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Short Communication

Simultaneous high-performance liquid chromatographic determination of altersolanol A, B, C, D, E and F

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ABSTRACT

A high-performance liquid chromatographic model for the simultaneous determination of altersolanol A-F by using a reversed-phase column with an acetonitrile-water gradient elution system is described. The analysis can be completed within 13 min, the detection limits are 0.2–0.5 pmol per injection (*5 μl*) and the relative standard deviations are 0.90–1.34%. The method was applied satisfactorily to the determination of altersolanols in culture media of a strain of ***Alternaria*** *solani* without any prepurification.

INTRODUCTION

The fungus *Alternaria solani,* a pathogen of early blight disease of tomato and potato [I], has been known to produce altersolanol A, B and C as metabolic pigments [2], which are rare but not unique examples of naturally occurring tetrahydroanthraquinones. These compounds have also been found as a phytotoxin from *Alternatria porri* [3] and *Dactylaria lutea* [4] and they exhibit phytotoxic activities in the seeds of lettuce and stone-leek [5]. Altersolanol A is a precursor [6] of altersolanol B, which is strongly cytotoxic to HeLa [7] and Ehrlich ascites carcinoma cells [8]. In a previous study we found that altersolanol A exhibited antimicrobial activity against Gram-positive and -negative bacteria and this activity was closely related with the interference of the respiratory chain in the bacterial membrane of *Pseudomonas aeruginosa* as an electron acceptor [9].

In a previous paper we reported the identification of the related tetrahydroanthraquinones altersolanol D, E and F, together with altersolanol A, B and C, and discussed the antimicrobial activities of altersolanol A-F against *Pseudomonas aeruginosa [IO]* (Fig. 1). Suemitsu and co-workers [11,12] reported the presence of altersolanol A–C and related anthraquinones in *Alternaria porri* using reversed-phase high-performance liquid chromatographic (HPLC) methods. However, these methods are not applicable to the simultaneous determination of altersolanol A-F. We therefore initiated the present

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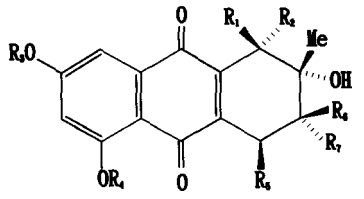

Altersolanol	R_1	R	R	R	R	R	R_7
A	OH	H	Me	H	OH	H	OH
B	H	H	Me	H	H	H	OH
C	OH	H	Me	H	H	H	OH
D	OH	H	H	Me	OH	H	OH
E	OH	H	Me	H	OH	OH	H
F	H	OH	H	Me	OH	H	OH

Fig. 1. Structures of altersolanols.

studies on the development of a simple and rapid method for the determination of altersolanol A-F in culture media of the strain of ***Alternaria solani.*** In this paper we describe the simultaneous HPLC determination of altersolanol A-F and the culture conditions for producing altersolanol A-F. With this method it was possible to study the production of altersolanol A-F in culture media.

EXPERIMENTAL

Materials

Altersolanol A-F were isolated from the culture liquid of the strain of ***Alternaria solani*** as metabolic pigments [10].

The water used for chromatography was deionized with a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile was of HPLC grade and other solvents and chemicals were of analytical-reagent grade, all of which were obtained from Wako (Osaka, Japan).

Sample preparation

A stock standard solution was made by diluting with methanol to give concentrations of 9.35 (altersolanol A), 8.74 (altersolanol B), 2.50 (altersolanol C), 8.06 (altersolanol D), 1.87 (altersolanol E) and 4.24 μmol/ml (altersolanol F).

The strain of ***Alternaria solani*** was isolated from a diseased tomato leaf in the greenhouse of the university. ***Alternaria solani*** (IFO 7516) was obtained from Institute for Fermentation, Osaka, and ***Alternaria porri*** (IF0 9762) was also obtained from the same Institute was a standard strain. The-morphological characteristics suggest that this strain was a synonym of ***Alternaria solani.*** The fungus was maintained on malt agar and cultured in the medium (20 ml per 100-ml flask) [10]. To assess the influence of detergent, dimethyl sulphoxide (DMSO) and N,N-dimethylformamide (DMF) were added at final concentrations of 2% and 1%, respectively. Cultivation was carried out at 25°C under continuous fluorescent lighting (43 $\mu E/m^2$, 3000 lux; 1 E = 6.02 $\cdot 10^{23}$ photons) or in the dark for 10 days. The culture liquid was filtered through a Millipore syringe filter unit (0.45 nm) and then a portion was injected into the HPLC column. For the calibration graph for altersolanols, standard solutions were diluted with methanol and the filtration was omitted.

Apparatus

HPLC was performed using a gradient system from Tosoh (Tokyo, Japan) with two CCPD pumps and a dynamic mixer, a Rheodyne Model 7125 syringe-loading sample injector equipped with a 5-μl sample loop and Tosoh UV-8000 UV-Vis detector set at 270 nm. The data were processed by means of a SIC Chromatocorder-II integrator to evaluate the peak areas. The purity of the chromatographic peaks was estimated using a Waters (Milford, MA, USA) M990J photodiode-array detector.

