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SEMI-QUANTITATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ECOTYPES OF *WITHANIA SOMNIFERA* CHEMOTYPE III

ROBERTO BESSALLE and DAVID LAVIE*
Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100 (Israel)
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SUMMARY

Eleven withanolides isolated from *Withania somnifera* L. (Dun.) were analyzed on a C_{18} reversed-phase column. Good separation factors were obtained, especially when analyzing the major component of the plant. The semi-quantitative determination of the main withanolides of chemotype III collected from four different locations (Yavne, Gedera, Farm-1 and Farm-2) was carried out in order to confirm the presence of ecotypes. The standards were obtained by isolation and purification of the withanolides from the ecotype identified as Farm-2 and our own collection. As a result of the semi-quantitative analysis, three ecotypes were found. They were identified according to the differences in concentration of their major component, and were related to the various locations.

INTRODUCTION

The withanolides, a group of naturally occurring ergostane type of steroids, have been the subject of extensive studies during the last 20 years. Several genera of plants were analyzed and a number of compounds possessing different structures were characterized[1,2].

In Israel, three chemotypes of *Withania somnifera* L. (Dun.) (I, II and III) were identified, each having a number of withanolides which present different functionalities. They all show a common structural pattern which was found to be characteristic for each chemotype[3–5]. In addition, sub-types found to grow in different locations were detected from chemotype III, here called ecotypes. These are characterized by possessing the same major components in the plant but differing in their relative concentrations[6].

Thus, in order to confirm the existence of such ecotypes and to determine with greater accuracy the range of concentration of the withanolides present in them, a semi-quantitative reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis of the major components of the ecotypes of chemotype III was carried out. Previous work has employed only analytical column chromatographic procedures[3–6] which led to errors due to losses during purification. Also a quanti-

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fication of withaferin A, the main component of chemotype I[4], and of *Acnistus breviflorus*[7] was performed.

EXPERIMENTAL

Isolation of withanolides from Withania somnifera chemotype III ecotype Farm-2

Plant material. Withania somnifera chemotype III (Farm-2) was collected at our experimental farm, dried at 45°C and stored.

Isolation procedure. Finely powdered crushed leaves (823 g) were exhaustively extracted with 5 l of methanol for 30 h in a Soxhlet apparatus and the extract was concentrated to a volume of 1 l. Distilled water (0.5 l) was added and the mixture was extracted in a 5-l funnel with hexane (2 l) and light petroleum (b.p. 40–60°C) (1 l) to remove chlorophyll and other pigments. The residual solution was then re-extracted with 3 l of dichloromethane, washed several times with distilled water and dried over sodium sulphate. The solvent was then evaporated to leave a green residue (*ca.* 28 g).

Chromatography 1. The crude residue was introduced at the top of a chromatographic glass column (110 × 6 cm) packed with 1 kg of silica gel 60 Merck (70–230 mesh) and the column was eluted with dichloromethane, ethyl acetate and methanol in different proportions, yielding eight major fractions (Table I).

TABLE I

ELUENTS AND WEIGHTS OF FRACTIONS USED FOR CHROMATOGRAPHY 1 OF CHEMOTYPE III

Fraction	*Eluent*			
	Dichloromethane	*Ethylacetate*	*Methanol*	*Weight (g)*
1– 4	7	3	–	0.728
5– 9	5	5	–	1.756
10–18	3	7	–	0.923
19–24	–	1	–	0.572
25–29	–	1	0.075	1.824
30–36	–	4	1	1.581
37–42	–	3	2	3.910
42–45	–	2	2	5.181

Chromatography 2. Each fraction was rechromatographed by flash chromatography with silica gel 60 Merck (230–400 mesh) using different solvent systems (Table II). The purified compounds obtained were crystallized and characterized by their m.p.s and ^{1}H NMR spectra[5,8,9]. Since 5α-Cl withanolide S (10) turned out to be a new compound its structure elucidation was undertaken and will be reported in a forthcoming publication[10].

Equipment and HPLC

The experiments were performed with an HPLC apparatus assembled from commercially available components. A solvent reservoir was connected to a Model A-30-S-2 pump (Eldex Labs., Menlo Park, CA, U.S.A.). From the pump outlet,

TABLE II

FLASH CHROMATOGRAPHY OF FRACTIONS 1–45

Fraction	*Withanolide*	*Eluent*			
		Hexane	*Ethyl acetate*	*Methanol*	*Dichloromethane*
1– 4	G-2	8	6	0.22	–
5– 9	L	8	6	0.33	–
5– 9	K	8	6	0.33	–
5– 9	J	8	6	0.33	–
10–18	J	4	7	0.15	–
10–18	G	4	7	0.15	–
19–24	6-Cl	0.4	6	0.11	–
25–29		0.4	6	0.11	
30–36	E	0.4	6	0.11	–
30–36	1,3 di-OH	0.4	6	0.11	–
30–36	5-Cl	4.0	7.5	1.0	–
37–42*	H	–	–	1.0	10
42–45	S	–	10	2.0	–

