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Simultaneous high-performance liquid chromatographic determination of visoltricin, acuminatopyrone and chlamydosporols in *Fusarium* cultures on maize

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Abstract

Visoltricin (VIS), acuminatopyrone (ACP), clamydosporol (CL), isochlamydosporol (ICL) and chlamydospordiol (DIOL), recently characterized *Fusarium* metabolites, were separated on a polymeric RP-18 column eluted with acetonitrile–0.01% ammonia solution (35:65) at 1 ml/min and detected with a diode-array UV detector. The presence of ammonia in the mobile phase improved the shape of the CL and VIS peaks. The use of a polymeric column was required owing to the basic pH of the mobile phase. Maize cultures of several strains of *F. tricinctum* and *F. chlamydosporum* were analysed with this procedure after extraction with aqueous methanol, partitioning with methylene chloride and clean-up with a C_{18} minicolumn. VIS was produced only by *F. tricinctum*, whereas ACP and chlamydosporols were produced by both *Fusarium* species.

Keywords: Fusarium; Visoltricin; Acuminatopyrone; Chlamydosporol; Isochlamydosporol; Mycotoxins; Chlamydospordiol

1. Introduction

Fusarium species are widely distributed in soil, plants and plant products and produce several mycotoxins and other metabolites with a variety of biological activity [1]. *Fusarium* mycotoxins such as trichothecenes and fumonisins are of concern for human and animal health owing to their toxicity and occurrence in foods and feeds. Several new *Fusarium* metabolites, including visoltricin (VIS), chlamydosporol (CL), isochlamydosporol (ICL), chlamydospordiol (DIOL) and acuminatopyrone (ACP) (Fig. 1), have been described recently [2–5]. The knowledge of secondary metabolites produced by different *Fusarium* species provides a useful tool for the evaluation of the toxic potential [6] and the chemotaxonomic characterization of the particular species within the genus *Fusarium* [7].

VIS, the first imidazole derivative produced by *Fusarium* species, was toxic to *Artemia salina* larvae, inhibited the growth of human tumor cell lines, produced a miotic effect on rabbit eye and showed anticholinesterase properties towards both human serum and pure enzyme [3,8]. CL occurs naturally as a mixture of two epimers, and has been shown to be toxic to *A. salina* larvae, HeLa cells, cultured mouse cells, human fibroblast and chick embryos. In addition, it caused feed refusal and mass loss in rats [9–11]. No

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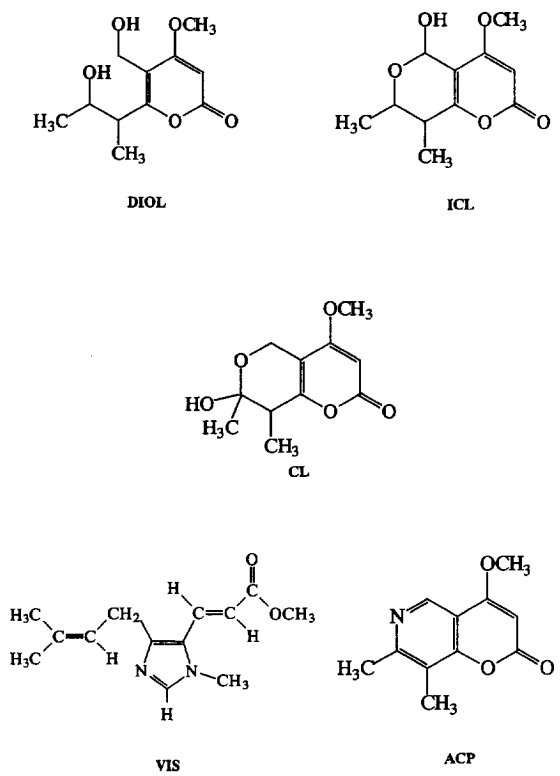

Fig. 1. Structures of chlamydosporol (CL), isochlamydosporol (ICL), chlamydospordiol (DIOL), acuminatopyrone (ACP) and visoltricin (VIS).

biological activity has been reported for ACP, ICL and DIOL. In this paper, we report a procedure for the simultaneous HPLC determination of these compounds in maize culture extracts of *Fusarium* species.

2. Experimental

2.1. Reference standards and culture materials

Visoltricin, acuminatopyrone and chlamydosporols were isolated from maize cultures of *F. tricinctum*, strain KF 260, and *F. chalmydosporum*, strain T-826, as described elsewhere [3–5]. The compounds were dissolved in acetonitrile at different concentrations and used as reference standards. *Fusarium* cultures on maize were prepared according to the procedure reported elsewhere [3].

