southern Canada as well as northern Mexico (4). Our previous large-scale phytochemical investigations on the species revealed the presence of an additional 22 withanolides; where subsequent cell-based screening of the major isolates determined that withalongolides A (**1**) and B (**3**), as well as withaferin A (**2**), exhibited potent anti-proliferative activity (5, 6). In this present study, we established a validated, quantitative HPLC-PDA method in order to determine the presence and quantity of these bioactive withanolides in *P. longifolia* extracts for future quality control and related biological applications.

Experimental

Instrumentation

The chromatographic separation was performed on an IRIS IProSIL120-5 C18 AQ column (4.6 × 250 mm, 5.0 μm), connected to an Agilent 1200 series system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, an autosampler and a PDA detector, and ChemStation software.

Materials and chemicals

(i) Aerial biomass of *P. longifolia* was used in this study. Its seed, collected in 2010 from wild samples gathered from Stafford county in Kansas, sown in the University of Kansas medicinal plant research garden, produced 2-month-old plant samples that were cultivated for this study.
All plant material was collected, cultivated and authenticated by plant taxonomist Dr. Kelly Kindscher of the Kansas Biological Survey, University of Kansas, Lawrence, Kansas, USA. Voucher specimens were deposited in the R.L. McGregor Herbarium of the University of Kansas.

(ii) HPLC grade solvents (acetonitrile, methanol) and analytical grade solvents (hexane, ethyl acetate, dichloromethane and methanol) were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA); HPLC grade water was prepared using a Millipore Milli-Q A10 system (Millipore Corp., Bedford, MA, USA).

Standards

Standard samples, withalongolide A (**1**), withaferin A (**2**) and withalongolide B (**3**), were isolated from *P. longifolia* in our laboratory. Their structures (Figure 1) were identified by UV, MS, X-ray crystallography, ^{1}H and ^{13}C NMR (5). The purities for these compounds were all >97% by HPLC analysis.

Method

Sample and standard preparation

Room temperature sonication-assisted 45 min extractions of 1.0 g of the dried and ground aerial parts of the title plant in 10 mL dichloromethane–methanol (1 : 1) was conducted. A 1 mL sample of the resulting filtered supernatant was evaporated to dryness under reduced pressure and subsequently dissolved in 1 mL HPLC grade methanol. The reference standard sample that generated the depicted chromatogram (Figure 2) was prepared by dissolving **1–3** in methanol to produce a withanolide methanolic solution containing 106.25, 78.75 and 76.25 μg mL^{-1}, of **1–3**, respectively. The resulting stock solution was further diluted to eight concentrations for creation of calibration curves through triplicate HPLC injections, where linear regression analysis was conducted by plotting the integrated peak areas (*Y*) versus the concentrations (*X*, μg mL^{-1}) of each generated solution. Each sample investigated was filtered through a 0.22-μm PTFE filter and then injected in10 μL aliquots for HPLC analysis.

Apparatus and chromatographic conditions

Comprehensive absorption analysis of the standards **1–3**, by the PDA detector across a 190–400 nm wavelength range, revealed

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220 nm as the optimal analytical detection wavelength. Chromatographic separation was achieved in a water (A)–acetonitrile (B) mobile phase by linear gradient (30–80% B; 0–18 min) followed by isocratic (80% B; 18–25 min) elution at a constant flow rate of 1.0 mL min^{-1}. Peak assignments were made by comparing retention times and characteristic absorption spectra from the PDA with those of the authentic standards.

HPLC method validation

Our newly developed HPLC method was validated in terms of linearity, precision and accuracy according to the ICH guidelines (7).

The limit of detection (LOD) and quantification (LOQ) for the standards were established by the minimum concentrations to have signal-to-noise ratios (S/N) of 3 : 1 and 10 : 1, respectively. They were detected by injecting a series of diluted mixed standard solutions with known concentrations of each standard.

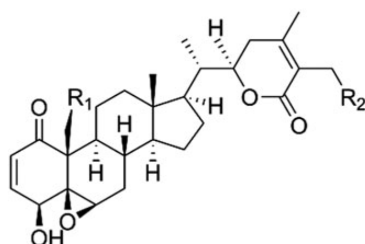
Linearity test solutions for the assay method were prepared (50–150%) with respect to the test concentration in the extract for each standard. The calibration curves were constructed by running the mixed standards at different concentrations in triplicate. The correlation coefficients were determined using a linear regression model.