* Fractionated by chromatography using silica gel Woelm (150–230 mesh).

stainless-steel tubing led to a sample injection valve (Model 7129; Rheodyne, Berkeley, CA, U.S.A.) with a loop of 20 μl and from there to the column inlet. The column was a stainless-steel tube (250 mm × 4.6 mm I.D.) packed in our department with LiChrosorb C_{18} of particle size 10 μm. The column outlet was connected to the inlet of a Waters Assoc. liquid chromatography detector (Model 440) with a flow cell having a pathlength of 10 mm adjusted to attenuation 0.1 and equipped with an interchangeable wavelength source set at 229 nm. The signal from the detector was recorded by a single-channel Rikadenki electronic recorder (Model B-141; Kogyo Co., Tokyo, Japan) set at 10 mV, with a speed of 0.5 cm/min, or to an OmniScribe recorder (Model EB5217-5; Houston Instruments, Austin, TX, U.S.A.) set at 10 mV and with a speed of 0.25 cm/min. A volume of 20 μl was injected for all samples. The column was washed with methanol after every four experiments in order to remove possible impurities. All the solvents used (methanol, dichloromethane, isopropanol) were Merck spectroscopic grade. Water was twice distilled and filtered.

Analytical thin-layer chromatography (TLC) was carried out using chromatoplates of silica gel F_{254} (Merck) with water–methanol (3:7) as the eluent. Acetone was used as a reference to calculate the capacity factor, k', since it absorbs in the UV and has no retention on the column.

Standard preparation and calibration curve

Pure crystalline compounds 1–11 (each 10 mg) were weighed accurately into a 100-ml volumetric flask. Dichloromethane–methanol (1:1) was used to dissolve and dilute the sample to the required volume. From each solution, series dilutions in the same solvent gave the desired concentrations. Withanolide I, withaferin A and withanolide D (3, 11a and 12a respectively) were taken from our collection and used as

standards. These solutions were used for the calibration curves (Figs. 4, 5 and Tables III, IV).

Sample preparation

Different ecotypes of chemotype III of *Withania somnifera* were collected in Yavne, Gedera and in the experimental farm of our Institute where it was cultivated experimentally. Chemotype I was collected in the village of Azaria and *Acnistus breviflorus* was taken from a stock of material grown experimentally and collected in the village of Sarona (lower Galilee).

Finely powdered dry leaves (1 g) were extracted with 100 ml of methanol during 48 h at room temperature. The supernatant was filtered and the residue washed with methanol (40 ml) and evaporated to dryness. The residue was dissolved in 30 ml of methanol and added to 100 ml of water containing 20% of sodium chloride for breaking subsequent emulsions. The solution was washed several times with 200 ml of dichloromethane which was later concentrated to about 50 ml and dried over sodium sulphate. The filtrate was evaporated, concentrated, filtered and redissolved in exactly 10 ml of dichloromethane–methanol (1:1). Different dilutions were prepared for each ecotype in order to obtain the highest possible area and so reduce errors. For each plant two different extractions with 1 g of material were carried out, and for each of them two analyses were performed.

The acetylation of withanolides A, D and chemotype I was carried as follows: 20 mg of pure crystalline compounds 11a and 12a and the dried purified extract of 1 g of leaves of chemotype I (as described above) were dissolved separately in 20 ml and 40 ml of dichloromethane–methanol (2:1), together with 2 ml of acetic anhydride, then some dimethylaminopyridine was added as a catalyst. The mixture was heated at 70°C for 3 h using a magnetic stirrer. Then the reaction quenched in 150 ml of ice-water and extracted with 120 ml of dichloromethane. The solvent was evaporated and the residue redissolved in 5 ml of the same solvent. An aliquot was injected for HPLC.

RESULTS AND DISCUSSION

Since the withanolides are highly oxygenated steroids and differ principally in the positions of the hydroxyl groups and in the locations of the double bonds, they display a wide range of polarity which enables good separation by HPLC. Until now, only HPLC analyses of artificial mixtures and of different genera of plants containing withanolides have been carried out[11–16]; no systematic quantitative analyses were done for the different chemotypes of *W. somnifera*.