2.2. Extraction and clean-up

DIOL, ICL, CL, ACP and VIS were extracted from maize cultures according to a previously reported procedure [4]. After solvent evaporation the residue was made up to 1 ml with MeOH.

The clean-up of sample extracts was performed on a 500-mg Bond-Elut C_{18} minicolumn (Varian, Harbor City, CA, USA). In particular, 50 μl of methanolic extract, corresponding to 500 mg of culture material, were forced by aspiration through the minicolumn previously conditioned with 2×2 ml of MeOH and 2×2 ml of MeOH–H_2O (20:80). The column was washed with 2 ml of MeOH–H_2O (20:80) and the compounds were eluted with 2×2 ml of MeOH–0.01% ammonia solution (80:20). Each sample, after evaporation of the solvent, was dissolved in an appropriate amount of acetonitrile and analysed by reversed-phase HPLC.

2.3. Apparatus

The HPLC apparatus was a Waters 625 LC system equipped with a Hewlett-Packard HP 1040 diode-array UV detector connected to an HP 9000 Series 300 computer. A polymeric-based RP-18 column (PLRP-S, pore size 10 nm, 150×4.6 mm I.D., 5 μm) preceded by a poly(styrene–divinylbenzene) guard cartridge (5×3 mm I.D.) with the same packing material (Polymer Laboratories, Shropshire, UK) was used. Quantitation of the *Fusarium* metabolites (VIS, ACP, DIOL, ICL and CL) was performed by comparison of peak areas with reference standards. Confirmation of the compounds was performed by comparison of retention times and UV spectra with reference standards prepared according to the literature [3–5]. The presence of interfering compounds which may co-elute with the metabolites analysed was excluded by performing a peak purity test. Peak spectra match values ranged from 994 to 1000.

3. Results and discussion

The optimum isocratic composition of the HPLC mobile phase for the separation of DIOL, ICL, CL (with the two epimers eluting as a single peak), ACP and VIS in culture extracts was acetonitrile–0.01% ammonia solution (35:65) using a polymeric-based RP-18 column. The elution sequence of compounds from this column was DIOL, ICL, CL, ACP and VIS. The separation of the two CL epimers, not required for the purpose of this study, can be achieved by using a stationary phase with a high percentage of free silanols and a quaternary mobile phase, as described elsewhere [12]. The ability of the diode-array detector to record and store spectral information was useful for identification of the optimum wavelength for each compound, in order to obtain the best sensitivity. DIOL, ICL and CL, which have similar UV spectra, gave the maximum response at 210 nm, ACP at 220 nm and VIS at 301 nm. Figs. 2 and 3 report the chromatograms relevant to culture extract of *F. tricinctum* and *F. chlamydosporum*, respectively.

Preliminary attempts performed with several methanol–water isocratic compositions using a Spherisorb ODS II column (125 × 4.6 mm I.D., 5 μm) showed major problems (in terms of res-