Chromatographic conditions

The reversed-phase column was made of stainless steel (150 × 4.6 mm I.D.) and packed with Wako Wakosil-II 5C18 HG (5 μm). The separation was carried out using a linear gradient programme with the following eluents: O-3 min, acetonitrile-water (18:82); 3-20 min, linear change to acetonitrile–water (90: 10); 20–23 min, acetonitrile-water (90: 10). A re-equilibration period of 12 min was used between individual runs. The flow-rate was maintained at 1.0 ml/min and the temperature at 20–25°C.

RESULTS AND DISCUSSION

HPLC was carried out on a Wako Wakosil-II 5C18 HG reversed-phase column, which was selected because of its large number of theoretical plates, its specific characteristics towards polyketides and the avoidance of poor peak shapes of altersolanols. Fig. 2A shows the separation of a standard mixture

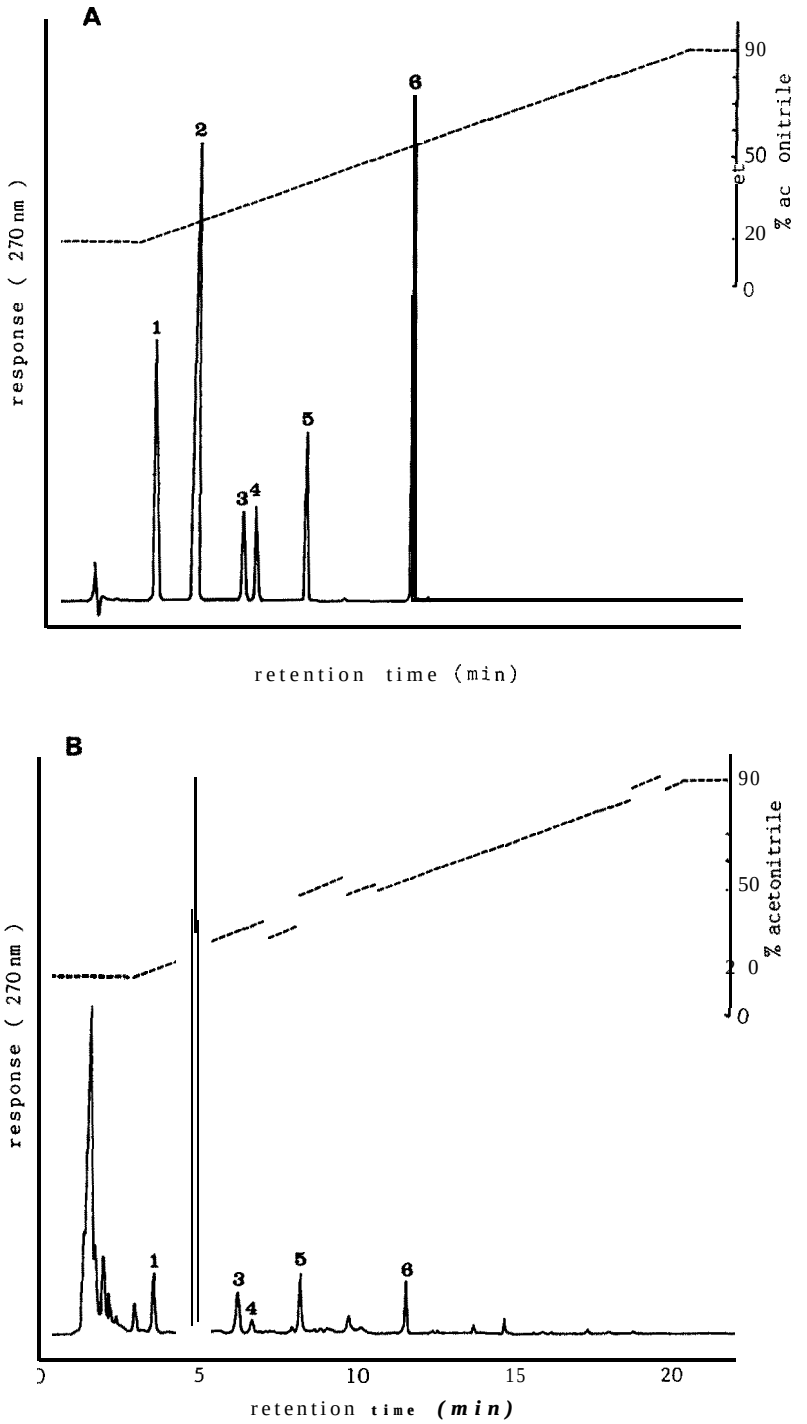

Fig. 2. Separations of altersolanols in (A) standard mixture and (B) the strain of *Alternaria solani*. Concentrations: (A) 1 = 80.6, 2 = 93.5, 3 = 42.4, 4 = 18.7, 5 = 25.0, 6 = 87.4 μM; (B) 1 = 69.8, 2 = 467.0, 3 = 72.5, 4 = 7.5, 5 = 43.5, 6 = 47.2 μM. Peaks: 1 = altersolanol D; 2 = altersolanol A; 3 = altersolanol F; 4 = altersolanol E; 5 = altersolanol C; 6 = altersolanol B.