Most of the previous HPLC work on withanolides was carried out in the normal-phase mode. Hunter *et al.*[11] described the separation of six withanolides from an artificial mixture, among which withanolides J, G and E belong to chemotype III. A good separation was obtained but the analysis time was extremely long (20 h). In addition, a crude extract of chemotype I was analysed, giving a retention time of 6 h for withaferin A. Tishbee and Kirson[12] were able to separate withanolide E and withaferin A from a crude extract of *Physalis peruviana* in approximately 30 min. The reversed-phase analysis of withanolides belonging to *Physalis minima*[15] and *Acnistus breviflorus*[16] has been reported. Both studies used a column packed with silica

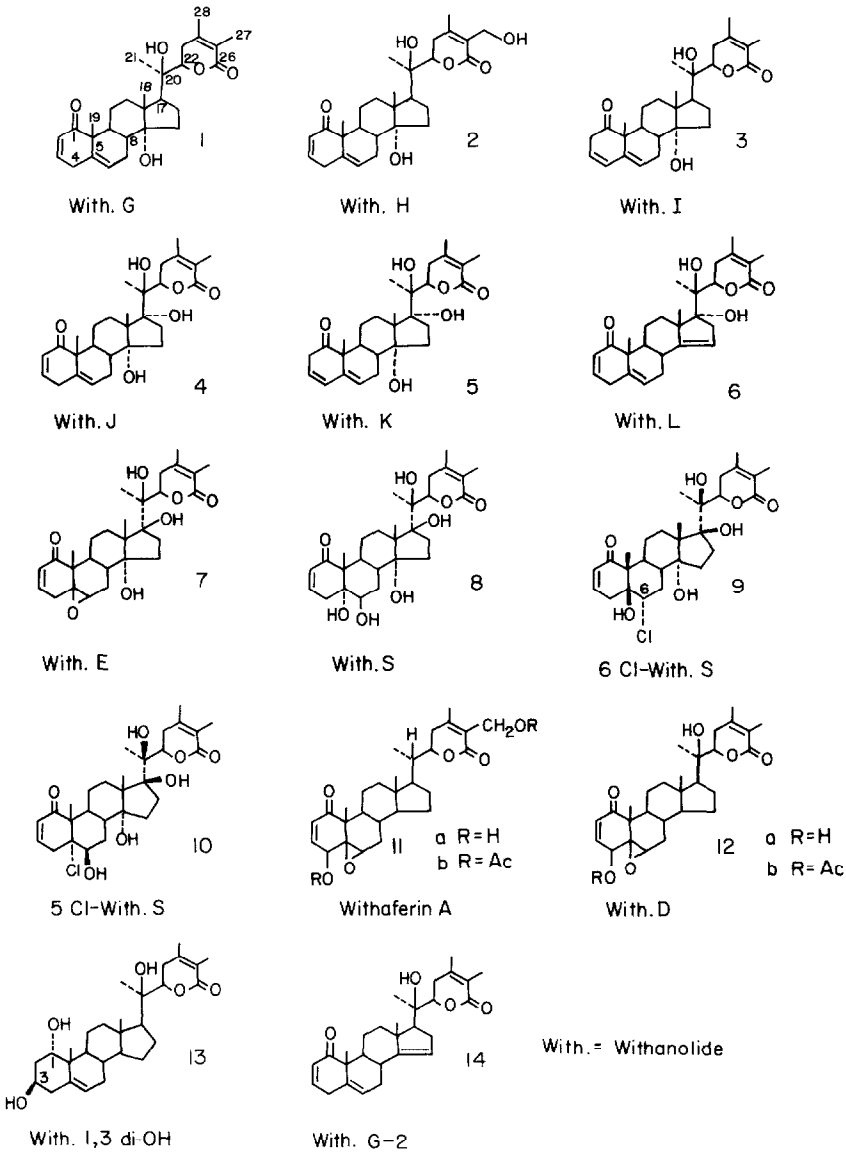
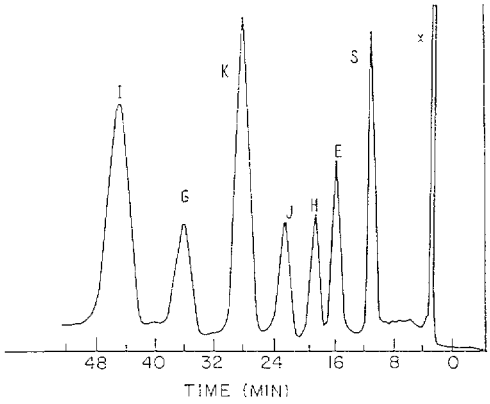


Fig. 1. Withanolide structures of compounds 1–14. Ac = Acetyl.

gel containing octadecylsilane (ODS) and methanol–water as the eluent. Rosazza and co-workers[13] reported no separation of derivatives of withaferin A using a C_{18} reversed phase. In our experiments we used a high sensitivity detection mode at 229 nm which enabled all the withanolides analyzed to be detected at the ng level.