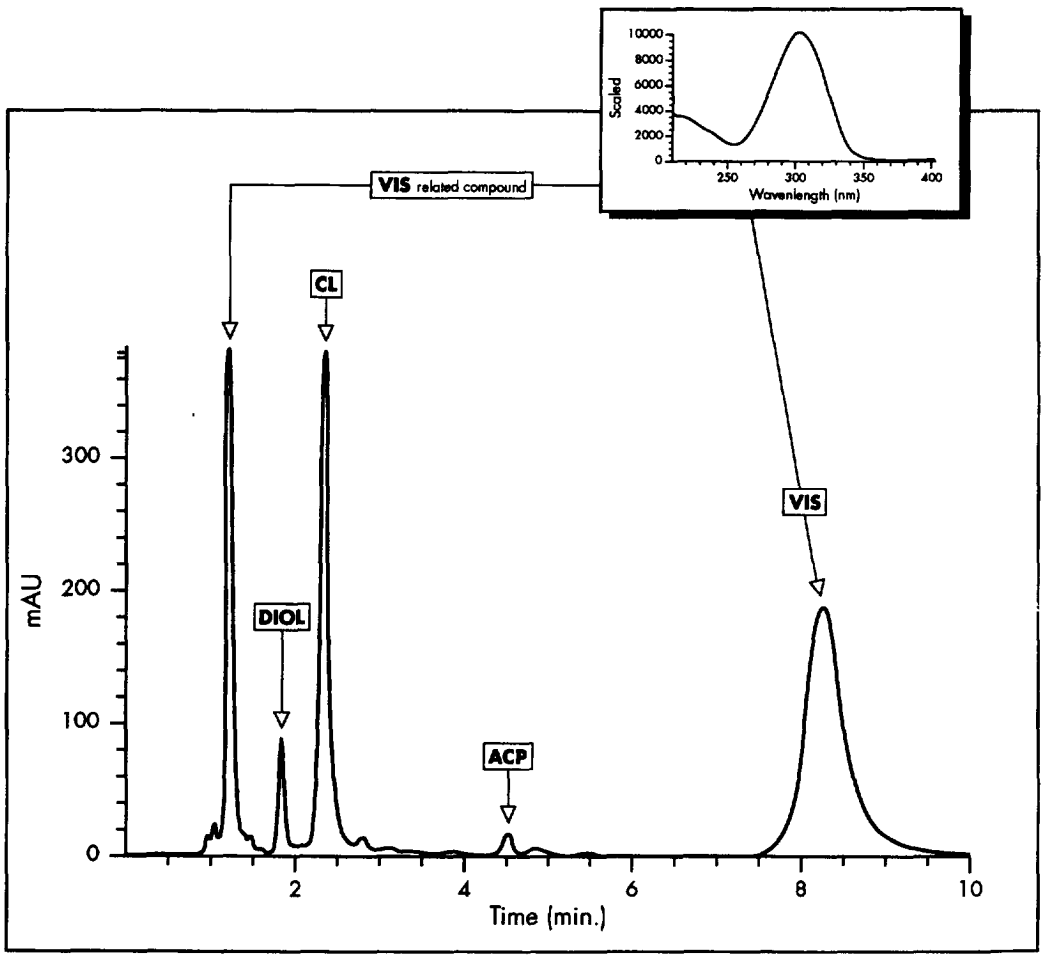

Fig. 2. Chromatogram of a maize culture extract of *F. tricinctum* containing DIOL, CL, ACP and VIS at levels of 110, 220, 10 and 1350 μg/g, respectively. Dry mass of culture equivalent injected, 10 mg; column, polymeric RP-18 (150 × 4.6 mm I.D., 5 μm); mobile phase, acetonitrile–0.01% ammonia solution (35:65); flow-rate, 1 ml/min; detection, diode-array UV at 301 nm.