The intraday precision was examined within 1 day for six different times at ambient temperature. The interday precision was calculated over six consecutive days with the standard solutions stored at 4°C.

The recovery results were performed on the calculated amount of the authentic standards added, which is the difference between the spiked and the unspiked samples analyzed, with the real weight added.

Analysis of P. longifolia samples

The newly established HPLC-PDA method was applied for the simultaneous determination of three withanolides in *P. longifolia* crude extracts. Withanolide contents were calculated on averaged triplicate measurements and standard curves.



	R_1	R_2
1 Withalongolide A	OH	OH
2 Withaferin A	H	OH
3 Withalongolide B	OH	H

Figure 1. Structures of standard withanolides **1**–**3**.

Table I
Linear Range, LOD and LOQ of the Investigated Withanolides (**1**–**3**)

Analytes	Equation	R^2	Linear range (μg mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
1	$y = 20.428x - 2.1179$	0.9995	10.625–106.250	3.188	10.630
2	$y = 22.669x - 5.342$	0.9996	7.875–78.750	5.906	19.690
3	$y = 16.838x - 0.8902$	0.9995	7.625–76.250	2.288	7.625

Table II
Intra- and Interday Precision of the Investigated Withanolides (**1**–**3**) for HPLC-PDA Method

Analytes	Intraday RSD (%) ($n = 6$)	Interday RSD (%) ($n = 6$)	Recovery and RSD (%) (mean, $n = 6$)
1	1.571	0.614	96.72, 3.793
2	1.254	0.426	95.43, 2.686
3	1.670	0.697	96.18, 3.968

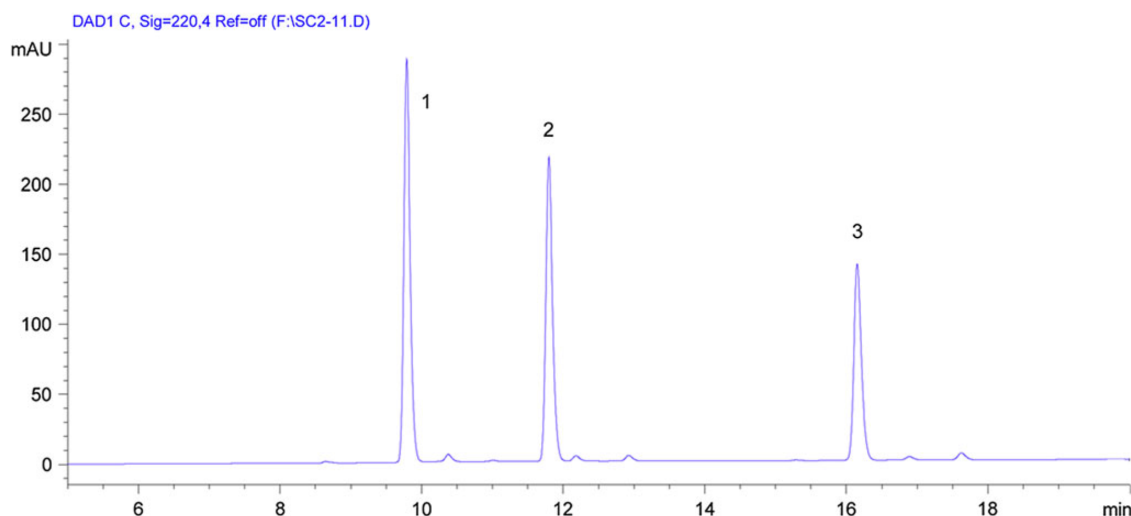


Figure 2. HPLC chromatogram of the mixed standards **1**–**3**.