Of the several solvent systems tested, methanol–water provided the best results. Thus, an artificial mixture containing all the major components of chemotype III was well resolved in less than 1 h using methanol–water (57:43) as the eluent at a flow-rate of 0.8 ml/min (Fig. 2). The capacity factor, k', separation factor, α, and



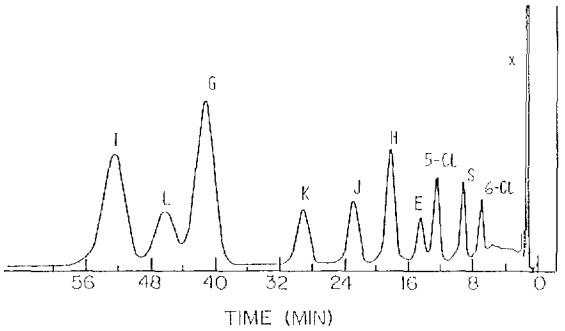
Withanolide	t'_R	k'	α
S	8.00	2.85	
			1.61
E	12.84	4.59	
			1.25
H	16.04	5.73	
			1.22
J	19.60	7.00	
			1.31
K	25.60	9.14	
			1.30
G	33.20	11.86	
			1.26
I	41.72	14.90	

Fig. 2. Chromatogram of an artificial mixture of withanolide S (8), E (7), H (2), J (4), K (5), G (1) and I (3). Column: LiChrosorb C_{18} (RP), 10 μm. Eluent: methanol–water (57:43); flow-rate, 0.8 ml/min. Detection: 229 nm, chart speed 0.25 cm/min. x = Acetone.

retention time, t'_R (min), are also given in Fig. 2. When the main minor components of chemotype III, *i.e.*, coumpounds 6, 9 and 10, were added to the above artificial mixture the polarity of the solvent (47:53) and the flow-rate (1.5 ml/min) were increased. The separation factors were slightly smaller but the analysis time was kept to the minimum (Fig. 3).

Exponential relationships were found for the capacity factor, k', and retention time when increasing the concentration of methanol, similar to those reported by Sen *et al.*[15].

As is seen in Fig. 2, the best resolved withanolides were S and E with a separation factor, α = 1.61. The other withanolides displayed approximately the same values, α = 1.22–1.30. In Fig. 3 the best separation was between K and G, 6α-Cl and S, and S and 5α-Cl, all around α = 1.4. Withanolide L appears as a trace in some extracts, suggesting that it may be an artifact formed by elimination of the labile tertiary 14-OH group in withanolide J[17]. The remaining withanolides display separation factors of 1.1–1.3.



Withanolide	t'_R	k'	α
6-Cl	5.64	3.92	
			1.40
S	7.92	5.50	
			1.41
5-Cl	11.20	7.78	
			1.18
E	13.24	9.19	
			1.28
H	16.96	11.78	
			1.26
J	21.36	14.83	
			1.30
K	27.76	19.28	
			1.44
G	39.92	27.72	
			1.13
L	44.96	31.22	
			1.14
I	51.28	35.61	

Fig. 3. Chromatogram of an artificial mixture of withanolide 6-Cl (9), S (8), 5-Cl (10), E (7), H (2), J (4), K (5), G (1), L (6) and I (3). Eluent: methanol–water (47:53), flow-rate, 1.5 ml/min. Other details as in Fig. 2.

Surprisingly, 6α-Cl (9) appears to be more polar than withanolide S itself (8), which in turn is more polar than 5α-Cl (10); subsequently withanolide E is eluted. Indeed compound 9 has a different configuration of the A/B rings (5β) compared to 8 and 10 (5α). This difference may be one of the factors responsible for the different elution order of these withanolides. Withanolide E (7) is eluted subsequently, probably due to its less polar 5β,6β-epoxy group. It is noteworthy that all the withanolides which have an α-oriented side chain are more polar, and therefore are eluted first.

The elution order of the remaining withanolides (Fig. 3) (expressed in relation to withanolide J) is: withanolide J (4), less polar than withanolide E (7) because it has a double bond at Δ^5 instead of the epoxide; withanolide K (5) which is Δ^3 withanolide J (a conjugated double bond with a ketone is more polar than a simple conjugated double bond); withanolide G (1) (17-deoxy withanolide J); withanolide

TABLE III

CALIBRATION DATA FOR WITHANOLIDES FROM CHEMOTYPE III

Withanolide	*Concentration (μg)*									*r*	*Slope*	*Intercept*
	0.25	*0.50*	*1.0*	*1.50*	*2.00*	*3.00*	*5.00*	*10.00*	*15.00*			
G	0.56	1.17	2.25	3.49	4.46	6.34	10.50	22.00	33.12	0.9998	2.2006	−0.031337
J	0.47	0.90	1.44	2.31	2.82	4.33	7.76	16.06	22.45	0.9990	1.5268	0.015747
H	0.49	0.93	1.89	2.89	4.15	5.99	10.28	21.32	30.09	0.9993	2.0461	−0.025937
E	0.28	0.59	1.11	1.88	1.95	3.24	5.55	12.34	16.90	0.9981	1.1581	−0.050660
S	0.44	0.85	1.85	2.58	3.44	4.9	8.03	16.17	24.87	0.9998	1.6394	0.047101
5α-Cl	0.25	0.48	0.96	1.79	2.37	3.67	5.81	9.95	16.12	0.9978	1.0495	0.139640
I	0.55	1.16	2.43	3.69	5.25	7.44	12.49	24.98	34.57	0.9987	2.3477	0.306890