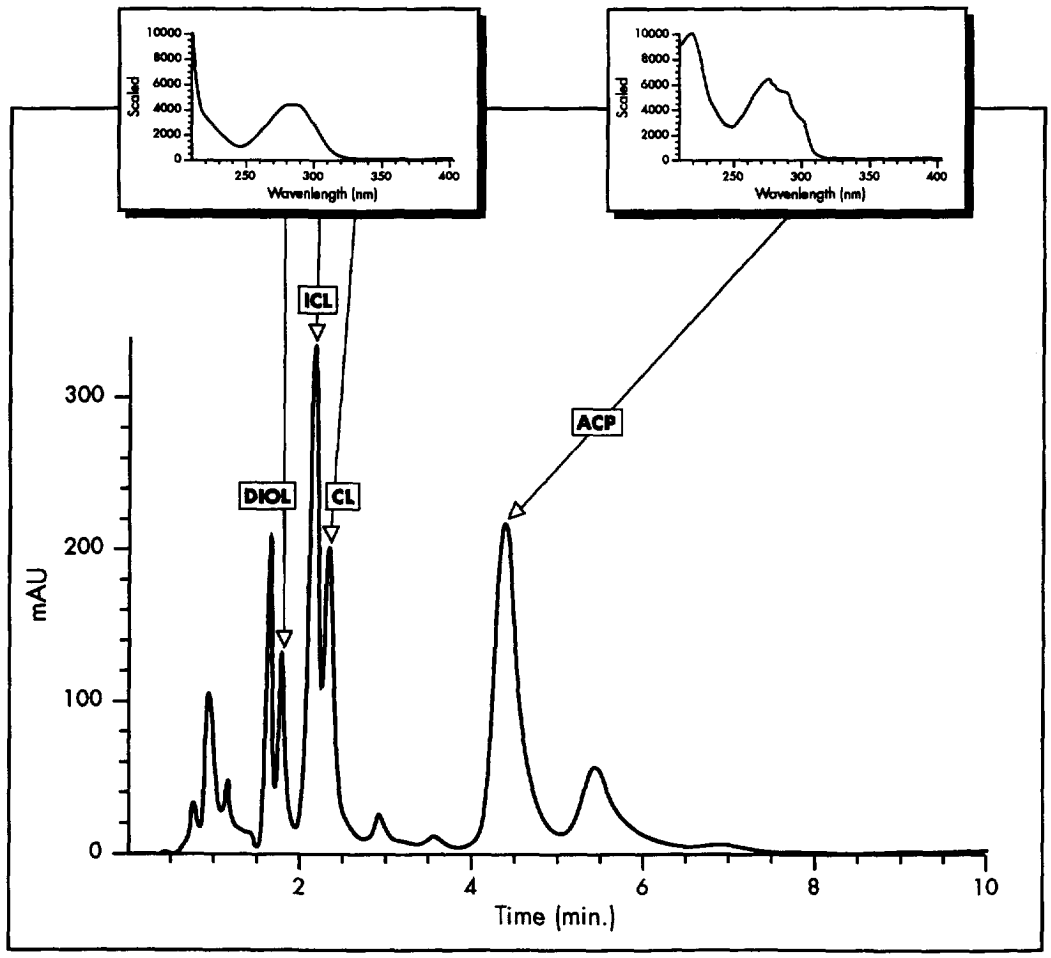

Fig. 3. Chromatogram of a maize culture extract of *F. chlamydosporum* containing DIOL, ICL, CL and ACP at concentrations of 1200, 1800, 1400 and 1460 μg/g, respectively. Dry mass of culture equivalent injected, 10 mg; chromatographic conditions as in Fig. 2; detection, diode-array UV at 220 nm.

olution and sensitivity) in the detection of VIS and CL owing to pronounced peak tailing. These problems were resolved by adding a small amount of ammonia to the mobile phase. With this approach, the CL and VIS signals appeared as well defined (narrow and symmetrical) peaks, and a good resolution was obtained between the ICL and CL signals [4]. The presence of ammonia in the HPLC mobile phase generated high pH values (>8), with the consequent risk of dissolution of the silica backbone of the stationary phase. In order to avoid this problem, a polymeric-based C_{18} column, having a polystyrene-based stationary phase instead of silica, was used. A suitable separation of the compounds of interest was obtained with this column despite the lower number of theoretical plates compared with silica-based C_{18} columns. Moreover, acetonitrile instead of methanol was used in the mobile phase in order to improve the detection sensitivity at lower wavelengths. In addition to VIS, ACP, CL and ICL, a peak with the same UV spectrum as VIS was observed at a retention time of about 1.2 min in the chromatogram of *F. tricinctum* extract (Fig. 2). This suggests that a new compound, with a structure

similar to that of VIS but more polar, is produced by *F. tricinctum*. It would be interesting to isolate, characterize and test this compound for biological activity. The use of the HPLC system with diode-array detection would be appropriate for monitoring this compound during the purification steps.

The production of DIOL, ICL, CL, ACP and VIS by several strains of *F. tricinctum* and *F. chlamydosporum* was tested, and the results of the producing strains are reported in Table 1. VIS was only produced by *F. tricinctum*, whereas DIOL, ICL, CL and ACP were produced at different levels by both *F. tricinctum* and *F. chlamydosporum*. All isolates producing DIOL also produced ACP, whereas the isolates lacking the capability to synthesize DIOL did not make ACP either. The co-occurrence in the same fungal cultures and the similarity of the structures imply that DIOL is an intermediate in the biosynthesis of ACP and CL [5]. The HPLC method described in this paper can be used for chemotaxonomic purposes in order to characterize different *Fusarium* species on the basis of the differential production of the secondary metabolites considered here.

Table 1
Production of DIOL, ICL, CL, ACP and VIS by strains of *F. chlamydisporum* and *F. Tricinctum* inoculated on autoclaved maize kernels, previously brought to 45% moisture, and incubated for 30 days at 25–27°C

Strain[a]	Concentration (μg/g)				
	DIOL	ICL	CL	ACP	VIS
F. chlamydosporum					
7-729	2200	800	3200	1800	nd[b]
T-731	260	900	400	459	nd
T-826	1200	1800	1400	1460	nd
T-513	170	nd	nd	441	nd
T-669	777	nd	nd	230	nd
T-746	353	nd	nd	36	nd
F. tricinctum					
T-460	74	nd	nd	37	nd
T-226	40	nd	40	6	40
T-387	225	200	200	13	29
T-511	130	650	780	3	271
T-693	110	nd	220	10	1350
T-823	70	nd	40	6	22
KF 260	50	80	110	20	100
T-545	nd	nd	nd	nd	30
T-904	nd	nd	nd	nd	1165

[a] T, *Fusarium* Research Center, University Park, PA, USA; KF, Katedra Fitopatologii, Agricultural University, Warsaw, Poland.

[b] nd = Not detected; <3 μg/g for DIOL, ICL, CL and ACP and <1 μg/g for VIS.

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