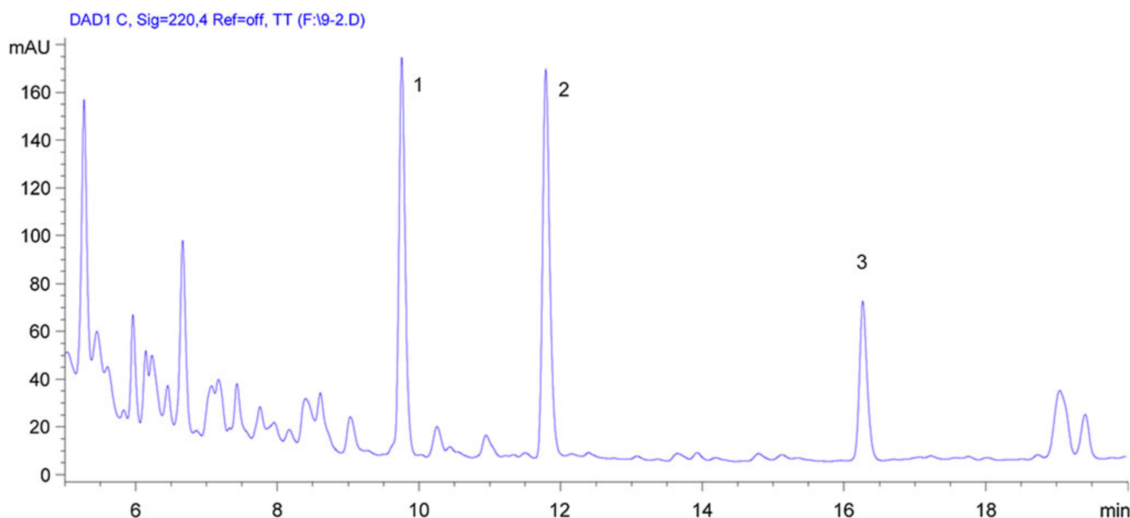


Figure 3. HPLC chromatogram of crude extract of *P. longifolia.*

Table III
The Contents of Withanolides **1**–**3** in *P. longfolia* ($n = 3$)

Analytes	Contents (µg g^{-1})	RSD (%)
1	560.3	0.801
2	547.3	2.284
3	320.6	1.218

Results

Optimization of extraction conditions

Analysis of different candidate extraction solvents (dichloromethane, ethyl acetate, hexane and methanol) found extraction efficiency and baseline stability in dichloromethane–methanol (1 : 1) solutions. In addition, plant material to solvent ratio (w/v) analysis revealed that the ratio 1 : 10 produced the greatest relative stable baseline and concentration linear range of all ratios examined (1 : 5, 1 : 10 and 1 : 15). Sonication-assisted extractions were evaluated at 20, 30, 45 and 60 min time intervals. As a result, the optimized extraction method was determined to be a 45 min sonication-assisted extraction of 1 g biomass in 10 mL of dichloromethane–methanol (1 : 1, v/v) solution.

HPLC method validation

Linear ranges (µg mL^{-1}), determination coefficients (r^2), LOD and LOQ (ng mL^{-1}), as well as intra- and interday precisions for the analyses of the three standards are summarized in Tables I and II.

The LODs of withalongolide A (**1**), withaferin A (**2**) and withalongolide B (**3**) were 3.188, 5.906 and 2.288 ng mL^{-1}, respectively. The LOQs of **1**, **2** and **3** were 10.630, 19.690 and 7.625 ng mL^{-1}, respectively.

Linearity was evaluated by a triplicate analysis of the working standard solutions of the withanolides **1**–**3** at eight different concentrations. Good linearity ($r^2 \geq 0.9995$) of the integrated peak areas (Y) versus the concentrations (X, µg mL^{-1}) was obtained for each standard by analyzing the eight concentrations (10.625, 21.250, 31.875, 42.500, 53.125, 63.750, 85.000 and 106.250 µg mL^{-1} of **1**; 7.875, 15.750, 23.625, 31.500, 39.375, 47.250, 63.000 and 78.750 µg mL^{-1} of **2**; 7.625, 15.250, 22.875, 30.500, 38.125, 45.750, 61.000 and 76.250 µg mL^{-1} of **3**).

The relative standard deviations (%RSD) of **1**–**3** in the intra- and interday precision studies were within 1.670 and 0.697%, respectively. The higher intraday precision RSD observed is likely caused by storage of samples at ambient temperature when formation of artificial withanolide may occur (8). The observed results confirm the precision of the liquid chromatography method we developed for withanolide-containing samples.

The percentage recovery rates between 95.43 and 96.72% are also indicative that the developed method is reproducible with good recovery and high accuracy.

Conclusion

Organic extracts of *P. longifolia* examined in this study produce a complex HPLC profile, with the three quantified bioactive withanolides as the major components as depicted in Figure 3. The quantity of each component identified is summarized in Table III. This HPLC-PDA method was validated for accuracy, precision, limits of detection and quantification. It also represents the first successful quantification of withanolides **1**–**3** in *P. longifolia* samples. Such an approach is therefore applicable for the rapid detection, as well as qualitative and quantitative assessment, of withanolides **1**–**3** in various *P. longifolia* plant collections (9).

Acknowledgments

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