TABLE IV

CALIBRATION DATA FOR WITHAFERIN A FROM CHEMOTYPE I

Concentration (μg)									*r*	*Slope*	*Intercept*
.00	*2.00*	*4.00*	*6.00*	*10.0*	*12.0*	*15.0*	*18.0*	*20.0*			
.89	3.82	7.46	11.10	18.38	22.02	27.41	32.93	36.32	0.9998	1.8145	0.183710

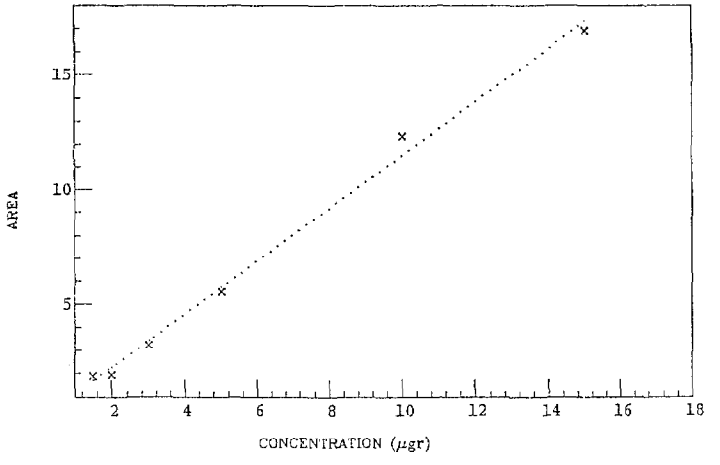


Fig. 4. Calibration curve for withanolide E (7). $y = 1.1581\ x - 0.05066$, $r = 0.9981$.

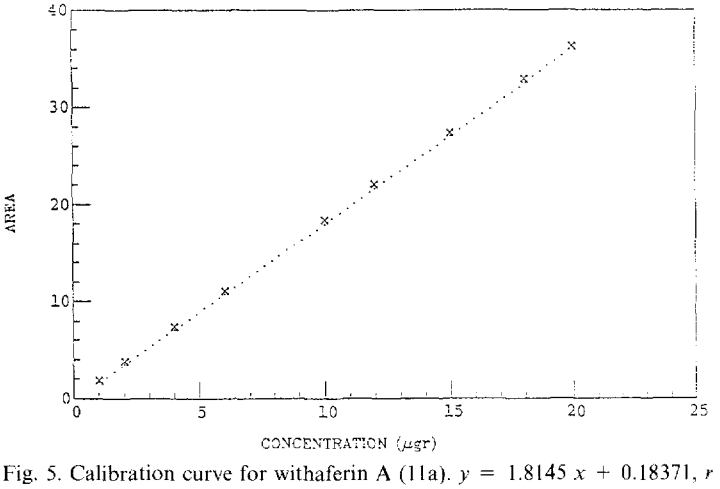


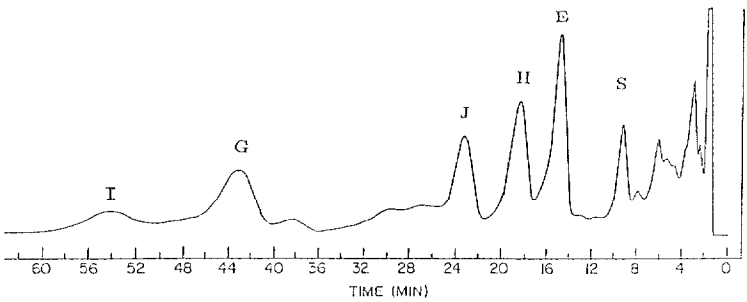
Fig. 5. Calibration curve for withaferin A (11a). $y = 1.8145\ x + 0.18371$, $r = 0.9998$.

L (6) (Δ^{14} withanolide J) and withanolide I (3) (Δ^{3}-17-deoxy withanolide J) respectively. Withanolides G and L both have one hydroxyl group less than withanolide J, but the latter possesses an extra double bond by elimination of the 14-OH group which makes it less polar than the former. Finally, withanolide I also has one hydroxyl group less than J but is less polar due to the Δ^{3} double bond as mentioned above.

In order to carry out the semi-quantitative work, it was first necessary to study the linearity of the detector response with respect to the concentrations of the withanolides. A linear relationship was observed with a correlation factor, r, greater than 0.9970 in all cases (Table III). Then calibration curves were constructed and fitted in the concentration range of the natural mixture to be injected, in order to minimize the errors. Nine points of calibration were taken in the range of 0.25–15 μg for chemotype III (Table III) and 1.0–20.0 μg for chemotype I (Table IV). The calibration curves for two withanolides, E (7) (chemotype III) and withaferin A (11a) (chemotype I) are shown in Figs. 4 and 5 respectively.