TABLE I

RETENTION TIMES AND CAPAICTY FACTORS FOR ALTERSOLANOLS

Compound	t_R (min)	k'
Altersolanol D	3.60	1.31
Altersolanol A	4.75	2.04
Altersolanol F	6.19	2.97
Altersolanol E	6.61	3.24
Altersolanol C	8.18	4.24
Altersolanol B	11.54	6.40

of altersolanols and Table I lists retention times (t_R) and capacity factors (k') of these compounds. The chromatogram of these compounds from a culture medium is shown in Fig. 2B. HPLC can be accomplished by applying a culture liquid without any prepurification and the determination of altersolanols can be achieved within 13 min. The detection limits (signal-to-noise ratio = 3) of altersolanols were 0.2–0.5 pmol per injection. There was good linearity from 0.4 to 1 μM of altersolanol A-F with correlation coefficients of 0.999-1.000. The relative standard deviations (n = 10) were 1.34 (altersolanol A), 1.24 (altersolanol B), 1.10 (altersolanol C), 1.20 (altersolanol D) and 0.90% (altersolanol E and F) using a standard mixture as shown in Fig. 2A.

Recovery tests were performed by adding known amounts of altersolanols to culture liquid (1 ml). The mixture was filtered and assayed according to the above procedure. Table II summarizes the recoveries of the altersolanols, which were ⩾91.6%.

TABLE II

RECOVERY AND PRECISION FOR ALTERSOLANOLS

Altersolanol	Initial amount (μg)	Added (μg)	Recovery (%)
A	36.96	10.84	103.6 ± 2.0
		21.68	104.1 ± 1.2
B	6.78	9.92	91.6 ± 1.4
		19.85	95.5 ± 1.5
C	4.93	7.58	98.2 ± 0.7
		15.17	93.0 ± 0.9
D	9.04	10.16	103.7 ± 0.8
		20.32	102.2 ± 1.3
E	2.79	4.35	95.9 ± 2.6
		8.70	96.3 ± 1.1
F	7.46	8.02	100.4 ± 0.5
		16.03	98.1 ± 1.7

[a] Results are means ± standard deviations from three independent experiments.

Altersolanols in culture media were determined using the proposed method. Usually, light exposure during the growth phase inhibits the accumulation of polyketides in *Alternaria alternate* [13]. However, the results in Table III demonstrate that light is nec-

TABLE III

CONTENT OF ALTERSOLANOLS IN CULTURE MEDIA OF *ALTERNARIA* SPECIES

Alternaria species	DMSO (%)	Altersolanol (μmol per flask)											
		Light						Dark					
		A	B	C	D	E	F	A	B	C	D	E	F
Strain of *A. Solani*	0	n d	tr[b]	nd	nd	nd	tr	nd	nd	nd	nd	nd	nd
	2	36.40	2.50	1.84	3.12	0.58	2.96	0.72	0.06	0.10	0.01	0.02	0.01
A. solani	0	0.08	0.26	0.08	nd	0.02	0.02	0.01	nd	tr	nd	nd	nd
	2	0.2	0.14	0.04	nd	0.02	0.04	0.18	nd	tr	nd	nd	nd
A. porri	0	*28.74*	nd	0.02	1.58	0.18	1.72	19.36	0.02	0.06	0.36	0.12	0.40
	2	0.38	nd	tr	nd	nd	nd	1.00	nd	tr	nd	nd	nd

[a] Not detected.
[b] Trace.

essary for a high productivity of altersolanols in a culture medium of the strain. Photosporogenesis is one of the most important in the morphological differentiation in fungi [14], which is closely related to the production of secondary metabolites. The secondary metabolites produced are often stored within the fungal cells. Application of an agent that releases the metabolites into the surrounding medium results in increased productivity. Table III shows that the production of altersolanols by *Alternaria porri* was completely inhibited by light exposure and addition of 2% DMSO, whereas *Alternaria solani* produced altersolanols under the same conditions. In the present experiment, however, the culture medium of the strain of *Alternaria solani* contained 4-14 times more altersolanols than the other on addition of either 2% DMSO or 1% DMF under light exposure. Comparison of altersolanols between *Alternaria porri*, *Alternaria solani* and the strain of *Alternaria solani* showed that a higher amount of altersolanols in the strain of *Alternaria solani* was induced by light exposure and detergent than the others in both mycelia and culture liquid. Hence this treatment presents a useful means for the production of altersolanols.

CONCLUSION

A reliable method for the simultaneous determination of altersolanol A-F in the strain of *Alternaria solani* has been developed. This method is excellent for identifying altersolanols in culture media of *Alternaria* species without any prepurification.

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