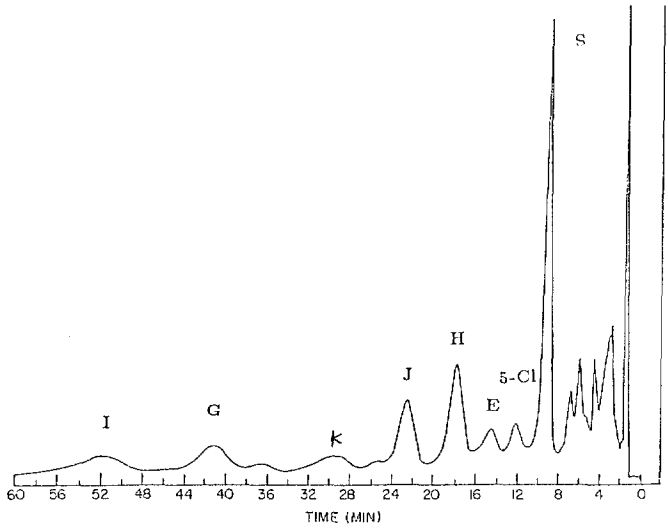
In order to detect possible differences in concentration from the same place of collection, five samples were collected from each ecotype and analyzed by HPLC, and similar concentrations were observed. Then, two of them were taken and two analyses were done for each one ($n = 4$). To determine whether the methanol extraction was complete, the residual powdered leaves were extracted under the same conditions with dichloromethane and analysed by HPLC; no significant concentration of withanolides was detected.

Figs. 6–9 show one example out of the four chromatograms for each ecotype and the statistical analyses where n is the number of analyses carried out for each withanolide of the respective ecotype, $\bar{x}$ is the average of the area per mg of sample,



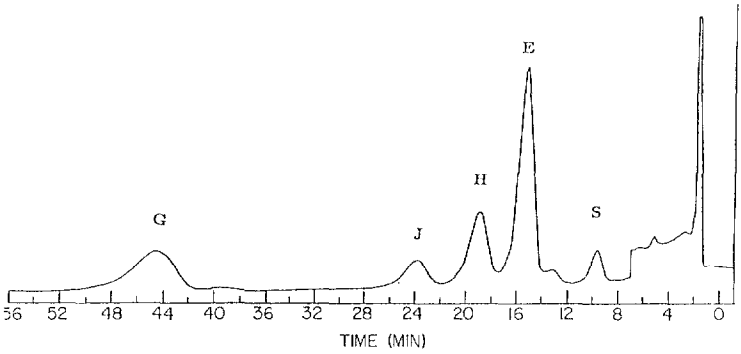
Withanolide	*n*	$\bar{x}$	$n-1$	*C.V. (%)*	*g%*	*Average (%)*
G	4	2.70	0.36	13.33	0.12	13.2 ± 1.8
J	4	2.40	0.44	18.33	0.16	17.6 ± 3.2
H	4	2.79	0.20	7.17	0.14	15.4 ± 1.1
E	4	4.88	0.57	11.68	0.43	47.3 ± 5.5
S	4	1.13	0.18	15.93	0.06	6.6 ± 1.1

Fig. 6. Chromatogram of a partially purified mixture of the Yavne ecotype; flow-rate 1.3 ml/min. Details as in Fig. 3.



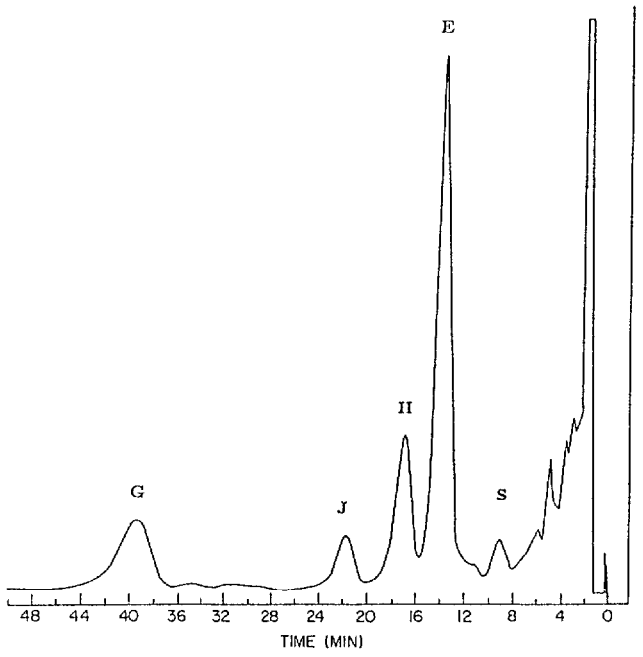
Withanolide	*n*	$\bar{x}$	*n* − *1*	*C.V. (%)*	*g%*	*Average (%)*
G	4	1.86	0.18	9.68	0.08	7.2 ± 0.7
J	4	2.93	0.32	10.92	0.19	17.1 ± 1.9
H	4	3.40	0.44	12.94	0.17	15.3 ± 2.0
E	4	0.47	0.19	40.43	0.05	4.5 ± 1.8
S	4	7.68	1.27	16.54	0.46	41.4 ± 6.9
5α-Cl	4	1.08	0.37	34.26	0.09	8.1 ± 2.8
I	4	2.31	0.22	9.52	0.07	6.3 ± 0.6

Fig. 7. Chromatogram of a partially purified mixture of the Farm-2 ecotype; flow-rate 1.2 ml/min. Details as in Fig. 3.



Withanolide	*n*	$\bar{x}$	*n* − *1*	*C.V. (%)*	*g%*	*Average (%)*
G	4	6.63	1.42	21.42	0.30	18.6 ± 4.0
J	4	1.34	0.46	34.33	0.09	5.6 ± 1.9
H	4	3.54	0.71	20.06	0.18	11.2 ± 2.3
E	4	11.01	0.93	8.45	0.96	59.6 ± 5.0
S	4	1.39	0.34	24.46	0.08	5.0 ± 1.2

Fig. 8. Chromatogram of a partially purified mixture of the Farm-1 ecotype; flow-rate 1.3 ml/min. Details as in Fig. 3.



Withanolide	*n*	$\bar{x}$	$n-1$	*C.V. (%)*	*g%*	*Average (%)*
G	4	4.18	0.37	8.85	0.19	11.6 ± 1.0
J	4	1.44	0.34	23.61	0.09	5.5 ± 1.3
H	4	3.81	0.36	9.45	0.19	11.6 ± 1.1
E	4	13.07	2.64	20.20	1.13	68.9 ± 13.9
S	4	0.77	0.42	54.55	0.04	2.4 ± 1.3

Fig. 9. Chromatogram of a partially purified mixture of the Gedera ecotype; flow-rate 1.2 ml/min. Details as in Fig. 3.

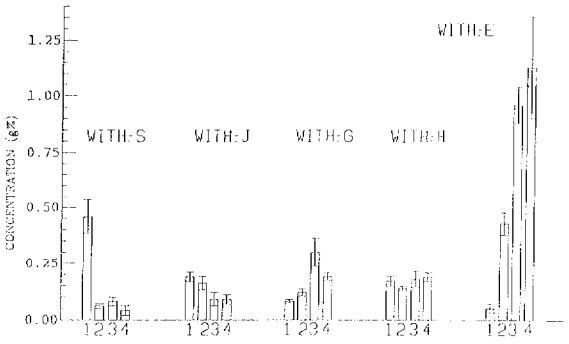


Fig. 10. Concentrations of withanolides S, J, G, H and E by ecotypes in an error bar diagram. 1 = Farm-2; 2 = Yavne; 3 = Farm-1; 4 = Gedera.

$n - 1$ is the standard deviation and C.V. is the coefficient of variation. According to these results the samples collected in Gedera and at the experimental farm (Farm-1) appear to be the same ecotype, presenting a similar relative concentration of withanolides, taking into account the C.V. of the analyses (see Fig. 10). This is not surprising since Farm-1 was a plant grown from seeds of the Gedera type at the experimental farm of the Institute, with the aim of improving the amount of withanolide E in the plant required for biological testing. The other two ecotypes, Farm-2 and Yavne, presented large differences between their major components. Farm-2 has withanolide S in the largest amount, whereas the Yavne type possesses withanolide E as the main withanolide; actually the Yavne ecotype has about half of the amount of withanolide E compared to Farm-1 and Gedera ecotypes, 0.43, 0.96 and 1.13 g% respectively. These relationships are plotted in Fig. 10. In each ecotype we always find a major component having an α-side chain which accounted for more than 40% of all the withanolides, which is a characteristic for chemotype III. The best example is Gedera (Fig. 9) which contains 69% of withanolide E (not 90% as reported previously[6]). The other withanolides, J, G and H, display similar concentrations for all the ecotypes.

Whilst looking for chemotypes in the neighbouring villages of Rehovot, in Azaria we found a plant belonging to the *Withania somnifera* species which was identified as chemotype I by TLC and HPLC analysis. The TLC analysis was done using hexane–ethyl acetate–methanol (2:8:1) as the eluent which gave a good separation between withaferin A (11a), withanolide D (12a) and withanolide E (7). When RP-HPLC was carried out on this sample an identification problem involving chemotypes I and II was encountered. No separation could be achieved in less than 1.30

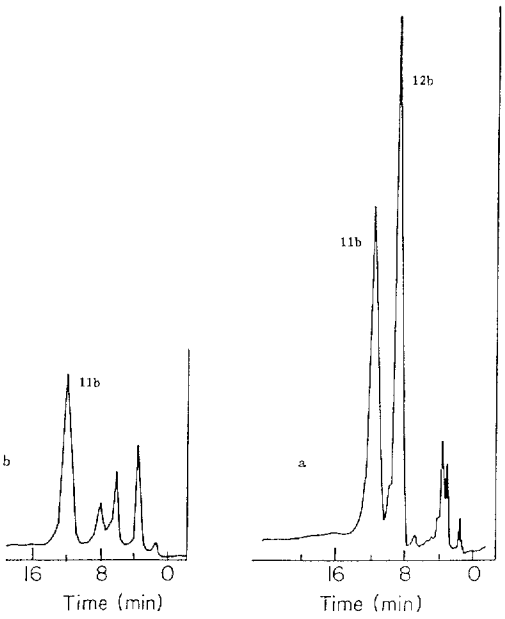


Fig. 11. (a) Separation of an artificial mixture of withaferin A acetate (11b) and withanolide D acetate (12b). (b) Chromatogram of a partially purified acetylated mixture of chemotype I. Eluent: methanol–water (70:30); flow-rate 1.3 ml/min. Other details as in Fig. 3.

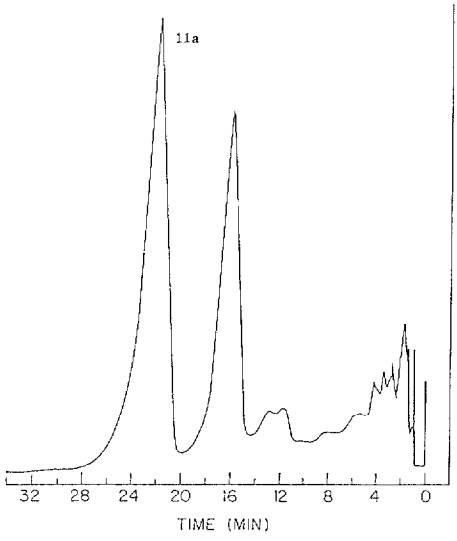
h when analyzing an artificial mixture containing withaferin A and withanolide D, the major components present in each of these two chemotypes. Unsuccessful attempts were made using water, methanol, acetonitrile and isopropanol in different combinations and proportions. Taking advantage of the fact that withaferin A (11a) has two hydroxyl groups which can be acetylated and withanolide D (12a) has only one, acetylation of an artificial mixture was carried out and the acetates were resolved in less than 13 min using water–methanol (3:7) as the eluent (Fig. 11a). The purified extract of the plant collected in Azaria was then acetylated and withaferin A was positively identified according to its retention times, t'_R = 12 min (Fig. 11b), and by coinjection with the acetylated standard.

The semi-quantitative analyses of *Acnistus breviflorus* and *W. somnifera* chemotype I are shown in Figs. 12 and 13 respectively, using the non-acetylated samples and standards. Chemotype I has about twice the concentration of withaferin A compared with *Acnistus breviflorus*. A similar concentration of withaferin A in the latter plant was reported previously[7].

Compounds 13, 14 were isolated in very low yields, thus no HPLC analyses are provided.

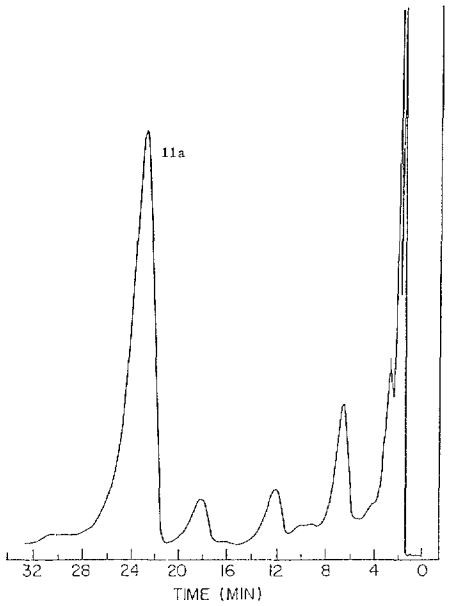
CONCLUSIONS

The aim of this work, rather than an accurate quantitative study, was to carry out qualitative and semi-quantitative HPLC analyses in order to determine the range



Compound	*n*	$\bar{x}$	*n* − *1*	*C.V. (%)*	*g%*
Withaferin A	4	16.36	0.73	4.46	0.88

Fig. 12. Partially purified mixture of *Acnistus breviflorus* containing withaferin A (11a). Eluent: methanol–isopropanol–water (45:5:55); flow-rate 1.5 ml/min. Other details as in Fig. 3.



Compound	*n*	$\bar{x}$	$n-1$	*C.V. (%)*	*g%*
Withaferin A	4	15.49	1.65	10.65	1.68

Fig. 13. Partially purified mixture of chemotype I containing withaferin A (11a). Details as in Fig. 12.

of concentrations of withanolides present in the plant. As a result, three ecotypes were identified possessing different relative amounts of the major component.

Generally, this method enables one to determine in which cases certain specific compounds may occur in large quantities in the chemotypes, and thereby obtain compounds required for bio-assays or any other general use. The production of withanolides in the plant could be monitored through seasonal changes or growth periods. It should also be an important tool in the study of biosynthetic pathways and genetic inheritance of hybrids.

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